Investigation of the therapeutic and prophylactic potential of omega-3 polyunsaturated fatty acids in head and neck cancer

Thesis submitted in accordance with regulations for the degree of Doctor of Philosophy

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ABSTRACT

Squamous cell carcinomas (SCCs) of the aerodigestive tract often recur because of incomplete excision or the appearance of second primary or second field cancers. Recent evidence suggests that the omega-3 polyunsaturated fatty acids (PUFA) have antitumorigenic activities. In the present study the potential of omega-3 PUFA to act as selective chemopreventive and therapeutic agents against oral and epidermal SCCs was tested and the mechanism of action was investigated. The effect of omega-3-PUFA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on oral and epidermal malignant SCC and pre-malignant cell lines and also normal keratinocytes were examined. The PUFA inhibited growth dose-dependently after 4 days, as measured by MTT cell viability assays. The PUFA appeared to be more selective against malignant and premalignant than normal keratinocytes. It was demonstrated that PUFA caused apoptosis by the annexin V apoptosis assay and cleavage of caspase 3 by western blotting. The cleavage of caspase 9 and 8 demonstrated the involvement of the intrinsic and extrinsic apoptotic pathways, respectively. Moreover, DHA and EPA decreased cell proliferation by the ^{3}H-thymidine uptake assay. PUFA appeared to increase ROS production and DNA damage after 16 hours, especially at the higher concentrations. However, the use of anti-oxidants could not rescue the cell. Furthermore, the role of telomerase in PUFA mechanism of action was not confirmed as overexpression of *TERT*, *TERT-HA* and *CMYC* did not protect the cells from the growth inhibitory effect of PUFA and serum albumin was identified as an antagonist of PUFA inhibitory effect. PUFA caused a rapid and sustained phosphorylation of ERK1/2 which is inhibited by MEK and EGF receptor inhibitors. The phosphorylation of ERK1/2 was accompanied by an increase in COX-2 expression. An increase in the phosphorylation of JNK, especially in higher doses, but no effect on Akt phosphorylation, was observed. It is hypothesised that PUFA may secrete a ligand which causes the suprastimulation of EGFR and over-activation of ERK1/2 pathway which leads to apoptosis. In summary, the omega-3-PUFA DHA and EPA display a marked anti-tumour effect against SCC keratinocytes at concentrations that do not eliminate normal cells, thus giving them a significant potential as future therapeutic and prophylactic tools against head and neck cancer.

Author's declaration

I declare that I am the sole author of this thesis and that is the result of my own independent work unless otherwise stated.

This thesis has not been submitted for consideration for any other degree in this, or any other university.

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Abbreviations

AA	arachidonic acid
Ab	antibody
ACBPs	acyl-CoA binding proteins
ACSL	long chain fatty acyl-CoA synthetase
AgRP	Agouti-related protein
Akt (PKB)	protein kinase B
ALA	alpha-linolenic acid
ALT	alternative lengthening of telomeres
Apaf-1	apoptotic protease activating factor 1
ATP	adenosine-5'-triphosphate
BA	bovine albumin
Bak	Bcl-2 homologous antagonist/killer
Bcl-2	B-cell lymphoma 2
BHT	butylated hydroxytoluene
BPE	bovine pituitary extract
BSA	bovine serum albumin
Cdc42	cell-division cycle 42
COX-1	cyclooxygenase-1
COX-2	cyclooxygenase-2
CPM	counts per minute
CTLs	cytotoxic T cells
DAPI	4',6-diamidino-2-phenylindole
DCF	2',7'-dichlorofluorescein
DGLA	dihomogamma-linolenic acid
DHA	docosahexaenoic acid
DNA	deoxyribonucleic acid
DPA	docosapentaenoic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EIF-2	eukaryotic initiation factor-2
EPA	eicosapentaenoic acid
ER	endoplasmic reticulum

ERK	extracellular signal-regulated kinase
EtOH	ethanol
FA	fatty acid
FABPs	fatty acid binding proteins
FACS	fluorescence-activated cell sorting
FADD	Fas-Associated Death Domain protein
FAMEs	fatty acid methyl esters
FATPs	fatty acid transport proteins
FBS	foetal bovine serum
FFA	free fatty acids
FGF-a	acidic fibroblast growth factor
FGF-b	basic fibroblast growth factor
FHIT	fragile histidine triad
FITC	fluorescein isothiocyanate
GC	gas chromatography
GC–MS	chromatography coupled to mass spectrometry
GLA	gamma-linolenic acid
GPCRs	G-protein-coupled receptors
GSK3β	Glycogen synthase kinase 3 β
H$_2$DCFDA	2′,7′-Dichlorodihydrofluorescein Diacetate
^{3}H-TdR	tritiated thymidine
HA	hemaglutinin
HA	human serum albumin
HE	hydroethidine
HNSCC	head and neck cancer squamous-cell carcinoma
HPV	human papilloma virus
HRP	horseradish peroxidase
IGF-1	Insulin growth factor type 1
IκBα	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
IL-1	interleukin-1
IL-6	interleukin-6
IPS	ichthyosis prematurity syndrome
Jak	janus kinase

JNK	c-Jun NH2-terminal kinases
KGM	keratinocyte growth medium
K-SFM	keratinocyte serum free medium
KO	knockout
LA	linoleic acid
LCFA	long chain fatty acid
LOX	lipoxygenase
LT	leukotriene
LXA$_4$	lipoxin A$_4$
MAPK	mitogen-activated protein kinase
MeOH	methanol
MetS	metabolic syndrome
MKP-1	MAP kinase phosphatase 1
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAFLD	nonalcoholic fatty liver disease
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK cell	natural killer cell
NO	nitric oxide
NPY	neuropeptide Y
NR2B	N-methyl D-aspartate receptor subtype 2B
NSAID	nonsteroidal anti-inflammatory drugs
OD	optical density
8-OGG1	8-oxoguanine DNA glycosylase 1
OSCC	oral squamous-cell carcinoma
PBS	phosphate buffered saline
PBN	N-tert-butyl-alpha-phenylnitrone
PDK	3-phosphatidylinositol dependent protein kinase
PI	phosphatidylinositol
PI3K	phosphatidylinositol-3 kinase
PIP$_2$	phosphatidylinositol-4,5-bisphoshate
PG	prostaglandin
PGI	prostacyclin
PH	pleckstrin homology
PKC	protein kinase C

PLA2	phospholipase-2
PMN	polymorphonuclear leukocytes
PPARγ	peroxisome proliferator-activated receptor gamma
PS	phosphatidylserine
PTEN	phosphatase and tensin homolog
PVDF	polyvinylidene fluorid
PUFA	polyunsaturated fatty acids
PUMA	p53 up-regulated modulator of apoptosis
qPCR	quantitative polymerase chain reaction
RA	retinoic acid
RAR	retinoic acid receptor
Rho	Ras homologue
RIPA	radio-immunoprecipitation assay
rpm	revolutions per minute
ROS	reactive oxygen species
RS	reactive species
RT	room temperature
RvD	D series of resolvins
RvE	E series of resolvins
SCC	squamous-cell carcinoma
SEM	standard error of means
shRNA	short hairpin RNA
siRNA	small interfering RNA
SDS	sodium dodecyl sulfate
SFT	second-field tumours
SPM	specialised pro-resolving mediators
SPT	second primary tumours
Stat	signal transducers and activators of transcription
T3s	tocotrienols
TA	transit amplifying
TAK	TGFβ-activated kinase
TBS-T	Tris-Buffered Saline and Tween 20
TBHP	*tert*-butyl hydroperoxide
TERC	Telomerase RNA component

TERT	Telomerase Reverse Transcriptase
TG	triglyceride
TGF-α	transforming growth factor alpha
TLC	thin layer chromatography
TLRs	toll-like receptors
TNF-α	tumor necrosis factor α
TNFR-1	tumor necrosis receptor I
TRAIL	TNF-related apoptosis inducing ligand
Toc	tocopherols
TPA	12-O-tetradecanoylphorbol, 13-acetate
TRAIL	TNF-related apoptosis-inducing ligand
TRAP	telomere repeat amplification protocol
TX	thromboxane
TSG	tumour suppresor gene
VEGF	vascular endothelial growth factor
VLCFA	very long chain fatty acid
WT	wild type

Chapter 1

INTRODUCTION

Chapter 1: Introduction

1.1 Oral mucosa

All covering and lining tissues of the body are composed of two layers: a surface epithelium and an underlying fibrous connective tissue separated by a basal membrane. The primary function of the epithelium is to create a barrier from the external environment, protecting the underlying tissues and organs from mechanical force, dehydration and harmful chemicals and pathogens. The connective tissue provides mechanical support and nutrients for the epithelium. The oral mucosa, as well as the mucosa of the esophagus, is very different from the other mucosae of the body, for example the digestive tract. It has more similarities with the skin than with the intestinal mucosa (figure 1-1). The two layers of the oral mucosa are specifically called oral epithelium and lamina propria (the oral connective tissue) (figure 1-1B). Skin, oral mucosa, and esophagus are covered by a stratified epithelium (figure 1-1) composed of multiple layers of cells that show various patterns of differentiation (or maturation) between the deepest cell layer and the surface. The oral mucosa mainly consists of squamous (flat and thin) epithelial cells. The main cells are the keratinocytes, which are filled with cytokeratins and are tightly linked with each other by desmosomes. The covering tissue of the stomach and the intestines, on the other hand, consists of a simple epithelium with only a single layer of cells, which enables the absorption of the nutrients. In many regions of the oral cavity, such as the cheeks, the lips, and parts of the hard palate, there is a layer of a loose fatty or glandular connective tissue, called submucosa, under the lamina propria. This tissue contains the major blood vessels and nerves of the oral mucosa and determines the flexibility of its attachment to the underlying structures. In other areas of the oral mucosa, such as the gingiva and parts of the hard palate, the submucosa is absent, resulting in a firm and inelastic attachement of the oral mucosa directly to the surface of the bone (periosteum), which is called mucoperiosteum (Liu *et al*, 2010; Squier & Kremer, 2001). Many regions of oral and esophageal epithelium contain cell types other than the epithelial cells, including melanocytes, Langerhans' cells, Merkel

cells, and inflammatory cells such as lymphocytes. These cells are calculated to represent about 10% of the cell population in the oral epithelium. The melanocytes

A.   **B.**

epidermis

dermis

hypodermis

epithelium

basal layer

lamina propria

submucosa

macrophages

capillaries   fibroblasts

Figure 1-1. The structure of skin and oral mucosa

The diagrams show the main layers and tissue components of the skin and oral mucosa and illustrate the similarities but also the differences of the two types of mucosa.

(A) The diagram illustrates the structure of the skin that consists of the epidermis, the dermis and the hypodermis layer. The sweat glands, the hair follicles, nerves and blood vessels are also included in the picture (adapted from http://health.allrefer.com/pictures-images/skin-layers.html)

(B) The diagram depicts the structure of the oral mucosa consisting of the oral epithelium, the fibrous lamina propria (oral connective tissue), and the submucosa that includes the blood vessels and the nerves (modified from (Sonis, 2004).

(specialised pigment cells) produce melanin in melanosomes and are situated in the basal layer of the oral epithelium and the epidermis. Melanin contributes to the color of the oral mucosa together with the hemoglobin of the blood. Langerhans' cells sometimes appear in the suprabasal layers of epidermis and oral and esophageal epithelium. These are dendritic cells coming from the bone marrow and can move in and out of the epithelium and migrate to the lymph nodes. They are immune cells that recognise and process antigens that enter the epithelium, and present them to helper T lymphocytes. The Merkel cells are situated in the basal layer of the oral and esophageal epithelium and epidermis. The Merkel cells are sensory cells responding to touch. They are characterised by small, membrane-bound vesicles in their cytoplasm, sometimes adjoining to a nerve fiber. Finally, the transient presence of inflammatory cells has been reported in the nucleated cell layers. These cells can be lymphocytes (most frequently), macrophages, leukocytes or mast cells (Harmse *et al*, 1999; Squier & Kremer, 2001).

The soft tissues of the oral cavity and esophagus consist of a layer of a stratifying squamous epithelium. This epithelium serves to protect the underlying tissue from the mechanical forces of mastication, any possible fluid loss or harmful environmental agents entering the oral cavity with the food like microbial toxins, enzymes, pathogens and carcinogens. It is classified into i) the keratinized, ii) the non-keratinized and iii) the specialised epithelium. In areas that come in primary contact with food and are subject to mechanical forces associated with mastication, such as the gingival and the hard palate, there is a keratinized epithelium tightly attached to the underlying tissues by the collagenous lamina propria. These areas are called masticatory mucosa and are very similar to the epidermis of the skin. A non-keratinized epithelium covers other areas, like the floor of the mouth, the esophagus and the cheeks, providing them with the flexibility they require for talking, chewing or swallowing because of the elastic connective tissue of the lining mucosa. A specialised epithelium covers the upper side of the tongue, which is as a mixture of keratinized and non-keratinized epithelium attached tightly to the tongue muscle (Liu *et al*, 2010; Squier & Kremer, 2001). The distribution of the three different types of oral mucosa is shown in figure 1-2. The lining mucosa represents approximately 60% of the oral mucosa, the masticatory mucosa 25% and the specialised mucosa 15% (Collins & Dawes, 1987; Squier & Kremer, 2001).

Keratinized, stratified, squamous
Hard palate
Gingivae

Nonkeratinized, stratified, squamous
Soft palate
Floor of mouth
Cheek
Underside of tongue
Inside of lips

Keratinized and nonkeratinized, stratified, squamous
Tongue

Figure 1-2. The distribution of the different types of mucosa within the oral cavity

The detailed diagram shows the anatomic location and extent of masticatory (keratinized epithelium), lining (nonkeratinized epithelium) and specialised mucosa (keratinized and nonkeratinized epithelium) in the oral cavity (Wilson, 2008).

1.1.1 The epithelium

The epithelium of the masticatory mucosa (keratinized) contains four distinct layers: the basal layer (stratum basale), the spinal layer (stratum spinosum), the granular layer (stratum granulosum) and the surface cornified layer (stratum corneum) (figure 1-3). The cells of the basal layer are cuboidal or columnar. The basal cells are separated from the connective tissue by a membrane called basal lamina. Stratum spinosum is characterised by many layers of oval to polygonal cells. The stratum granulosum consists of cells that contain keratohyalin granules. Finally, the stratum corneum contains thin, flat cells without nuclei and filled with soft keratin (Avery, 2000; Berkovitz *et al*, 2009)

The epithelium of the lining mucosa (non-keratinized) is composed of three layers: the stratum basale, the stratum spinosum and the stratum superficiale. The cells of the stratum superficiale generally consist of flattened cells with small oval nuclei. The lamina propria of this mucosa is composed of the papillary and reticular layers (Avery, 2000; Berkovitz *et al*, 2009).

The tongue is covered with the specialised mucosa which contains 3 types of epithelial structures called papillae. The vast majority are filiform papillae, which are threadlike keratinized extensions of the epithelium. The other type is the fungiform papillae, which are a few mushroom-shaped structures near the tip of the tongue. There are only 10-14 circumvallate papillae located between the body and the base of the tongue. Finally 4-11 foliate papillae are located on the lateral posterior sides of the tongue and contain taste buds. The taste buds are barrel-shaped structures located among the papillae of the tongue although some also appear on the soft palate, epiglottis, larynx and pharynx. They are the sense organs of the chemical sense of taste (Avery, 2000; Berkovitz *et al*, 2009).

The different oral epithelium layers represent a progressive process of cell maturation. The cells are moving to the upper layers as they differentiate and mature, so cells from the surface layer are continuously removed and replaced by others. The

A.   **B.**

Figure 1-3. The structure of the oral mucosa

The diagram (A)(Avery, 2000) and a tissue section (B) (Berkovitz et al, 2009) show the structure of the oral mucosa and the different layers of the oral epithelium. For the tissue section the letters represent: A= stratum basale; B= stratum spinosum; C= stratum granulosum; D= stratum corneum.

turnover time varies depending on the region. The turnover time for the epithelia adjusted to the tooth surface is about 5 days, for the lining mucosa it is about double that time (10 days) and for the masticatory mucosa a little more than that (Berkovitz *et al*, 2009).

The feature of the epidermis and the oral epithelium that enables them to maintain tissue homeostasis, repair after injury and, also for the epidermis, regenerate hair, is stem cells. Many definitions of the stem cell have been suggested. Slack defines them as cells that are able to reproduce themselves and give rise to differentiated cells throughout the life span of the animal (Slack, 2000). The stem cells of the epidermis are located in the adult hair follicle, the sebaceous glands and also the basal layer of the epidermis. The skin tissue is very similar to the oral tissue, so the stem cells of the oral mucosa are most likely to be located in the basal layer of the epithelium. The stem cells were previously thought to constantly give rise to transit – amplifying (TA) cells which migrate to the surface, while undergoing terminal differentiation (Calenic *et al*, 2010; Fuchs, 2008; Potten, 1974) but this has recently been questioned (Jones *et al*, 2007; Lopez-Garcia *et al*, 2010). The exact location of these stem cells is not known, as there are no specific stem cell markers. There have been several attempts of development of stem cell markers like adhesion markers such as β1-integrins, β-catenin, keratins 15 and 19 which some researchers claim can be used to identify stem cells in the oral mucosa and the skin (Calenic *et al*, 2010; Izumi *et al*, 2000; Slack, 2000; Squier & Kremer, 2001). They are thought to reside mainly at the bottom of the epithelial ridges that project into the lamina propria were most of the dividing cells tend to appear (Berkovitz *et al*, 2009; Squier & Kremer, 2001). Cell colonies *in vitro* that have a high capacity of self-renewal are called holoclones. The clones progressively lose their clonogenic capacity and form paraclones, which eventually terminally differentiate and this may be related to stem cell ageing (Barrandon & Green, 1987; Gemenetzidis *et al*, 2010; Ressler *et al*, 2006).

1.2 Head and neck cancer

1.2.1 Frequency and risk factors

Head and neck cancer, including oral cancer, is the sixth most frequently occurring malignancy in the United Kingdom and worldwide. The most common type of head and neck cancer is squamous-cell carcinoma (HNSCC). More than half a million patients are diagnosed with HNSCC worldwide each year (Haddad & Shin, 2008). Despite the fact that oral cancer represents just 0.6% to 5% of the cancers in Western Societies, it is very prevalent in countries like India, where it accounts for nearly 45% of all cancers (Kirsch, 2007). Current therapeutic approaches like surgery, chemotherapy and radiotherapy often result in cytotoxic effects and development of resistance to therapy. Unfortunately, advances in current treatment have not led to a significant improvement in the patients' survival rates (Hsu *et al*, 2004; Hunter *et al*, 2005). Approximately 83% of the patients with cancer of the oral cavity and pharynx survive for 1 year. The 5 year and 10 year survival rates are 61% and 50% respectively (American Cancer Society, 2010).

The main risk factors for HNSCC are tobacco usage and alcohol consumption, accounting for approximately 80% of oral cancer cases in Western countries (Hunter *et al*, 2005), (Morita *et al*, 2010). Oral cancer shows an association to alcohol in never smokers and with tobacco smoking in moderate drinkers. Moreover, it is suggested that the two factors have a synergistic effect when combined. Heavy consumption of both alcohol and tobacco results in an over 48-fold increased risk in young people (Rodriguez *et al*, 2004). Smoking is a very severe problem, as more than 47 million adults and 4 million non-adults smoke cigarettes in the USA only. The rates of smoking have decreased significantly lately; however, it still represents a major health issue and it still causes severe chronic diseases and many deaths around the world (http://www.netwellness.org/healthtopics/smoking/). Diet characterised by low consumption of fruits and vegetables is another important factor implicated in the aetiology of HNSCC (Macfarlane *et al*, 1995). Other risk factors may be the use of marijuana and tobacco chewing in countries such as India and Indonesia, where chewing of betel quid is prevalent (Hunter *et al*, 2005). It has

also been suggested that human papilloma virus (HPV) has a role in HNSCC pathogenesis (Gillison *et al*, 2000; Mork *et al*, 2001) because viral DNA, mostly of HPV type 16 (HPV-16), has been found in tumour tissue and patients who are positive for HPV antibodies have an increased risk of HNSCC (D'Souza *et al*, 2007).

1.2.2 Oral pre-malignancies and genetic alterations

Patients with HNSCC develop a series of premalignant lesions (dysplasias) and malignant lesions. There are two main types of HNSCC premalignancies, which are called leukoplakia and erythroplakia. They can and should be identified by oral examination during regular visits to the dentist. Leukoplakias are white patches and erythroplakias are red patches in the mouth (figures 1-4) (Hunter *et al*, 2005). Erythroplakias are much more prone to progress to squamous cell carcinoma (SCC) (more than 50% in a decade) than leukoplakias (2–5% in the same period) (Mashberg, 1977). However, most patients present initially with carcinomas because of infrequent routine dental checkups, or the possibility that dysplasias are not recognisable macroscopically.

A. *B.*

Figure 1-4. Oral pre-malignant dysplasias

Photographs of (A) an area of leukoplakia on the floor of the mouth and part of the tongue and (B) an area of erythroplakia on the right tonsillar pillar (Hunter et al, 2005).

In HNSCC, chromosomal changes increase at each progression step, from benign hyperplasia to dysplasia, to carcinoma *in situ*, to invasive cancer (Hunter *et al*, 2005). Some of these mutations are mentioned here. The most common changes occur at chromosomes 9p (*CDKN2A*, encoding *INK4A* and *ARF*), 3p and 17p (*TP53*) (Califano *et al*, 1996). *TP53* is one of the earliest Tumour Suppressor Genes (TSG) discovered and it is involved in apoptosis and cell cycle regulation (Ha *et al*, 2008). The silencing of the *TP53* gene due to point mutations leads to tumour development in HNSCC (Somers *et al*, 1992). As regards chromosome 3p, the genetic changes that occur at two main regions in HNSCC are: a deletion at 3p14 which might be the site of the candidate suppressor gene, fragile histidine triad (*FHIT*) and the changes in the 3p21-p26 region, one of which might contain a telomerase-repressor gene (Cuthbert *et al*, 1999). Other genetic changes in oral dysplasias have also been identified at chromosome 8q24, which is probably the *MYC* locus (Garnis *et al*, 2004b), 11q13 which is probably the gene encoding cyclin D1, and 7p11 which is probably the epidermal growth factor receptor (*EGFR*) gene (Garnis *et al*, 2004a). *EGFR* is an oncogene which is overexpressed in the majority of HNSCC tumours. Loss of the *FAT* gene at 4q35 was also detected in HNSCC. FAT is thought to play a role in cell-cell adhesion within the cadherin family (Nakaya *et al*, 2007). In addition, 8-oxoguanine DNA glycosylase 1 (*OGG1*) is a DNA repair enzyme whose reduction in function has been shown to be a risk factor in head and neck cancer (Paz-Elizur *et al*, 2006). Another possible oncogenes involved in HSNCC include c-Jun NH2-terminal kinases (*JNK*), which are involved in T cell differentiation and apoptosis (Gross *et al*, 2007). Finally, it was reported that there is an accumulation of mitochondrial mutations in HNSCC (Ha *et al*, 2008).

1.2.3 Treatment, recurrence and 'field cancerisation'

Patients with stage I or II head and neck cancers are usually treated with surgery or radiation, while a combination of surgery, chemotherapy or radiation is used to treat advanced stage III or IV of the disease. Surgery is one of the main therapeutic

approaches and continues to evolve towards being as minimally invasive as possible. Furthermore, improved reconstruction and tissue transfer methods are being used. Radiotherapy is improving with the use of intensity-modulated radiation therapy which delivers radiation more precisely to the tumour than the normal surrounding tissues. Chemotherapy, e.g cisplatin, is usually used in advanced stages of the disease before radiotherapy, or in conjunction with radiation (concurrent chemo-radiotherapy). Finally, some targeted agents have been used in combination with radiotherapy or not. One of them is cetuximad, a monoclonal antibody which blocks EGFR and it is used in combination with radiotherapy or as a single agent in patients resistant to cisplatin (Haddad & Shin, 2008; Vermorken *et al*, 2007).

The biggest problem in HNSCC treatment is the recurrence of the tumours. The frequency of second primary tumours (SPTs) in patients with upper aerodigestive tract cancer is approximately 20%. Many of the secondary tumours that frequently develop throughout the head and neck region are genetically related (Hunter *et al*, 2005). More than 50 years ago, Slaughter *et al*, used the term 'field cancerisation' for the first time, in a study of 783 patients with oral cancer (Slaughter *et al*, 1953). He suggested that 'abnormal' or pre-neoplastic tissue exists somewhere in the oral mucosa of the patient. These abnormalities might be due to epigenetic changes induced in a field of cells, or due to spread of genetically altered cells within the oral cavity (Jang *et al*, 2001). Nowadays, field cancerisation is defined as the presence of fields consisting of genetically altered epithelial cells which are linked to the carcinogenesis process. Also, the field lesion has a monoclonal origin, and does not show invasive growth and metastatic behavior, the hallmark criteria of cancer (Braakhuis *et al*, 2003).

According to the 'carcinogenesis model', initially, a stem cell acquires one (or more) genetic alterations (such as *TP53* mutations) and forms a patch with genetically altered daughter cells. Subsequent genetic alterations result in the escape of the stem cell from normal growth and its development into an expanding clone. The patch gradually converts into a field into the normal epithelium. As the lesion becomes larger, the accumulation of additional genetic alterations gives rise to various subclones within the field which share a common clonal origin (Tabor *et al*, 2001). Eventually, a subclone evolves into invasive cancer which is called a second-

field tumour (SFT). Alterations in the *cyclin D1* gene, located at 11q13 were shown to be important for the progression from field to cancer (Izzo *et al*, 1998) (figure 1-5). The chance of an SFT to develop in a patient will be proportional to the number of affected stem cells and additional genetic hits.

Figure 1-5: Proposed model of HNSCC carcinogenesis

Firstly a patch develops, consisting of TP53-mutated cells (17p chromosomal location). Next the patch expands to a field, consisting of cells with cancer-related genetic alterations (the chromosomal locations of which are: 3p, 9p, 8p, 18q), which continues expanding. Finally the field progresses to cancer. The amplification of 11q13 is considered to be important for that transformation (Braakhuis et al, 2003).

The theories which have been proposed to explain the origin of multiple secondary tumours are the following (Braakhuis *et al*, 2003; Braakhuis *et al*, 2002): firstly, two tumours can develop independently and have a different molecular fingerprint. These are the SPTs. Secondly, the second tumour can develop from the first tumour (metastasis). Single cells or small clusters may migrate through the submucosa or are shed in the oral cavity at one place and re-grow at another (Califano *et al*, 1999). According to the third theory, multiple clonally related neoplastic lesions develop from a genetically altered field of the epithelium (Simon *et al*, 2001; Tabor *et al*, 2002). These tumours are called SFTs (figure 1-6).

Figure 1-6: The three theories of the development of second oral tumours

A. The new tumour develops independently from the previous one and is called second primary tumour (SPT).

B. The second tumour comes from the first tumour after cells or clusters are spread in the oral cavity.

C. Multiple clonally related tumours develop from a genetically altered field of the epithelium which expands, so these tumours are called second field tumours (SFTs).

(Braakhuis et al, 2002)

1.3 Cancer and nutrition

It has been reported that 30–40% of cancers are directly linked to nutrition (Weisburger & Horn, 1982). Since there is a direct link between diet and cancer incidence, several studies are trying to identify natural chemopreventive agents from nutritional sources. In addition to cancer chemoprevention strategy investigations, the development of effective therapeutic treatments for cancer is also essential. Current chemotherapeutic treatments are not selective for malignant cells and display cytotoxic effects on normal cells, decreasing the patients' quality of life (Constantinou *et al*, 2008). Recent evidence in the literature suggests that natural dietary products, such as polyunsaturated fatty acids, have antitumourigenic activities so they might be successful chemopreventive compounds and adjuvant agents for effective cancer treatments, when combined with chemotherapeutic drugs.

1.3.1 Long chain omega-3 polyunsaturated fatty acids

Fatty acids are essential for our body structure and metabolism. They are oxidised in the mitochondria producing energy. They can either be incorporated into phospholipids participating into plasma membrane structure or be packaged into triglycerides for storage and use (Molendi-Coste *et al*, 2010). Polyunsaturated fatty acids (PUFA), specifically, are important compounds in all organisms and display a large number of important biological functions. The most important PUFA series are the omega-3 and the omega-6 fatty acids. The omega-3 (or n-3) fatty acids refer to a class of PUFA which have the first double bond in the n-3 position (three carbons from the methyl end of the carbon chain). Omega-3 fatty acids must be obtained from the diet since they cannot be synthesised by mammals (Biondo *et al*, 2008). Omega-3 fatty acids, like alpha-linolenic acid, are found in soybeans, walnuts, dark green leafy vegetables, seeds and their oils (flaxseed, mustard seed and canola oil) (Berquin *et al*, 2008). Dietary omega-3 fatty acids include the cis-5,8,11,14,17-eicosapentaenoic acid (EPA) which contains 20 carbon atoms and five double bonds

(20:5n-3) and the cis-4,7,10,13,16,19-docosahexaenoic acid (DHA) which contains 22 carbon atoms and 6 double bonds (22:6n-3) (figure 1-7).

A.

DHA   EPA

B.

DHA

C22:6n-3 (docosahexaenoic acid)

EPA

C20:5n-3 (eicosapentaenoic acid)

Figure 1-7. The structure of DHA and EPA

(A) The 3-dimensional models of DHA (http://www.3dchem.com/molecules.asp?ID=23) and EPA (http://www.3dchem.com/molecules.asp?ID=239) and (B) the chemical structure of DHA and EPA (Biondo et al, 2008).

These long chain omega-3 PUFA are contained in oily cold-water fish such as salmon, trout, mackerel, anchovies and sardines. Fish take DHA and EPA from phytoplankton and zooplankton. The amount of omega-3 fatty acids in fish vary widely depending on the type of fish and habitat in which they live. Generally, higher concentrations of EPA and DHA are found in sardines, salmon, mackerel, herring and rainbow trout (Table 1-1) (Larsson *et al*, 2004).

FISH	total fat g/100 g	α-LNA	EPA	DHA	n-3/n-6
		g/100 g (% of total fatty acids)			
Cod, Atlantic	0.7	traces	0.06 (13.2)	0.17 (34.4)	11.11
Haddock	0.6	traces	0.05 (12.2)	0.10 (24.4)	7.67
Herring, Baltic	9.3	0.29 (3.5)	0.56 (6.7)	0.83 (9.9)	2.94
Herring, Pacific	18.5	0.32 (1.9)	1.03 (6.2)	1.63 (9.8)	5.88
Mackerel, Atlantic	16	0.29 (2.0)	0.89 (6.2)	1.56 (10.8)	7.14
Perch, all varieties	1.3	0.01 (1.6)	0.08 (8.7)	0.19 (21.4)	4
Pike	0.7	0.01 (1.1)	0.04 (7.6)	0.16 (33.0)	7.14
Salmon, Atlantic	12	0.18 (1.7)	0.49 (4.5)	1.33 (12.3)	3.85
Salmon, Pacific	5.2	0.05 (1.1)	0.63 (13.5)	0.88 (18.9)	16.67
Sardines (in tomato sauce)	14.8	0.22 (1.6)	1.24 (8.8)	1.77 (12.6)	11.11
Trout, rainbow	9.6	0.15 (1.7)	0.60 (7.0)	1.76 (20.4)	5.26
Tuna, in water	1.2	0.01 (1.6)	0.09 (11.3)	0.16 (19.4)	6.67

Table 1-1. Omega-3 fatty acid composition of fish from different species or geographical areas

Amounts of total fat (fatty acids), a-linolenic acid (LNA), EPA, DHA, and ratios of omega-3 (n-3) to omega-6 (n-6) fatty acids in different species of fish or fish from a different habitat are shown. The percentage of total fatty acids are shown in parentheses and traces ≤ 0.005 g/100 g (Larsson et al, 2004).

1.3.2 PUFA biosynthesis and transport

Humans are able to produce EPA and DHA from the omega-3 fatty acid α-linolenic acid (LNA), which serves as a precursor. The omega-3 and omega-6 PUFA biosynthetic pathways (figure 1-8) are characterised by a series of chain desaturation and elongation steps in the endoplasmic reticulum, until 24:5n-6 and 24:6n-3. In more detail the omega-3 biosynthetic pathway starts with the desaturation of LNA followed by elongation. Then Δ5-desaturase catalyses another desaturation that produces EPA, which is further elongated, and after another desaturation produces 24:6n-3. Then, 24:5n-6 and 24:6n-3 are translocated to the peroxisome, where the chains are shorted by C2 by one cycle of the β-oxidation pathway to form 22:5n-6 and 22:6n-3 (DHA), respectively, which are translocated back to the endoplasmic reticulum for esterification into aminophospholipids (Dyall & Michael-Titus, 2008). However, only 5–10% of LNA is converted to EPA (Jump, 2002), so EPA and DHA are acquired mainly through dietary consumption.

PUFA have the ability to signal through G-protein-coupled receptors (GPCRs), causing insulin release and the toll-like receptors (TLRs) of the immune system in some types of cells and tissues (Doege & Stahl, 2006; Li, 2004; Steneberg *et al*, 2005). However, they generally need to cross the plasma membrane in order to elicit their diverse effects. The uptake of fatty acids from the circulation into cells includes the following steps: adsorption, transmembrane movement and desorption. In more details (1) the PUFA are generated, mostly through hydrolysis of triglyceride (TG)-rich lipoproteins by lipases inside the endothelial lumen, and then the majority of them bind to albumin. Next (2) they must dissociate from albumin in order to bind to the plasma membrane proteins or integrate into the lipid bilayer and (3) be transported across the plasma membranes, into the cytoplasm (4) where they are associated with fatty acid binding proteins (FABPs) and long chain fatty acyl-CoA binding proteins (ACBPs) (Abumrad *et al*, 1998; Storch & Thumser, 2000). The mechanism by which fatty acids (FA) cross the plasma membrane is not yet completely clear. There has been a debate about this for several years and different ways of transfer have been proposed.

Omega-6   Omega-3

LA	18:2n-6	18:3n-3	LNA

Δ6 Desaturase

GLA 18:3n-6   18:4n-3

Elongase

DGLA 20:3n-6   20:4n-3

Δ5 Desaturase

AA 20:4n-6   20:5n-3 EPA

Elongase

Endoplasmatic Reticulum 22:4n-6   22:5n-3 DPA

Elongase

24:4n-6   24:5n-3

Δ6 Desaturase

24:5n-6   24:6n-3

Translocation

Peroxisome 24:5n-6   24:6n-3

β-Oxidation

22:5n-6   22:6n-3 DHA

Translocation

Endoplasmatic Reticulum 22:5n-6   22:6n-3

Figure 1-8. Omega-3 PUFA biosynthesis and dietary sources

A summary of the biosynthetic pathways of omega-3 and omega-6 PUFA (modified from (Dyall & Michael-Titus, 2008) where LA= Linoleic acid, GLA= Gamma-linolenic acid, DGLA=Dihomogamma-linolenic acid, AA=Arachidonic acid, DPA=Docosapentaenoic acid and LNA= Alpha-linolenic acid.

At first, it was suggested that PUFA are transferred through the lipid bilayer by passive diffusion (flip-flop), without any involvement of protein mediators (figure 1-9) (Hamilton *et al*, 2002; Hamilton & Kamp, 1999). However, it was demonstrated that protein-mediated transport takes place in tissues with high PUFA metabolism and storage such as skeletal muscle, adipose tissue, liver, and heart (Doege et al, 2006; Doege & Stahl, 2006; Schaffer & Lodish, 1994; Stremmel, 1989) (figure 1-10). The crucial role of proteins for efficient PUFA uptake has been supported by a number of knockout (KO) or over-expression model systems with impaired or enhanced fatty acid (FA) transport (Binas *et al*, 1999; Martin *et al*, 2003; Newberry *et al*, 2006). The occurrence of both PUFA uptake mechanisms is now widely accepted and is likely to be dependent on different cell types and tissues. Several PUFA carrier- proteins have been proposed, such as FA translocase (FAT/CD36) (Coburn *et al*, 2001), FABPs (Storch & McDermott, 2009; Storch & Thumser, 2010), long chain fatty acyl-CoA synthetases (ACSL) (Gargiulo *et al*, 1999), ACBP (Knudsen *et al*, 2000) and FA transport proteins (FATPs) (Doege & Stahl, 2006). Moreover, lipid rafts are also involved in FA influx and efflux (Ehehalt *et al*, 2006).

The family of FABPs are intracellular proteins which are expressed in many tissues having tissue specific homologs. In humans nine FABPs have been identified (Storch & McDermott, 2009; Storch & Thumser, 2010). All FABPs have a high affinity binding site for a saturated or unsaturated long-chain PUFA, except from liver FABP (LFABP), which binds two FAs (Storch & Thumser, 2010). Tissues that exhibit high rates of FA uptake and metabolism appear to have higher expression of FABPs. The large diversity of FABPs correlates with the diversity of their functions in the different types of tissues (Storch & McDermott, 2009; Storch & Thumser, 2010). *In vitro* studies revealed that different FABPs transfer FA to membranes by two different ways. The majority of FABPs, such as the adipocyte, keratinocyte, intestinal, brain, myelin, and heart/muscle types, transfer their FA by direct interaction with the membrane, while LFABP transfers their ligand to and from membranes by aqueous phase diffusion (Storch & Thumser, 2000). LFABP has been hypothesised to be involved in lipid absorption by the enterocyte and hepatocytes. *Lfabp* KO mice show a defect in FA β-oxidation, which might represent a defect in FA transport (Martin *et al*, 2003). Adipocyte FABPs (AFABP) and the keratinocyte FABP (KFABP) are both expressed in adipocytes and macrophages.

Figure 1-9. Uptake of PUFA into the cells

In order to cross the plasma membrane, PUFA can diffuse through the lipid bilayer or be transported by a protein. Free diffusion occurs by flip-flop of the un-ionised form of free FAs (FFA) across the plasma membrane. As regards the protein-mediated transport, the anionic form of FFAs binds to a transmembrane protein either from the external phase or via the membrane lipid phase (Hamilton & Kamp, 1999).

KFABP is also expressed in skin, liver, brain, lung, and cancerous tissue (Storch & McDermott, 2009). KFABP binds not only fatty acids (FA) but also retinoic acid (RA) which may explain its suggested role in cancer. RA can either inhibit cell growth by binding to the nuclear RA receptor (RAR); or promote cell growth by binding and activating PPARβ/γ (Schug *et al*, 2007). RA binding to either PPARβ/γ or RAR depends on the ratio of KFABP/cellular RA binding protein II (CRABPII). When the ratio is high, RA activates PPARβ/γ, when the ratio is low it results in

RAR activation. In a breast cancer mouse model resistant to RA, when the KFABP-CRABPII ratio in mammary tissue decreased, then RA was diverted from PPARβ/γ to RAR and suppressed tumour growth (Schug *et al*, 2008; Storch & McDermott, 2009). The small intestine is responsible for the absorption of the dietary lipids and the reuptake of bile acids via the enterohepatic circulation (Storch & Thumser, 2010). Small intestinal enterocytes express IFABPs (intestinal FABPs) and LFABPs. *Ifabp*$^{-/-}$ mice showed increased triacylglycerol levels in serum, weight gain, hepatic steatosis, and insulin resistance demonstrating the role of IFABP in lipid processing, and suggesting its protective role against metabolic syndrome (Newberry *et al*, 2006). In muscle tissue, the most important FABP is HFABP (heart FABP) (Storch & Thumser, 2010). The central nervous system expresses also HFABP in the adult brain, whereas BFABP (brain FABP) and KFABP are expressed in the pre- and perinatal whereas myelin FABP (FABP8) is found predominantly in the peripheral nervous system (Storch & Thumser, 2010). The HFABP plays an important role in the transport and metabolism of FA, as *Hfabp-/-* mouse showed dramatic reduction in FA uptake into the heart and skeletal muscle and reduced muscle FA oxidation (Binas *et al*, 1999).

The family of FATPs consists of six members (FATP1-6) that are similar in humans and mice. There is an ongoing debate about the function of FATPs: 1) FATPs might be just transmembrane FA transport proteins that associate with other proteins, such as ACSLs, or 2) membrane-bound long-chain (LC) and very-long-chain (VLC) acyl-CoA synthetases which role is to trap long chain FA in the cytoplasm after FA diffusion across the plasma membrane, or 3) combine both the transport function with the acyl-CoA synthetase activity for optimal uptake, or, 4) are multifunctional proteins that also mediate long chain FA transport (Doege & Stahl, 2006). FATP1 is mainly expressed in adipose tissue but also found in skeletal muscle and, to a lesser extent, in the heart. Heart-specific overexpression of FATP1 in a transgenic mouse model caused eightfold increase in FATP1 expression in heart muscle, long chain fatty acid (LCFA) accumulation, increased cardiac lipid metabolism and, finally, lipotoxic cardiomyopathy (Chiu *et al*, 2001). On the other hand, FATP1 deletion *in vivo* caused reduced triglyceride accumulation and dietary FA deposition in skeletal muscle and adipose tissue and also demonstrated that FATPs are predominantly involved in the uptake, but not in the export of the LCFA (Wu *et al*, 2006). FATP2 is

found almost exclusively in liver and kidney while FATP3 shows a broader expression pattern, with higher expression levels in the lung (Stahl, 2004). FATP4 is the only FATP protein in the small intestine localised at the area responsible for the absorption of dietary lipids. FATP4 is also expressed in adipose tissue, brain, liver, skin, and heart (Doege & Stahl, 2006). FATP4 deletion studies demonstrate the importance of this protein for early development and skin lipid metabolism, which will be discussed later (chapter 7). Also, FATP4 is involved in fat absorption in early embryogenesis, while its depletion results in early embryonic lethality in mice (Cunningham & McDermott, 2009; Gimeno *et al*, 2003). FATP5 is expressed only in the liver and exhibits FA transport activity *in vitro*. Long chain FA uptake in primary hepatocytes isolated from FATP5 KO mice is reduced by 50% (Doege *et al*, 2006). Finally, FATP6 is expressed predominately in the heart (Stahl, 2004).

Work *in vitro* and *in vivo* has provided evidence that FAT/CD36 (CD36) has a role in FA transport. CD36 is expressed and involved in FA transport in various tissues such as adipose tissue, stomach, upper intestine, heart and skeletal muscle (Coburn *et al*, 2001) and also have been found in placental tissues extracts (Cunningham & McDermott, 2009; Larque *et al*, 2006). Also CD36 is co-expressed and co-regulated with FABP in various tissues (Spitsberg *et al*, 1995). FAs induce CD36 expression in preadipocytes (Amri *et al*, 1995) and in neonatal cardiomyocytes (van der Lee *et al*, 2000) and in the heart of mice fed a high-fat diet (Heuckeroth *et al*, 1987). Transgenic mice with CD36 overexpression in muscle (MCK/CD36) have less body fat, and lower serum FAs, triglycerides and cholesterol (Ibrahimi *et al*, 1999). FA uptake by heart, skeletal muscle, and adipose tissues from CD36-null mice is significantly reduced (50–80%) while that of glucose is highly increased (Febbraio *et al*, 1999).

ACSL (long chain fatty acyl-CoA synthetase) catalyses the esterification or activation of long-chain FAs (10–20 carbons) (Gargiulo *et al*, 1999). In this initial step, free FAs are quickly activated and coupled to coenzyme A (CoA) by the catalysis of ACSLs or by FATPs. ACBP or FABPs facilitate the intracellular unloading of the transporters and the synthetases and can also function as an intracellular FA buffer (Doege & Stahl, 2006). The acyl-CoA esters are later substrates for β-oxidation, phospholipid and triglyceride biosynthesis (Gargiulo *et al*,

1999). ACS1 is highly expressed in the heart and is also found at the plasma membrane of cultured adipocytes, where it functions in coordination with FATP1 to facilitate long chain FA movement across the plasma membrane of mammalian cells (Gargiulo *et al*, 1999). Transgenic mice with overexpression of ACSL in the heart showed marked cardiac myocyte triglyceride accumulation and developed cardiomyopathy (Chiu *et al*, 2001). ACBP (long chain fatty acyl-CoA binding protein) is an 86–103 residue protein with a highly conserved amino acid sequence. In mammals, l-ACBP is predominately found in the liver, evenly distributed in all hepatocytes and also expressed in other high energy metabolism tissues, such as steroid-producing cells of the adrenal cortex and testis (Bovolin *et al*, 1990; Knudsen *et al*, 2000). l-ACBP binds medium- and long chain acyl-CoA esters with very high affinity, with a preference for C:14-C:22 acyl-CoA esters (Faergeman *et al*, 1996). The second isoform is the testis specific t-ACBP and the third one is the brain specific b-ACBP. The fourth group of ACBP is a group of longer sequences, with up to 533 amino acids. Some of these longer sequences are suggested to be membrane-bound ACBP domain proteins (Knudsen *et al*, 2000). Generally, ACBP is thought to act as an intracellular acyl-CoA transporter and affect FA-mediated regulation of gene expression (Knudsen *et al*, 2000).

1.3.3 The role of PUFA in disease

Long chain PUFA are essential dietary components and they play a significant role in a variety of physiological processes. As mentioned before, they are involved in plasma membrane synthesis and metabolic energy production and storage, and also affect gene expression (Doege & Stahl, 2006; Jump & Clarke, 1999). Moreover, they influence innate immune response through TLR signalling (Li, 2004) and cause insulin release via GPCR activation (Steneberg *et al*, 2005). PUFA play a highly significant role in foetus development. DHA is essential for the development of the brain and retina of the foetus making it a necessary component of the pregnant women's diet (Cunningham & McDermott, 2009; Herrera, 2002).

Figure 1-10. A model for the protein mediated PUFA transport

Extracellular PUFA might bind to FATP (FA transport protein) and be transported into the cell or could firstly bind to CD36 which transfers them to FATP dimers. When PUFA reach the cytoplasm, they are coupled to coenzyme (CoA) by ACSL (long chain fatty acyl-CoA synthetase) which prevents their efflux. FABPs (FA binding proteins) act as a cytoplasmic buffer by incorporating the intracellular PUFA (Stahl, 2004).

Not only the absolute intake of omega-3 fatty acids but also the ratio of omega-6 to omega-3 fatty acids plays a significant role in general health. Lately, the total fat intake and the ratio of omega-6 to omega-3 fatty acids have increased in the Western diet. This ratio is between 15–20 : 1 in western Europe and the USA, whereas during evolution it was 1 : 1 or even less (Simopoulos, 2004). Because of the physiological significance of long-chain PUFA, lipid imbalances can cause a plethora of abnormalities and pathologies, like hyperlipidemia, obesity, type 2 diabetes, non-alcoholic fatty liver disease (NAFLD), cardiovascular problems and cancer (Kushi & Giovannucci, 2002; Lichtenstein *et al*, 1998; Marchesini *et al*, 2001; Masuzaki *et al*, 2001; Saltiel & Kahn, 2001). All these pathologies could be summarised by the term of metabolic syndrome (MetS) which has reached epidemic proportion nowadays (Molendi-Coste *et al*, 2011).

This also means that omega-3 fatty acids may play an important role in decreasing the incidence and development of these diseases, including cardiovascular, inflammatory, neurodegenerative and immune disorders, and cancer. There is an inverse correlation between diets high in fish and fish oil and coronary heart disease (Madsen *et al*, 2001) and biomarkers of inflammation like tumour necrosis factor-α (TNFα) and interleukin-6 (IL-6) (Lopez-Garcia *et al*, 2004; Zhao *et al*, 2004). Human trials have confirmed that PUFA supplements significantly reduce triglyceride levels in MetS patients (Mattar & Obeid, 2009) and lower the mortality of patients with cardiovascular problem or MetS (Ebrahimi *et al*, 2009). Recently, it was also shown that DHA and EPA signal through GPR120, mediating anti-inflammatory effects by inhibiting both TLR and TNF-α pro-inflammatory signalling pathways. Since chronic tissue inflammation is related to insulin resistance in obesity, a GPR120 knock out model was used to demonstrate that the anti-inflammatory action of omega-3 PUFA also led to insulin sensitising effects *in vivo* (Oh *et al*, 2010). Moreover PUFA play an important role in the immune system development from the fetal stage (Enke *et al*, 2008). As mentioned, PUFA decrease the production of pro-inflammatory cytokines like IL-1 and TNF, having an important protective effect against inflammation and immune disorders like asthma, rheumatoid arthritis, inflammatory bowel disease (Crohn's disease and ulcerative

colitis) and psoriasis (Simopoulos, 2002). The first evidence for this was the observation of the low incidence of autoimmune and inflammatory disorders in a population of Greenland Eskimos compared to a population from Denmark (Kromann & Green, 1980; Simopoulos, 2002). Generally, Greenland Eskimos and the Japanese have a diet enriched in omega-3 PUFA from seafood which leads to a low incidence in inflammatory, autoimmune and heart diseases (Hirai *et al*, 1980; Simopoulos, 2002).

PUFA are also involved in neurological diseases as the nervous system and especially the brain are enriched in PUFA and need high levels of the omega-3 and omega-6 fatty acids in order to function normally (Dyall & Michael-Titus, 2008). The accretion of DHA during development is essential for preventing neuronal cell death and supports neuronal differentiation. Also, DHA is crucial for neuronal and retinal functions (Kim, 2007). It was suggested that there is a relationship between higher levels of omega- 3 PUFA and a decreased appearance of dementia and cognitive decline with age (Johnson & Schaefer, 2006; Kalmijn *et al*, 2004). Specifically, there is evidence that links DHA and EPA levels and Alzheimer's disease (Corrigan *et al*, 1998) and a decrease in DHA levels correlate with the severity of the disease (Tully *et al*, 2003). In animal models, dietary omega-3 PUFA depletion caused decrease of NR2B, which is a receptor associated with age-related loss of memory and learning ability (Calon *et al*, 2005) while a diet enriched in DHA and EPA could reverse the decrease in NR2B (Dyall *et al*, 2007). Moreover, there are studies which suggest that PUFA may be beneficial in other neurological diseases, like Parkinson's disease, Huntington's disease and multiple sclerosis (reviewed in Dyall & Michael-Titus, 2008). Omega-3 PUFA also show a significant neuroprotective and neuroregenerative effects after acute neurological injury such as spinal cord injury. DHA administrated intravenously 30 min after injury, caused a significant reduction of the inflammation and improved neuronal survival and mobility (Huang *et al*, 2007; King *et al*, 2006).

1.3.4 PUFA in cancer: *in vivo, in vitro* and human studies

PUFA appear to have a great potential to become useful prophylactic and therapeutic tools in cancer. EPA and DHA have been observed to induce apoptosis in human cancer cell lines including breast cancer (Schley *et al*, 2005) colon cancer (Chen & Istfan, 2000; Eitsuka *et al*, 2005) and lymphoma cell lines (Heimli *et al*, 2002). The pro-apoptotic effect of omega-3 PUFA has also been observed in a number of other types of cancer cells such as leukemia cells (Finstad *et al*, 1998) lung, pancreatic and prostate cancer cell lines (Edwards *et al*, 2008; Merendino *et al*, 2005; Serini *et al*, 2008), and different molecular mechanisms of action have been proposed.

Animal studies provide evidence that there is a negative relationship between diets rich in omega-3 PUFA and respectively a positive relationship between diets with a high proportion of omega-6 PUFA and breast, prostate, and colon cancer (Edwards & O'Flaherty, 2008; Tsubura *et al*, 2009). Fish oil with high levels of omega-3 PUFA, such as EPA and DHA, reduced the risk of mammary and colon carcinogenesis in an animal model (Bartsch *et al*, 1999). EPA and DHA suppressed mammary and colon cancer cell growth (Yuri *et al*, 2003) and the incidence and multiplicity of these specific types of cancers were lower in rats fed a diet high in EPA (Minoura *et al*, 1988; Sakaguchi *et al*, 1984). Moreover, dietary EPA and DHA suppressed breast tumour growth and lung metastasis in athymic mice transplanted with human breast cancer cells (Rose *et al*, 1996). EPA-enriched diet appeared to suppress liver metastasis of ACL-15 colon carcinoma cells (Iwamoto *et al*, 1998). In rats orally administrated with PUFA, DHA in particular, but also EPA, significantly decreased lung metastasis compared with control rats (Suzuki *et al*, 1997). In xenograft models of prostate cancer, diets rich in omega-3 PUFA inhibited tumour growth (Kobayashi *et al*, 2006).

Recently, Kang *et al*, generated a *fat-1* transgenic mouse expressing the Caenorhabditis elegans *fat-1* gene which is absent in mammals and encodes for an omega-3 fatty acid desaturase that converts omega-6 to omega-3 fatty acids (Kang *et al*, 2004). The *fat-1* transgenic mice are capable of producing omega-3 fatty acids from the omega-6 type, causing abundant omega-3 fatty acids and reduced levels of

omega-6 fatty acids in their organs and tissues, without the need of a specific diet. The *fat-1* transgenic mouse is now being used widely and is a novel tool for studying the benefits of omega-3 fatty acids and the molecular mechanisms of their action. Several studies with *fat-1* mice demonstrated that an increased tissue status of omega-3 fatty acids, as well as decreased omega-6/omega-3 ratio, protect against inflammation. DSS-induced colonic inflammation was significantly less severe in *fat-1* than wild type (WT) mice which correlated with enhanced formation of anti-inflammatory derivatives of omega-3 fatty acids and down-regulation of pro-inflammatory factors/cytokines (Hudert *et al*, 2006). Also, protection from inflammatory injury was observed in chemically-induced hepatitis *fat-1* mice (Schmocker *et al*, 2007). *Fat-1* mice with implanted mouse melanoma B16 cells showed a striking reduction in melanoma development and progression. The levels of omega-3 fatty acids and their anti-inflammatory metabolite PGE3 were much higher but the omega-6/omega-3 ratio was much lower in the tumour and surrounding tissues of *fat-1* mice compared to WT. Also, the tumour suppressor PTEN gene was up-regulated in the *fat-1* mice. These results were confirmed *in vitro* (Xia *et al*, 2006). It was also demonstrated that *fat-1* transgenic mice had lower incidence and growth rate of colon tumours (Nowak *et al*, 2007) and reduced risk of breast cancer (Liu *et al*, 2007b; Ma *et al*, 2006). Furthermore, a *Pten*-knockout plus *fat-1* mice suppressed tumour growth and had an extended lifespan compared to *Pten*-knockout mice (without *fat-1*) (Berquin *et al*, 2007).

Several human studies have shown that consumption of a diet enriched in omega-3 PUFA may offer protection against a number of malignancies, including breast (Sasaki *et al*, 1993), prostate (Terry *et al*, 2004) and colon cancer (Yang *et al*, 2003). Many of these studies have relied on estimates based on national consumption or dietary intake information from self-reported questionnaires. However, some of them have used the levels of fatty acid in tissues as a measure of exposure to dietary fats (Edwards & O'Flaherty, 2008). The large study of Simonsen et al. provided evidence that the balance between omega-3 and omega-6 PUFA probably plays a role in breast cancer. Adipose tissue from breast cancer patients and healthy donors was examined, and showed that the ratio of long chain omega-3 to omega-6 PUFA was inversely associated with breast cancer in four out of five centers (Simonsen *et al*, 1998). When human prostate tissue was examined, it was demonstrated that lower

levels of EPA and DHA, as well as lower omega-3 to omega-6 PUFA ratios were associated with cancer compared to benign prostate hyperplasia (Mamalakis *et al*, 2002). These results were supported by the analyses of fatty acid profiles in serum from patients with prostate disease (Yang *et al*, 1999). In pancreatic cancer patients, 3 months of dietary supplementation with EPA and DHA led to a significant weight gain, accompanied by a stabilization of resting energy expenditure (Wigmore *et al*, 1996). Additionally, 60 patients with generalised solid tumours were given dietary supplements containing either fish oil or a placebo daily until death. Omega-3 PUFA restored the immunodeficiency of the patients and prolonged their survival (Gogos *et al*, 1998).

Several studies have also demonstrated that omega-3 PUFA are able to sensitise tumour cells to anticancer drugs *in vitro* and in cancer animal models. *In vitro*, DHA and EPA improved the cytotoxic effects of several anticancer chemotherapy agents in breast, colon, bladder, neuroblastoma, and glioblastoma human cancer cell lines (Biondo *et al*, 2008; Lindskog *et al*, 2006; Maheo *et al*, 2005). In animal cancer models, chemotherapy in combination with dietary supplementation of DHA and EPA decreased tumour size, prolonged survival and reduced side effects (Cha *et al*, 2002; Colas *et al*, 2006; Hardman *et al*, 2002). Few clinical trials have been conducted to examine the effects of omega-3 PUFA on tumour response to chemotherapy with human cancer patients. EPA and DHA enriched supplements consumed by patients with advanced colorectal cancer had as a result an increase in body weight and energy levels during chemotherapy (Read *et al*, 2007). Finally, breast cancer patients with higher concentrations of DHA in breast adipose tissue at the time of cancer diagnosis were reported to respond better to chemotherapy (Bougnoux *et al*, 1999). The phase II clinical trial evaluated the safety and efficacy of the addition of 1.8 g DHA daily to an anthracycline-based chemotherapy in breast cancer patients. Median overall survival was 22 months and reached 34 months in the sub-population of patients with the highest plasma DHA incorporation. They concluded that DHA during chemotherapy was devoid of adverse side effects and could improve the outcome of chemotherapy when it is highly incorporated supporting its potential to specifically chemosensitise tumour (Bougnoux *et al*, 2009).

1.3.5 Molecular mechanisms involved in the effects of PUFA

Several molecular mechanisms have been reported to be involved in the apoptotic effect of PUFA in cancer cells. PUFA have been reported to increase the levels of the pro-apoptotic proteins Bak and Bcl-xS and reduce those of the anti-apoptotic proteins Bcl-2, Bcl-xL (Calviello *et al*, 2005; Danbara *et al*, 2004) in colon cancer cells. Also, PUFA have been reported to increase the activity of caspase-3 and -9 in lymphoma cells (Heimli *et al*, 2002). Moreover they increased the expression of both caspases 8 and 9 and cytochrome c release from mitochondria in leukaemia (Arita *et al*, 2001). This demonstrates that omega-3 PUFA can cause apoptosis through activation of caspase-8 of the extrinsic pathway as well as the intrinsic mitochondrial pathway.

Apart from the effect of PUFA on mediators of apoptosis, they also affect several signalling pathways. Ras plays a key role in tumour development and progression, since Ras proteins (H-Ras, K-Ras, N-Ras) promote cell growth, survival and resistance to apoptosis. Several studies have reported effects of omega-3 PUFA on Ras signalling, such as decreased H-ras activity and levels, and decreased Ras localization to the plasma membrane (Collett *et al*, 2001). It has been also suggested that PUFA can promote apoptosis through the mitogen-activated protein kinase (MAPK) pathway, which is linked to Ras signalling, causing the up-regulation of MAPK phosphatase 1 (MKP-1) and down-regulation of phospho-extracellular signal regulated kinases 1/2 (p-ERK1/2) and p-p38 expression (Serini *et al*, 2008).

The phosphatidylinositol 3-kinase (PI3K) pathway regulates a number of cellular functions including proliferation, apoptosis, differentiation, and its activity is elevated in many types of human cancer. Akt is a primary mediator of the PI3K signalling pathway. EPA and/or DHA have been reported to prevent Akt phosphorylation and activity in several cell types (Lee *et al*, 2003; Schley *et al*, 2005).

Long-chain omega-3 PUFA also decrease NFκB activity and expression (Narayanan *et al*, 2003; Schley *et al*, 2005). Active NFκB promotes cellular survival and inhibits

apoptosis, promoting tumourigenesis. It has been suggested that these fatty acids modulate upstream signalling involved in the activation of NFκB, such as Akt activation (Lee *et al*, 2003; Schley *et al*, 2005), TNF-α signalling (Novak *et al*, 2003; Weber *et al*, 1995), phospholipase C activation (Weber *et al*, 1995) and IκBα phosphorylation (Novak *et al*, 2003). Moreover, it has been reported that down-regulation of COX-2 may be a crucial mechanism underlying the apoptotic effect of omega-3 PUFA in colon cancer cells (Narayanan *et al*, 2004).

In addition, it has been suggested that omega-3 PUFA may enhance the efficacy of chemotherapy drugs by increasing the reactive oxygen species levels, thus causing elevated apoptosis (Sturlan *et al*, 2003).

Furthermore, it has been proposed that the omega-3 PUFA inhibit telomerase in cell-free assays and *in vitro* systems, using DLD-1 human colorectal adenocarcinoma cells. Telomerase is the enzyme that maintains the ends of mammalian chromosomes inhibiting senescence and apoptosis and consists of a catalytic subunit called *hTERT* and an RNA component, *hTERC*. Omega-3 PUFA appeared to inhibit the expression of the *hTERT* gene, in parallel with *CMYC* and protein kinase C (*PKC*) (Eitsuka *et al*, 2005).

In summary, there are many different signalling pathways potentially linked to the effect of omega-3 PUFA and various suggested mechanisms which may be cell type and/or context dependent. These mechanisms involved in the effect of omega-3 PUFA are mentioned in Chapters 4- 6 in more detail.

1.3.6 Omega-3 PUFA derivatives

AA and EPA are the precursors of lipid mediators that consist of 20 carbon atoms and are called eicosanoids. These are the prostaglandins (PG), thromboxanes (TX), prostacyclins (PGI) and leukotrienes (LT). The omega-6 AA is the precursor of the 2-series of prostanoids (PG, TX and PGI) and the 4-series of leukotrienes. The omega-3 EPA and DHA are precursors of the 3-series of prostanoids and the 5-series of leukotrienes (figure 1-11) (Simopoulos, 1991).

Cyclooxygenase (COX) enzymes catalyse the conversion of AA to prostanoids such as prostaglandins and thromboxane A_2. Leukotrienes are biosynthesised through the lipoxygenase (LOX) pathway. Generally, the majority of the eicosanoids produced by AA are pro-inflammatory and cancer-promoting. PGE2 produced by AA is pro-inflammatory and has a predominant role in promoting cancer (Wang & Dubois, 2010). It is the most abundant prostaglandin found in human tumours like colon (Rigas *et al*, 1993), lung (McLemore *et al*, 1988), breast (Wang & Dubois, 2004) and HNSCC (Camacho *et al*, 2008). Moreover, AA-derived LTB4 and LTD4 promote tumour growth and metastasis (Wang & Dubois, 2010). LTB4 is increased in human colon (Dreyling *et al*, 1986) and prostate cancer (Larre *et al*, 2008), and inhibition of LTB4 synthesis by an LT4 hydrolase inhibitor reduced oesophageal adenocarcinoma in a rat model (Chen *et al*, 2003). The CysLT1 receptor expression is increased in human colon and prostate cancers, which correlates with the ability of LTD4 to induce proliferation and inhibit apoptosis.

Nowadays, omega-6 fatty acids are the predominant PUFA in all diets in the developed countries, especially Western diets. When diets are supplemented with omega-3 fatty acids, they partially replace the omega-6 fatty acids in the membranes of the cells. There is a competition between the omega-6 and omega-3 fatty acids in the formation of prostaglandins. Specifically, EPA competes with AA for prostaglandin and leukotriene synthesis through the COX and LOX pathways respectively (Simopoulos, 2002). Omega-3 PUFA metabolism produces less inflammatory or anti-inflammatory eicosanoids (3-series prostanoids and 5-series leukotrienes) (Molendi-Coste *et al*, 2010). EPA and DHA consumption causes a

decreased production of PGE2, thromboxane A2 and LTB4 formation, and results in an increase in thromboxane A3 (a weak platelet aggregator and a weak vasoconstrictor), PGI3 (active vasodilator and inhibitor of platelet aggregation) and LTB5 (a weak inducer of inflammation and a weak chemotactic agent) (Simopoulos, 1991).

Recently, new omega-3 lipid mediators have been discovered, called resolvins, protectins and maresins, as well as lipoxins, which are termed specialised pro-resolving mediators (SPM) (figure 1-11). Acute inflammation can progress to chronic inflammation or to complete resolution, which results in the return of the local tissue to the normal non-inflamed state. Resolution was previously thought to be a passive process; however, it is an active process that involves the biosynthesis of local mediators such as SPM (Serhan, 2009; Serhan & Chiang, 2008). Generally, the catabasis of the tissue to the homeostatic healthy state is accompanied by lipid mediator switching from pro-inflammatory PGs and LT to the biosynthesis of anti-inflammatory and pro-resolving mediators, which include the SPM. This process is called 'eicosanoid class switching' (Serhan & Chiang, 2008). The first pro-resolving mediators identified were lipoxins (Serhan, 2005) such as lipoxin A_4 (LXA_4) and LXB_4, which are anti-inflammatory. Lipoxins are lipoxygenase-derived eicosanoids produce from omega-6 AA. During resolution, lipoxins signal macrophages to phagocyte the remainings of apoptotic and necrotic cells in the area of inflammation (Godson *et al*, 2000). They exert their anti-inflammatory effect in picogram and nanogram levels in tissues (Serhan & Chiang, 2008). Resolvins and protectins are local lipid mediators identified in the resolving exudates of inflammation. Resolvins (resolution phase interaction products) are bioactive mediators biosynthesised from the omega-3 fatty acids EPA and DHA. The E series of resolvins (RvE) derives from EPA and the D series (RvD) from DHA (Serhan *et al*, 2002). Resolvins can also be produced by a COX-2-dependent pathway, in the presence of aspirin, generating their aspirin triggered form. Growing evidence indicates that resolvins exert anti-inflammatory and immunoregulatory actions, such as blocking the production of pro-inflammatory mediators and regulating leukocyte trafficking (Serhan, 2009; Serhan & Chiang, 2008). Protectins are biosynthesised from DHA via a separate pathway (Serhan *et al*, 2006), and their name comes from the observed protective and anti-inflammatory action of these molecules (Hong *et al*, 2003). The term neuroprotectin

refers to the protectins found in neuronal tissue such as neuroprotectin D1, which show neuroprotective action (Serhan *et al*, 2006). Protectins have been demonstrated to reduce polymorphonuclear leukocytes (PMN) infiltration *in vivo* and also act in an additive way with resolvins to stop the inflammation process (Hong *et al*, 2003). The most recent members of SPM are maresins (macrophage mediators in resolving inflammation). Serhan *et al* identified a new biosynthetic pathway for these novel, anti-inflammatory and pro-resolving mediators derived from DHA by macrophages (Serhan *et al*, 2009). They have similar anti-inflammatory potency with resolvins and protectins, and they are produced via the 14-LOX pathway.

In summary, the omega-3 PUFA DHA and EPA not only possess therapeutic actions on their own, but also produce mediators which are anti-inflammatory and play a significant role in the resolution of inflammation.

Figure 1-11: Metabolism of omega-6 and omega-3 PUFA

The metabolism of omega-6 and omega-3 PUFA after the activation of phospholipase (PLA2) which causes their release from the membrane phospholipids. PUFA then act as substrates for different enzymes, a process which leads to the production of the final lipid mediators including eicosanoids and the novel pro-resolving mediators lipoxins (lipoxygenase-derived eicosanoids), resolvins (resolution phase interaction products), protectins and maresins (macrophage mediators in resolving inflammation) via COX and LOX pathways (Molendi-Coste et al, 2011).

1.4 Aims of the study

The aims of this project were:

- To test the ability of omega-3 PUFA to specifically inhibit neoplastic keratinocyte growth including epidermal and oral malignant keratinocytes.
- To test the effect of omega-3 PUFA on normal keratinocytes.
- To investigate the biological mechanism of any growth inhibition (inhibition of cell proliferation or cell death).
- To investigate the molecular pathways involved in any growth inhibition observed.

Chapter 2

MATERIALS AND METHODS

Chapter 2. Materials and Methods

2.1 Cell lines

Malignant and normal keratinocyte cell lines were used in this study. SCC-13 and SCC-25 are tumourigenic keratinocyte lines, epidermal (facial epidermis) and oral (tongue) respectively (Rheinwald & Beckett, 1981), and SVHFK is an SV40 virus-transformed epidermal keratinocyte line that is not tumourigenic at early passage (Brown & Gallimore, 1987) and can be considered pre-malignant. NHEK-131 and HEK-127 (Invitrogen, Paisley, UK) are normal foreskin epidermal keratinocyte lines. The five cell lines were maintained in keratinocyte basic medium (KBM) (Cambrex-Lonza, Walkersville, MD) supplemented with bovine pituitary extract (BPE) (0.03 mg/ml), human epidermal growth factor (EGF) (0.1 ng/ml), insulin (5 g/ml), hydrocortisone (0.5 g/ml), and antibiotics/antimycotics GA-1000 (gentamicin at 50 g/ml and amphotericin B at 50 ng/ml) to make Keratinocyte Growth Medium (KGM). The cells were maintained in a humidified atmosphere with 5% CO_2 and 95% air, at 37°C. The cells were passaged before they became more than 50% confluent. In order to investigate the effect of PUFA in a serum medium, SCC-25 were maintained in Flavin Adenine Dinucleotide medium (FAD) which consisted of 3 parts of Dulbecco's Modified Eagle Medium (DMEM) 4.5 g/L glucose (Gibco-Invitrogen) and 1 part of Ham's F12 (Gibco-Invitrogen) with 10% (v/v) foetal bovine serum (Hyclone FetalClone II) (Thermo Fisher Scientific, Loughborough, UK), 20 mM HEPES, 100 U/mL penicillin, 100 U/mL streptomycin and 2 mM L-glutamine (Lonza), supplemented with 1.8 x 10-4 M adenine, 5 µg/mL insulin, 5 µg/ml transferrin, 0.4 µg/ml hydrocortisone and 8.4 ng/ml cholera toxin (Sigma-Aldrich, Gillingham, UK).

Three oral dysplasia cell lines were also used: D19, which is a severe erythroleukoplakia of lateral tongue, D20, which is a lateral tongue moderate leukoplakia, and D17, which is buccal mucosa mild/moderate leukoplakia (McGregor *et al*, 2002). D19 progressed to SCC within 6 months of the biopsy being taken (Dr Keith Hunter, University of Sheffield - personal communication) and is

invasive in certain organotypic cultures. Two normal oral mucosa immortal cell lines were also included in the study; OKF6/TERT-1 (Dickson *et al*, 2000) and OKF4/TERT/p53DD/Cdk4 (Rheinwald *et al*, 2002) both oral floor of mouth keratinocytes which were generous gifts of Dr James Rheinwald (Brigham and Women's Hospital- Harvard Medical School, Boston, U.S.A.). Moreover, three more oral normal primary cultures were used: NHOK-810, NHOK-846 and NHOK-881 (normal oral human keratinocytes) which were kindly provided from Dr Angela Hague (University of Bristol, UK). These normal cells and the dysplasias were maintained in 10% CO_2 in the Keratinocyte-Serum Free Medium (K-SFM) with L-glutamine which is supplemented with human recombinant 0.19 ng/ml EGF and 25 μg/ml BPE, 0.39 Mm calcium chloride (Invitrogen) and 10 μg/ml penicillin and streptomycin (Lonza).

Additionally, HaCaT cells were included which are immortalised human epidermal keratinocytes cells which are non-invasive and non-tumourigenic. HaCaT-TERT expressing wild-type TERT, HaCaT-TERT-HA expressing TERT-HA that cannot lengthen telomeres (non canonical function only) and the HaCaT-PURO with the empty vector (pBabePuro) were created by retroviral transduction (E.K.Parkinson – unpublished data) and the mRNA expression was tested by quantitative polymerase chain reaction (qPCR) (B.Cereser - unpublished data). HaCaT-MYC cells are HaCaT cells transfected with the *CMYC* oncogene (Cerezo *et al*, 2002) and are a generous gift from Prof. Petra Boukamp of the Deutche Krebsforschungzentrum in Heidelburg. DLD-1 was obtained from the American Type Culture Collection and is a colon cell line derived from human colorectal adenocarcinoma. It was used as a control, as there are published experiments with DHA and EPA performed using this cell line (Eitsuka *et al*, 2005). Cells were maintained at 37ºC in 5% CO_2 and 95% air in RPMI 1640 medium (Lonza, Walkersville, MD) containing 10% v/v foetal bovine serum (Hyclone FetalClone II) (Thermo Fisher Scientific) containing 2 mM L-glutamine, 25 mM HEPES and 10 μg/ml penicillin and streptomycin (Lonza).

The cells were passaged with 0.1% trypsin (Worthington, Lakewood, USA)/0.01% EDTA (Sigma-Aldrich, Gillingham, UK) in phosphate buffered saline (PBS) when they reached around 50% confluence. They were first washed with PBS and then incubated with the trypsin/EDTA mixture at 37ºC. The HACAT cells were first

incubated for 5 min with 0.02% EDTA at 37ºC and then incubated in 0.1% trypsin/ 0.05% EDTA until separated from the plate surface. Media with 10% FBS were added to neutralise the trypsin (1:5). The cells were then centrifuged at 800 rpm for 5 min and re-suspended in appropriate medium.

All cells were preserved in liquid nitrogen in a freezing medium (RPMI 1640, 10% DMSO (Sigma), 10% FetalClone II) after a gradual dropping in temperature at -80°C for 24 h.

2.2 Reagents

PUFA: The cells were treated with 5-8-11-14-17 eicosapentaenoic acid (EPA) and 4-7-10-13-16-19 docosahexaenoic acid (DHA) (NU-CHEK, Minnesota, USA). The free fatty acids arrived as pure oil and were diluted in ethanol under nitrogen at a stock concentration of 0.5 M. The stock was aliquoted in dark coloured glass vials with screw tops (Agilent Technologies, Wokingham, UK) to protect from light and oxidation, and stored at -20^{0}C for up to six months.

Tocotrienols: Pure tocotrienols α, γ, δ and Tocomin®, a natural full spectrum Tocotrienol/Tocopherol 50% complex, were a kind gift from Mr. WH Leong (Carotech BHD, Perak, Malaysia). Tocomin® is a vegetable oil suspension of a naturally occurring mixture of tocotrienols and tocopherols extracted and concentrated from palm fruits. It contains approximately 115 mg/ml α-tocotrienol, 15 mg/ml β-tocotrienol, 210 mg/ml γ-tocotrienol, 55 mg/ml δ-tocotrienol and 115 mg/ml α-tocopherol. Pure tocotrienols and Tocomin® were also diluted in ethanol to a stock concentration of 0.5 M, aliquoted in dark coloured glass vials with screw tops and stored at -20^{0}C.

Growth Factors: Insulin growth factor type 1 (IGF-1) (1-30 ng/ml), transforming growth factor alpha (TGF-α) (1-40 ng/ml), transforming growth factor beta 1 (TGF-β_1) (3-300 pg/ml), epidermal growth factor (EGF) (0.3-30 ng/ml) and acidic

fibroblast growth factor (FGF-a) (0.1–3 ng/ml), were obtained from the Sigma-Aldrich Co. Poole Dorset, UK. Basic fibroblast growth factor (FGF-b) (0.1-3 ng /ml) was obtained from Invitrogen, UK and added to the growth medium RPMI at the indicated final concentrations in the presence of 3% v/v FBS. Cultures of HaCaT cells either received 0.1% ethanol alone (vehicle control), 50 μM DHA or 50 μM EPA. PUFA were also added to medium containing 10% v/v FBS, as a further control of the reduced effect of the lipids in these conditions.

Antioxidants: The antioxidant n-tert-butyl-α-phenylnitrone (PBN) and α-tocopherol were obtained from Sigma (Poole Dorset, UK) and added to the growth medium (RPMI containing 3% v/v FBS or serum free KGM) to give final concentrations between 50 μM and 1 mM.

Albumin: Human and bovine serum albumin (BSA) were purchased from the Sigma-Aldrich Co. and added to the RPMI medium containing 3% v/v FBS to give final concentrations of 0.1%, 0.3%, 1% and 3% w/v.

Inhibitors: Several inhibitors were used in order to investigate the signalling pathways involved in PUFA effect. The caspase inhibitor Q-VD-OPh was from Calbiochem (EMD Biosciences, La Jolla, CA, USA). The MEK inhibitors, U0126 was from Cell Signaling Technology (Danvers, MA, USA) and AZD6244 was from Selleck Chemicals (Houston, TX USA). The EGF-receptor inhibitor AG1478 was from Invitrogen (Paisley, UK). The EGFR blocking antibody (EGFR Mouse anti-Human Monoclonal (Azide-free) (225) Ab) was from LifeSpan Biosciences (Seattle, WA, USA). The inhibitors were added in the culture for 1.5 hours before the addition of the EPA and DHA. After 2 hours since the PUFA were added, the lysates were obtained.

2.3 MTT Assay

The Thiazolyl Blue Tetrazolium Bromide (MTT) assay (Sigma) is a colorimetric assay which is based on the use of [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to determine the number of viable cells (Mosmann, 1983). It is based on the ability of viable cells to release the mitochondrial dehydrogenase enzyme which converts MTT to dark blue formazan crystals. The number of surviving cells is directly proportional to the level of formazan product generated. This assay was used to determine the number of viable cells after incubation in medium containing different concentrations of PUFA and tocotrienols. I have shown that the absorbance in the MTT assay has a linear relationship with the HaCaT cell number between 10^4 and 10^6 cells per well. To determine the sensitivity of the MTT assay in detecting growth inhibition, HaCaT cells treated with 1 ng/ml of transforming growth factor beta (TGF-β), a powerful inhibitor of proliferation in this cell line, were included as controls in all experiments.

A stock MTT (5 mg/ml) solution was prepared in sterile PBS and stored at 4ºC and used as needed. Then 0.5 mg/ml of MTT solution was prepared from the stock solution in serum free medium and filter sterilised. After the supernatants were aspirated, 1ml of the MTT solution was added into the culture wells of the 24-well plate (Nunc- VWR International Ltd, Lutterworth, UK) and the plate was incubated for 1 hour in a CO_2 incubator at 37ºC. The media containing MTT was removed carefully using an aspirator. The purple formazan dye was dissolved adding 0.5 ml or 1 ml of DMSO in each well. Triplicates of 200 µl dissolved coloured aliquots from each well were transferred to a 96-well plate (Nunc-VWR). This was read at 570 nm optical density (OD) using a FLUOstar OPTIMA BMG Labtech plate reader. The OD of the blank (just 200 µl DMSO) was subtracted from the sample OD. Cell viability was expressed as a percentage of the control cultures as:

$$\text{Viability} = \text{OD}_{(570nm)} \text{ Treated / OD}_{(570nm)} \text{ Untreated (Control)} * 100$$

2.4 Apoptosis Assay

In the early stages of apoptosis, changes occur on the cell surface. One of these changes is the translocation of phosphatidylserine (PS) from the interior side of the plasma membrane to the outer layer. Annexin V is a Ca^{2+}-dependent protein which binds to PS with high affinity and it is fluorescein-conjugated in this assay (Vermes *et al*, 1995). Since necrotic cells also expose PS as a result of lost membrane integrity, apoptotic cells must be differentiated from these necrotic cells. The simultaneous application of a DNA stain like DAPI allows the discrimination of necrotic cells from the annexin V positively stained cell cluster (figure 2-1).

The control and treated cells were trypsinised and pelleted including any cells in supernatants. They were re-suspended in 500 μl of annexin V Binding Buffer (Becton Dickinson, Oxford, UK). Then, 4 μl/ml of Annexin-V-FLUOS (Roche Diagnostics Ltd, Burgess Hill, UK) and 200 ng/ml of DAPI (Sigma) viability dye were added and the cells were incubated at room temperature (RT) for 15 min. The samples were analysed on a flow cytometer (LSR II BD Biosciences) collecting 10-20,000 events.

2.5 ^{3}H-thymidine incorporation assay

Cell proliferation was measured by incorporation of tritiated thymidine (^{3}H-TdR). Cells were seeded at $4x10^2$ cells/ml/well into a sterile, 96-well flat-bottom tissue culture plates (Fisher Scientific, Edmonton, Alberta, Canada) and treated with 3 μM of EPA and DHA as described for the MTT assay (sub-chapter 2.3). Cell cultures were pulsed with 0.5 μCi/well of ^{3}H-TdR (Amersham, UK) and incubated for 18 hours. Cells were then harvested onto filters (Wallac Perkin Elmer, USA) using a 96-well cell harvester (TOMTEC Harvester 96/Mach 3M). Scintillation fluid (Wallac) was added to the filter, which contains fluorophores that absorb radioactive energy and converts it into light that is then detected by the beta counter (Wallac). The results of the incorporated thymidine were expressed in counts per minute (CPM)

and were a mean of triplicate. CPM were then expressed as a percentage of the PUFA untreated control.

Figure 2-1. Schematic representation of the annexin V assay

Annexin V-FITC binds to the PS that translocate to the outer side of the membrane during apoptosis. The staining of the DNA with DAPI discriminates the early from the late apoptotic or necrotic cell as it can only penetrate the plasma membrane when its integrity is breached.

2.6 Detection of protein expression

2.6.1 Preparation of cell lysate

The medium was removed from the adherent cells and washed twice in cold PBS. Radio-Immunoprecipitation Assay (RIPA) lysis buffer (1% NP-40, 0.1% SDS, 50 mM Tris pH 7.3, 150 mM NaCl) (all Sigma, Poole, UK) and protease (cOmplete cocktail tablets) and phosphatase inhibitors (PhosSTOP cocktail tablets) (both Roche Diagnostics) were added to the cells on ice.

After 20-minute incubation on ice, the cells were scraped with rubber cell scrapers and centrifuged at 15,000 rpm for 20 min, at 4°C. The supernatant was collected and the protein concentration was determined by the DC protein assay (Biorad, Hemel Hempstead, UK), according to the manufacturer's instructions. The DC Protein Assay is a colorimetric assay for protein concentration following detergent solubilisation. The protein concentration in each sample was estimated according to the standard curve which was calculated from the bovine serum albumin (BSA) standards. Total cellular protein (10-35 μg) from the samples were boiled for 5 min at 100°C in SDS sample buffer (5X, 0.3 M Tris HCl, pH 6.8, 10% SDS, 50% glycerol, 20% 2-mercaptoethanol, 0.25% bromophenol blue) (all Sigma, Poole, UK). Lysates were stored at -80°C for western blot analysis.

2.6.2 Western blotting

Total cellular protein from the cell lysates were separated by SDS PAGE on 4-12% resolving gels under denaturing and reducing conditions (NuPAGE Novex Bis-Tris Pre-Cast Gels, Invitrogen) at 130 V. 1x Running buffer was prepared using the 20x NuPage sodium dodecyl sulfate (SDS) running buffer (Invitrogen). Seven μl of ladder (Dual colour, Biorad) were also used for the protein molecular weight. The

proteins on the gels were transferred to 0.45 μm Immobilon PVDF membranes (Millipore, Watford, UK) that had been soaked in methanol (VWR, Lutterworth, UK) for 1 min and then in transfer buffer. Protein transfer took place at 30 V at 4ºC for 90 min using a transfer buffer containing 25 mM Tris, 190 mM glycine, and 20% methanol. The membrane was then blocked in 5% low fat (<1.5%) milk in Tris-Buffered Saline and Tween 20 (TBS-T) (1 M TRIS pH 8.0, 5 M NaCl, 0.05% Tween 20) (all Sigma) at RT for 1 hr. Primary antibody was added in 5% milk in TBS-T or 5% BSA (PAA, Pasching, Austria) in TBS-T, according to the antibodies' manufacturer's instructions. The antibodies used are shown in Table 2.1. The membrane was incubated at room temperature for 2 hrs or overnight at 4°C and then washed extensively with TBS-T. The membrane was incubated in horseradish peroxidase-conjugated secondary antibody (Table 2.1) prepared in 5% milk in TBST at room temperature for 1 h and then washed four times with TBS-T. Antigen-antibody complexes were detected and visualised by Amersham ECL Chemiluminescent detection reagent (GE Healthcare Life Sciences, Little Chalfont, UK) for strong signal, by Amersham ECL Plus (GE Healthcare Life Sciences) for medium-weak signal or by SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Thermo Fisher Scientific) for very weak signals (according to the manufacturer's instructions) on Amersham Hyperfilm ECL (GE Healthcare Life Sciences). The films were scanned and, when needed, the quantification of the difference between protein expression was carried out by densitometry using Scion Image software (Frederick, Maryland, USA). Corrections for the background were made. The amount of proteins was normalised using the estimated expressed GAPDH in the loading control or the total protein.

Antibody	Supplier	Antibody type	Dilution
Cleaved caspase 3	Cell Signalling	1° rabbit polyclonal	1:1000
Cleaved caspase 8	Cell Signalling	1° rabbit monoclonal	1:1000
Cleaved caspase 9 (Asp330)	Cell Signalling	1° rabbit polyclonal	1:1000
Caspase 3	R&D Systems	1° goat polyclonal	1:1000
Caspase 8	Cell Signalling	1° mouse monoclonal	1:1000
Caspase 9	Cell Signalling	1° rabbit polyclonal	1:1000
phospho-p44/42 MAPK(Thr202/Tyr204) (phospho-ERK1/2)	Cell Signaling	1° rabbit monoclonal	1:1000
p44/42 MAPK (ERK1/2)	Cell Signaling	1° mouse monoclonal	1:2000
Anti-Akt/PKB[pS^{473}]	Biosource-Invitrogen	1° rabbit polyclonal	1:1000
Total Akt	Cell Signaling	1° rabbit polyclonal	1:1000
Cox-2	Santa Cruz Biotechnology	1° goat polyclonal	1:200
phospho-SAPK/JNK MAPK(Thr183/Tyr185)	Cell Signalling	1° rabbit monoclonal	1:1000
GAPDH	Abcam	1° rabbit polyclonal	1:1000
GAPDH	Abcam	1° mouse polyclonal	1:5000
Phospho-p90RSK	Cell signaling	1° rabbit polyclonal	1:500
RSK 1/2/3	Cell signaling	1° rabbit monoclonal	1:1000
HRP-conjugated anti-rabbit IgG	Pierce, Thermo Fisher Scientific	2°goat polyclonal	1:2500
HRP-conjugated anti-mouse IgG	Pierce, Thermo Fisher Scientific	2°goat polyclonal	1:3000
HRP-conjugated anti-goat IgG	Sigma	2° mouse monoclonal	1:10000

Table 2-1. Antibodies used for the western blot method
Antibodies and suppliers are listed. The antibody types and the dilution used are also mentioned. The blots were usually incubated in the primary (1°) antibodies overnight shaking in 4°C and for 1 h into the secondary (2°) antibodies at room temperature.

2.6.3 Densitometry

Scion Image software (Scion Corp, USA) was used for densitometry measures. Western blot films were scanned and uploaded in to Scion Image (figure2-2), the band of interest was selected and the mean intensity of the area (x) was measured (figure 2-2B). Similarly the background (y) was also measured (figure 2-2C), the results were displayed in a table (figure 2-2D) and the mean density of the band was calculated by subtracting the background (figure 2-2E). All western blot measurements were also corrected for the loading (GAPDH or total protein), which was measured as described in figure 2-2.

Figure 2-2. Measuring the density of band on a western blot band

(A) Western blot bands were uploaded in to scion image, (B) the density of the band was measured, (C) the density of background was measured, (D) results were displayed in a table and the (E) mean density was calculated by subtracting the mean background density from the mean density of the band.

2.7 Detection of reactive oxygen species (ROS) production

2.7.1 Immunocytochemistry with 8-oxo-dG Antibody

The anti-8-oxo-dG (Clone 2E2) antibody (Trevigen, Gaithersburg, USA) was used to detect reactive oxygen species (ROS) production. This mouse monoclonal antibody specifically binds to 8-hydroxy-2'-deoxyguanosine. It can be used to detect oxidative damage by immunocytochemistry.

The cells were plated on sterile chamber slides and were treated with PUFA. The staining was performed according to the manufacturer's instructions:

Cells were plated on sterile Lab-Tek™ chamber slides (Thermo Fisher Scientific). After 24 h the medium was aspirated and the positive control cells were treated with 100 μM tert-butyl hydroperoxide (*TBHP)* in medium, at 37°C for 30 minutes. Then the medium was removed and fresh medium was added overnight. The cells in other wells of the chamber slides were also treated with PUFA. Following this, the cells were washed 3x with PBS, and fixed with 1:1 MeOH, acetone for 20 min at -20°C and allowed to air dry. Fixed cells were then treated with 0.05 N HCl for 5 min on ice and washed 3x with PBS for 5 minutes each and incubated with 250 μl of 100 μg/ml RNAse in 150 mM NaCl, 15 mM sodium citrate for 1 hour at 37°C. The cells were then washed sequentially in PBS, 35%, 50% and 75% EtOH, for 3 minutes each and the DNA was denatured *in situ* with 250 μl 0.15N NaOH in 70% EtOH for 4 minutes. Following two washes with PBS, the cells were then washed sequentially in 70% EtOH containing 4% v/v formaldehyde, 50% and 35% EtOH, and 1X PBS for 2 minutes each. Following incubation with 250μl of 5μg/ml proteinase K in 20mM Tris, 1mM EDTA, pH 7.5 (TE) for 10 minutes at 37°C and several washes with PBS, the cells were incubated with 5% normal goat serum (Sigma) in PBS, 1 hour at room temperature to block non-specific binding. After that, the cells were washed 3x with PBS, and incubated with 250 μl anti-8-hydroxyguanine antibody at a concentration of 1:250 diluted in PBS containing 1% BSA (PAA), 0.01% Tween-20 (Sigma) at 4°C overnight in a humidified chamber. The next day, the cells were

washed several times with PBS containing 0.05% Tween 20 for 5 min each and incubated in 250 µl of fluorescent secondary antibody conjugate, goat anti-mouse IgG (Alexa Fluor 488) (Molecular Probes-Invitrogen) at 5 µg/ml in PBS containing 1% BSA for 1 hr in the dark, at room temperature. After several more washes with PBS containing 0.05% Tween 20, the DNA off the cells was stained with 0.2 µg/ml Hoechst 33258 (Invitrogen Molecular Probes, Eugene, Oregon, USA) in PBS for 10 minutes. Finally, the cells were washed several times with PBS, rinsed with de-ionised water, mounted with appropriate mounting media (Thermo Scientific, Runcorn Cheshire, UK) before the analysis by fluorescence microscopy and visualised with a Leica DM5000 epifluorescence microscope under the x40 objective lens and x100 oil emersion lens, and analysed with Metamorph imaging software (Sunnyvale, CA).

2.7.2 ROS detection by DCF or HE staining

2′,7′-Dichlorodihydrofluorescein Diacetate (H_2DCFDA) (Calbiochem-Merck, Nottingham, UK) is a cell-permeable fluorogenic probe that detects the reactive oxygen species (ROS) and nitric oxide (NO) in cells and it was used to determinate the overall oxidative stress in the cells. Upon cleavage of the acetate groups by intracellular esterases and oxidation, the nonfluorescent H_2DCFDA is converted to the highly fluorescent 2',7'-dichlorofluorescein (DCF) (Yuan *et al*, 1993).

Moreover, dihydroethidium (Invitrogen) was used to confirm oxidation. The dihydroethdium, also called hydroethidine (HE) is a superoxide indicator. Cytosolic HE exhibits blue fluorescence; however, once this probe is oxidised to ethidium, it intercalates within DNA, staining the cell nucleus a bright fluorescent red (Carter *et al*, 1994). HE is oxidised by superoxide to 2-hydroxyethidium and it is frequently used for mitochondrial superoxide detection (Zielonka *et al*, 2008).

DCF and HE were diluted in DMSO and stored at -20ºC in aliquots. The staining was performed as described below:

The cells were plated in 60 mm dishes (0.2×10^6 cells / dish). After two days, PUFA were added. The HFF cell line, which is a human fetal skin fibroblast cell line with low ROS levels, was used as negative control. Also, cells treated with 100 μM of *tert*-butyl hydroperoxide solution (TBHP), for 2 hours at 37ºC, were used as positive controls (as they cause oxidative stress). After the 2 hour treatment in TBHP, the medium was refreshed for the positive control cells. After 24 hours, the control and treated cells were trypsinised and pelleted, including any cells in supernatants. The cells were then re-suspended in medium with 1 μM of H_2DCFDA or 5 μM HE, and 250 ng/ml of DAPI in universal tubes and incubated for 30 min in 37^0C. After that, the cells were washed in PBS twice (800 rpm for 5 min) and then re-suspended in PBS. Finally, the samples were analysed on a flow cytometer (LSR II BD Biosciences) collecting 10-20,000 events.

2.8 Lipid analysis by gas chromatography

The determination of the fatty acid composition of the cells or media samples involves several steps. Firstly, the lipid component was extracted from the sample. Secondly, the phospholipids were isolated from the total lipid extract, and finally, the fatty acids of the phospholipids were transesterified to produce fatty acid methyl esters (FAMEs), which are suitable for analysis by gas chromatography.

Different types of lipid solvent extraction methods are being used depending on the type of sample. For tissues and cells the Folch *et al.* method is used (Folch *et al*, 1957), whereas the Bligh and Dyer method is more appropriate (Bligh & Dyer, 1959) for samples with a high water content, such as incubation media. Due to the highly peroxidisable nature of PUFA the exposure to oxygen was minimised during the procedure, and the antioxidant 2,6-di-tert-butyl-*p*-cresol (butylated

hydroxytoluene, BHT) was added to all solvents at 0.05%. Moreover, some of the reagents which are prone to oxidation were changed regularly.

2.8.1 Folch extraction

Lipids were extracted using the Folch method (Folch *et al*, 1957). The antioxidant 2,6-di-tert-butyl-p-cresol (BHT) was added to the solvents used. The solvents that were used (chloroform/BHT 0.05%, methanol/BHT 0.05%, chloroform/methanol/BHT 0.05% (2:1 v/v), H_2O/methanol, 0.88% KCl) were kept on ice during the procedure. The solvents were also stored in dark glass bottles and changed every two weeks.

The treated and control cells were scraped from the culture dish, and transferred to a glass test tube. After centrifugation, the supernatant was discarded and 1 ml methanol/ BHT 0.05% was added. Following this, the cells were homogenised with a Polytron PG Kinematic AG homogeniser for 30 sec. Then 2 ml of chloroform/BHT 0.05% were added and the process was continued for 2×30 sec, and the samples were centrifuged at 3500 rpm for 5 min at room temperature. Next, 2 ml of the supernatant were removed and added to a fresh glass test-tube. The pellet was re-suspended in 2 ml chloroform/methanol/BHT 0.05% (2:1 v/v) and the centrifugation was repeated. Another 2 ml of supernatant were removed by Pasteur pipette and combined with the first supernatant in the glass test-tube. 1 ml of 0.88% KCl was added, everything was mixed gently by inversion and centrifugation was repeated. Next, 0.8 ml H_2O/methanol was added to the combined supernatant and was mixed gently by inversion, and centrifuged. Again the upper layer was discarded. A sheet of Whatman phase separation filter paper (Whatman PS No 1) was then placed into a small glass funnel and was attached to a clamp above a 5 ml Reacti-Vial (Pierce, USA). The sample was evaporated under a stream of nitrogen using the Techne SC-3 Sample Concentrator in fume hood. To do this the vial was placed under a rod with a gentle flow rate to prevent spillage and ensure even dispersal. When the solvent had evaporated, yellow oily residue was visible at the bottom of the vial which was re-

suspended in 40 µl chloroform/BHT, capped under a gentle stream of nitrogen and stored at -20°C until the next step which was carried out the same day or the next day.

2.8.2 Thin layer chromatography of total phospholipids

The total phospholipid extracts were then separated by thin layer chromatography (TLC) (Manku *et al*, 1983) by elution with petroleum ether/diethyl ether/acetic acid/methanol (85:15:2.5:1). Prior to running the TLC, the tank was equilibrated by adding the eluent to a depth of approximately 1 cm and leaving in the fume hood for several hours. Merck silica 60 g thin-layer 20x20 cm pre-coated chromatography plates were used in this procedure. A baseline was marked 2 cm from the bottom edge of a silica plate, using a pencil. Also a solvent front barrier was marked 1.5 cm from the upper edge of the plate and twelve 1.5 cm sample lanes into the silica. The procedure was carried out into the fume hood to avoid exposure to the toxic silica dust. Next, 20 µl phospholipid were added on the baseline by superimposing successive 2 µl drops from a Hamilton 10 µl pipette onto the plate, and excess solvent was removed by cool air-drying every 3 to 4 spots with a hair-dryer and the plate was put into the tank for the 1 h elution (figure 2-3). The plate was removed, gently dried with cool air from the hair-dryer in the fume hood and then was sprayed with primuline at 0.1% in acetone/H_2O (60:40 v/v). The phospholipids were visualised on a UV Transilluminator at 365 nm (figure 2-3). Phospholipids remain at the origin, whereas other lipid components elute up the plate. When the primuline had dried, the spots were scraped into a 5 ml Reacti-vial.

2.8.3 Transesterification

The phospholipids form the silica plate spots were placed into 5 ml Reacti-Vials. The fatty acids were derivatised by transesterification (Morrison & Smith, 1964). The procedure was carried out in a fume hood wearing double gloves. 1 ml 14% BF_3/methanol (toxic) was added to the TLC phospholipid samples using a glass pipette, and capped under a gentle stream of nitrogen. The vials were placed in the heating block at 100°C for 20 min and then were allowed to cool for about 10 min. Next, 2 ml pentane and 1 ml H_2O were added and capped under nitrogen, and the vials were vortexed for 10 sec. The caps were loosened slightly to release pressure, and centrifuged at 3500 rpm for about 20 sec. The upper pentane layer containing the FAMEs (fatty acid methyl esters) was transferred to an Agilent GC sample vial and was evaporated under nitrogen. The fatty acids were re-dissolved in 60 μl of hexane/BHT (0.01% w/v). The samples where then stored at -20ºC until analysis by gas chromatography the next day.

2.8.4 Gas chromatography coupled with mass spectrometry (GC-MS)

Individual fatty acids were identified by gas chromatography coupled to mass spectrometry (GC–MS) (Agilent technologies, USA) using an SGE BPX70 capillary column (30m×0.25m×0.25mm i.d.). Lipid identity was confirmed by retention times compared to known standards and mass spectra comparison to the National Institute of Standards and Technology database. Quantification of peak area was by ChemStation software (Agilent Technologies, USA). Corrections were made for variations in the detector response and values of detected fatty acids were normalised to 100% and expressed as mol% (which were calculated by normalising the measured fatty acids values to 100% and then dividing by their molecular weights). Unless otherwise stated, all laboratory chemicals and reagents were of analytical grade, and purchased from Sigma–Aldrich (Poole, UK) or BDH (Poole, UK). Phospholipid and fatty acid standards were from Sigma–Aldrich.

Figure 2-3. Diagram of a TLC developing tank

After the samples were added on the baseline, the plate was put into the tank for the 1 h elution. Phospholipids remain at the origin, whereas other lipid components elute on the silicon plate. After the thin layer chromatography (TLC) the plate is sprayed with primuline and the position of the phospholipid spots can be visualised at 365 nm (Meyers & Meyers, 2008).

2.9 Statistical analysis

The non-parametric Wilcoxon Mann-Whitney Rank test was used for the analysis of the data and the comparison between the normal, malignant and pre-malignant cells and the DCF experiment. One way Anova followed by post hoc Bonferroni test was used for the MTT assay, the apoptosis assay and the proliferation assay results. Both tests were performed via the SPSS statistical software (Version 17, Chicago, IL, USA). A p value <0.05 was considered significant.

Chapter 3

EFFECT OF PUFA ON CELL GROWTH

Chapter 3. Effect of PUFA on cell growth

3.1 Background

Several studies have shown that omega-3 PUFA have an antitumour effect by inhibiting the cell growth of various types of cancer *in vitro*. EPA caused the inhibition of cell growth of hepatoma cells (Murata *et al*, 2001), the breast cancer cells MCF-7 (DeGraffenried *et al*, 2003) and the leukemic cell lines HL-60 and K562 (Chiu & Wan, 1999). Both EPA and DHA also showed an *in vitro* anti-cancer effect in various cancer cell lines, such as the colon adenocarcinoma DLD-1 (Eitsuka *et al*, 2005; Tsuzuki *et al*, 2007), CC531 and LT97 (Gutt *et al*, 2007; Habermann *et al*, 2009), breast cancer MDA-MB-231 (Schley *et al*, 2005), hepatocellular carcinoma (Schley *et al*, 2005) and lymphoma (Heimli *et al*, 2002). DHA has also inhibited the neuroblastoma cells SK-N-BE *in vitro* (Gleissman *et al*, 2010).

PUFA exert their anticancer effect through inhibiting proliferation or/and promoting cell death. There is accumulating evidence that they particularly promote apoptosis. But what exactly is apoptosis?

a) Apoptosis

The term 'apoptosis' comes from the Greek word which means 'drop off' or 'fall off' and was used to describe leaves falling from the trees and petals from flowers (Alenzi & Warrens, 2003). It was adopted by Currie *et al* in 1972 to describe a common type of cell death observed in various cells and tissues (Hengartner, 2000; Kerr *et al*, 1972). Actually, apoptosis is a highly organised type of programmed cell death used to eliminate harmful, damaged or unwanted cells by phagocytosis, with minimal damage to the surrounding tissue and avoiding inflammation (Logue & Martin, 2008). The apoptotic cells undergo specific morphological changes: cytoplasm and nuclear shrinkage, convolution and blebbing of the cell surface, condensation of the nucleus and DNA degradation. The blebs break off in 'apoptotic bodies', but the cells remain sealed so there is no leakage of the intracellular

components that could cause inflammation and damage to neighbouring cells (Alenzi & Warrens, 2003; Letai, 2008). The phosphatidyl serine (PS) that is normally restricted to the cytoplasmic face of the inner leaflet of the plasma membrane is exposed to the extracellular environment. PS and other induced signals allow recognition by phagocytic cells, which phagocytose apoptotic cells and rapidly remove them from their environment (Letai, 2008). The coordination and execution of the apoptotic process depend mainly on cystein aspartate-specific proteases known as caspases. They are expressed as inactive precursors (zymogens) and they become activated after proteolysis at internal aspartic acid residues (Logue & Martin, 2008). There are 14 known human caspases and the ones involved in apoptosis can be divided into two groups: the initiator caspases (caspases 2, 8, 9 and 10) which initiate the caspase activation cascades, and the effector caspases (caspases 3, 6 and 7) which are the executors of apoptosis and responsible for cell demolition.

There are two main pathways in apoptosis: the intrinsic mitochondrial pathway and the extrinsic death-receptor pathway. There is also a third one called the granzyme B pathway:

1. The intrinsic mitochondrial pathway

The intrinsic mitochondrial pathway is triggered from various forms of cellular stress such as DNA damage, heat shock, oxidative stress and other types of damage. This pathway converges on mitochondria often via activation of a pro-apoptotic member of the Bcl-2 family. The Bcl-2 family is a large family of proteins that are important in the regulation of cellular life and death decisions (Cory & Adams, 2002). Each Bcl-2 family member has at least one BH domain. There are the pro-survival members, Bcl-2, Bcl-XL, Bcl-w, Bcl-b, Mcl-1 and A1, which typically contain four BH domains, whereas the pro-apoptotic members contain either one BH-3 domain (Noxa, PUMA (p53 up-regulated modulator of apoptosis), Bad, Bim, Bid, Bmf, Hrk and Bik) (Petros *et al*, 2004) or BH domains 1–3 (Bax, Bak and Bok). The balance of pro- and anti-apoptotic Bcl-2 family proteins controls the permeabilization of the outer mitochondrial membrane and the release of cytochrome c and other proteins (Logue & Martin, 2008). Efflux of cytochrome *c* from mitochondria drives the assembly of a caspase-activating complex in the cytoplasm known as the mitochondrial apoptosome (Wang, 2001). Apaf-1 detects the release of cytochrome c

and serves as the scaffold around which the apoptosome is built and recruits and activates caspase 9, which then causes the activation of the cascade of caspases (Li *et al*, 1997). In the absence of cytochrome *c*, Apaf-1 is a monomeric protein not able to interact with caspase 9, whereas cytochrome *c* and dATP promote Apaf-1 oligomerization into a wheel-like structure which consists of seven Apaf-1 molecules and seven caspase 9 dimers (figure 3-1) (Acehan *et al*, 2002). Caspase 9 is directly responsible for the downstream activation of caspases 3 and 7 while caspase 3 propagates the cascade further by activation of caspases 2 and 6 and by promoting further processing of caspase 9 (Slee *et al*, 1999). During the final stage of this cascade, caspase 6 catalyses the activation of caspase 8 and caspase 10 (Cowling & Downward, 2002; Logue & Martin, 2008; Slee *et al*, 1999).

2. The extrinsic pathway

The extrinsic death-receptor pathway is triggered by members of the death-receptor superfamily which share a domain within their cytoplasmic tail called 'death domain'. Some of them are CD95 (Fas) and tumour necrosis receptor I (TNF-1). When the ligands of these receptors, such as FasL/CD96L, TNF and TRAIL (TNF-related apoptosis inducing ligand), bind to them, the death domain recruits adaptor proteins that recruit caspases into the receptor complex (Ashkenazi & Dixit, 1998; Logue & Martin, 2008). For example, when the CD95 ligand binds to CD95, it induces the formation of a death-inducing complex which binds to FADD (Fas-Associated Death Domain protein). This complex leads to procaspase-8 molecules recruitment and activation. Caspase 8 is the initiator caspase (or caspase 10 in some instances) and can subsequently cause the activation of executioner caspase-3, which results in apoptosis (Hengartner, 2000). This can be done by two alternative pathways downstream of caspase 8 activation. In most cells, stimulation of death receptors produces enough activated caspase 8 to cause sufficient caspase 3 cleavage and engage the full caspase cascade necessary to destroy the cell. Some cell types, though, do not manage to activate enough caspase 8 for the secure apoptosis of the cell. Therefore, the pro-apoptotic signal of caspase 8 causes the engagement of cytochrome c/Apaf-1 of the mitochondrial pathway after the proteolysis of Bid (Logue & Martin, 2008).

Figure 3-1. The three major pathways of apoptosis

The three apoptotic pathways (I) the extrinsic or death receptor pathway, (II) the intrinsic or mitochondrial pathway and (III) the granzyme B pathway, that cause caspase activation and programmed cell death (Logue & Martin, 2008).

3. The granzyme B pathway

A third pathway of apoptosis, called the granzyme B pathway, is initiated by the components of the cytotoxic granules released from the cytotoxic T cells (CTLs) and natural killer (NK) cells when they confront transformed or infected cells. These components include perforin, which is a protein that forms pores to facilitate the delivery of the other components into the target cells, and granzyme B, which is a serine protease that cleaves after aspartic residues thus has the ability to activate the caspase cascade (Logue & Martin, 2008; Trapani & Smyth, 2002).

b) Other forms of cell death

Necrosis is a form of cell death which follows damage such as mechanical injury, ischaemia, extreme heat or cytotoxic drugs, and is not required during development unlike apoptosis. The morphological features of the initial phases of necrosis include clumping of the chromatin, dilatation of the endoplasmatic reticulum, and swelling of the mitochondria. In the final phase, the lysosomes rupture and hydrolases are released and disintegrate the cell which leads to the release of the intracellular contents causing an inflammatory response in the neighbouring tissues (Alenzi & Warrens, 2003).

More recently, alternative mechanisms of programmed cell death have been described, supporting the idea that cells can 'commit suicide' by mechanisms other than apoptosis. These alternative ways are the programmed necrosis and autophagy. In programmed necrosis, specific signals can trigger signalling pathways of the cell which initiate the necrosis process rather than accidentally. These signals could be DNA damage or an apoptotic receptor ligation (TNFR, Fas or TRAIL), when apoptosis is blocked by an anti-apoptotic drug or a virus (e.g vaccinia virus) (Chan *et al*, 2003; Edinger & Thompson, 2004). Many human tumours carry mutations that inactivate the pathways of apoptosis so they must be more sensitive to programmed necrosis, explaining how chemotherapy drugs induce cancer cell death (Edinger & Thompson, 2004). Autophagy is a Greek word which means that the cell 'eats itself'. Autophagy involves the vesicular sequestration of cytoplasmic proteins and organelles such as mitochondria, which results in a double-membrane vesicle called an autophagosome. Autophagosomes fuse with lysosomes and degrade the autophagosome contents. Although apoptosis and necrosis cause death irreversibly,

autophagy could either lead to cell death or help cells escape cell death, paradoxically contributing to cell survival (Amaravadi & Thompson, 2007). It promotes survival by serving as an intracellular mechanism used by the cells to dispose of damaged organelles and proteins (Gu *et al*, 2004) thus enabling the recycling of macromolecules to maintain energy (Lum *et al*, 2005).

Several studies have demonstrated that EPA or DHA can cause apoptosis that may follow the extrinsic or intrinsic pathway. In colon cancer cells such as LS-174, colo 201, HT-29 and Caco-2, DHA or fish oil appeared to trigger the intrinsic pathway of apoptosis by increasing the levels of the pro-apoptotic proteins Bak and Bcl-Xs and decreasing those of the anti-apoptotic proteins Bcl-2, Bcl-xL (Calviello *et al*, 1999; Danbara *et al*, 2004). The cleavage of caspase 3 and 9 but not caspase 8 has been reported in hepatocellular carcinoma lines by EPA and DHA (Lim *et al*, 2009) and in the lymphoma Ramos cell line by EPA (Heimli *et al*, 2002). EPA caused apoptosis detected by annexin V flow cytometric assay but also necrosis in HL-60 leukemia cells, by decreasing Bcl-2 and Bax protein expression (Chiu & Wan, 1999). However, in human Caco-2 colon cancer cells (Narayanan *et al*, 2001), DHA increased the expression of both caspases 8 and 9, and the activation of cytochrome c. Moreover, Habermann showed trends of caspase 8 and caspase 9 involvement in the apoptosis of human colon adenoma LT97 and colon adenocarcinoma HT29 caused by EPA and DHA, by using caspase inhibitors, and also demonstrated cleavage of caspase 8 but also downregulation of Bid protein expression and Bcl-2 mRNA (Habermann *et al*, 2009). Finally, EPA induced cytochrome c release from mitochondria and mitochondrial membrane depolarization in HL-60 leukemia cells, but additionally enhanced the activities of both caspases 8 and 9 and cleaved Bid expression (Arita *et al*, 2001).

In summary, EPA and DHA and the combination of both (e.g in fish oil) can trigger cell death and specifically apoptosis in cancer cells. It is apparent that different pathways of apoptosis can be triggered by PUFA in different cells and by different types of PUFA and regimes of treatment.

3.2 Results

3.2.1 The effect of PUFA on SCC and normal keratinocyte growth

In order to test whether PUFA specifically inhibit SCC (squamous cell carcinoma) growth at physiological concentrations, a panel of SCC lines were initially used which included the epidermal SCC-13 and the oral SCC-25, and the pre-malignant cell line SVFHK. Normal keratinocytes (NHEK-131 and HEK-127) were also used as controls to test the cancer specificity of PUFA. The colorimetric MTT assay was used for this purpose. Specifically 10^5 cells (or $2x10^5$ normal keratinocytes) were plated in 24-well plates and after 3 days were treated with different doses of EPA or DHA. 1 μl/ml of ethanol was added to the vehicle control and did not inhibit cell growth. The KGM medium with or without PUFA were refreshed every 2 days.

DHA and EPA showed significant effects on the survival of malignant cells after only 4 days (figure 3-2). As regards oral cancer keratinocytes SCC-25 and premalignant keratinocytes SVFHK, nearly all the cells were dead at concentrations >3 μM. Epidermal squamous cell carcinoma cells SCC-13 were more resistant compared to the other two lines, however their growth was inhibited by the PUFA s, specifically at concentrations >3 μM for DHA and at concentrations >5 μM for EPA. There were only 37% and 34% live cells left at 3 μM of DHA and EPA, respectively, and only 22% and 26% live SCC-13 cells at 5 μM.

In the normal keratinocyte NHEK-131 and HEK-127 cultures (figure 3-2) there was very low viability at 10 μM, but 59% and 98% of NHEK-131 cells were viable in 3 μM of DHA and EPA, respectively. As regards HEK-127, 64% and 111% cells (slight proliferative effect) were alive in 3 μM of DHA and EPA, respectively. DHA appeared to decrease normal cell growth at 5 μM, while EPA left 72-94% of the cells alive. These results demonstrate that EPA and DHA are able to eliminate SCC cells at a physiological dose (3 μM) without killing normal keratinocytes ($P<0.05$). Also, EPA eliminated SCC cells without killing the normal cells at 5 μM ($P<0.05$).

A.

B.

Figure 3-2. The effect of PUFA on keratinocyte cell growth

*Means± S.E.M of 6 independent MTT experiments for SCC-13, SCC-25, SVFHK and 4 experiments for NHEK-131 and HEK-127. Each experiment was conducted in duplicate. Cells were incubated in different DHA (A) and EPA (B) concentrations for 4 days in KGM serum-free medium. S.E.M=Standard error of means. * is significantly different from the mean value of untreated control of each cell line (* = p<0.05 and ** = p <0.01 *** = <0.001 as measured by one-way ANOVA followed by post-hoc Bonferroni test).*

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We extended our study in three oral dysplasia (premalignant) cell lines D17, D19 and D20 (figure 3-3). DHA (figure 3-3A) strongly inhibited all the dysplasias at concentrations 3-10 µM except from D19 which was more resistant at 3 µM (76% viable cells) but was highly inhibited at 5-10 µM. As regards EPA (figure 3-3B), it was very potent inhibiting D20 cell growth at 3-10 µM while D17 and D19 were more resistant at 3 µM (53% and 57% viable cells respectively), but were eliminated at 5-10 µM.

More normal cells were also included in the study. Three normal oral keratinocytes, NHOK-846, NHOK-881 and NHOK-810, were used and also the normal immortal oral cell lines OKF4 (TERT p16$^{-/-}$ p53$^{-/-}$) and OKF6 (TERT p16$^{+/-}$ p53$^{+/+}$) (figure 3-4). DHA (figure 3-4A) appeared to inhibit all normal cells at concentrations higher than 5 µM. At 3 µM of DHA though, 73% of OKF6, 40% of NHOK-881 and 30% of OKF4 were viable. EPA (figure 3-4B) seemed friendlier to the normal cells compared to DHA at 3 µM, but affected cell growth more in concentrations higher than 5 µM. Even at that concentration of EPA, 46% of OKF4, 62% of OKF6, 28% of NHOK-881 and 26% of NHOK-810 were still viable.

Figures 3-5 and 3-6 summarise the effect of EPA and DHA, respectively, on the growth of all the cells used in the study at the concentrations of 3 µM and 5 µM for EPA and just 3 µM for DHA. Overall, the most potent concentration of DHA was 3 µM, which caused more death to malignant and premalignant cells (except from SCC-13 epidermal cells and D20 oral dysplasia) without completely eliminating all normal cells. As regards EPA, it appeared more selective, as it inhibited growth of premalignant and malignant cells more than in normal cells at 3 µM and 5 µM. Figure 3-7 compares the means±S.E.M of the considered malignant cells (SCC-13, SCC-25), premalignant (SVFHK, D17, D19, D20) and the normal cells (OKF4, OKF6, NHEK-131, HEK-127, NHOK-846, NHOK-881 and NHOK-810) showing selectivity of DHA (at 3 µM) and EPA (at 3 µM and 5 µM) in growth inhibition towards malignant and premalignant cells compared to normal keratinocytes. Figure 3-8 shows the epidermal (figure 3-8A) and the oral (figure 3-8B) keratinocytes separately. As regards the epidermal keratinocytes (figure 3-8A), the means± S.E.M of the malignant (SCC-13), premalignant (SVFHK) and the normal epidermal cells (NHEK-131, HEK-127) are demonstrated, showing selectivity of DHA (at 3 µM)

and EPA (at 3 μM and 5 μM) in growth inhibition towards malignant and premalignant cells compared to normal keratinocytes with the pre-malignant cells being more susceptible than the malignant ones. As regards the oral keratinocytes (figure 3-8B), the means± S.E.M of the malignant (SCC-25), dysplasias (D17, D19, D20), the normal immortal (OKF4, OKF6) the normal primary oral cells (NHOK-846, NHOK-881 and NHOK-810) are shown. There is selectivity of DHA (at 3 μM) and EPA (at 3 μM and 5 μM) in growth inhibition towards malignant and premalignant cells compared to normal keratinocytes, with the malignant cells being more susceptible than the dysplasias.

A.

B.

Figure 3-3. The effect of PUFA on cell growth in dysplasias

*Means± S.E.M of 3 independent MTT experiments for dysplasias D17, D19 and D20. Each experiment was conducted in duplicate. Cells were incubated in different DHA (A) and EPA (B) concentrations for 4 days in KGM serum-free medium. S.E.M=Standard error of means. * is significantly different from the mean value of untreated control of each cell line (* = p<0.05 and ** = p <0.01 *** = <0.001 as measured by one-way ANOVA followed by post-hoc Bonferroni test).*

A.

B.

Figure 3-4. The effect of PUFA on normal keratinocyte cell growth

*Means± S.E.M of 3 independent MTT experiments for normal keratinocytes OKF4, OKF6, NHOK-846, NHOK-881 and NHOK-810. Each experiment was conducted in duplicate. Cells were incubated in different DHA (A) and EPA (B) concentrations for 4 days in KGM serum-free medium. S.E.M=Standard error of means. * is significantly different from the mean value of untreated control of each cell line (* = p<0.05 and ** = p <0.01 *** = <0.001 as measured by one-way ANOVA followed by post-hoc Bonferroni test).*

A.

B.

Figure 3-5. Overview of the effect of EPA on keratinocyte cell growth

Overview of the means± S.E.M of the MTT viability test with EPA 3 μM (A) and 5 μM (B) for malignant cells, dysplasias/pre-malignant and normal keratinocytes used. S.E.M=Standard error of means.

Figure 3-6. Overview of the effect of DHA on keratinocyte cell growth

Overview of the means± S.E.M of the MTT viability test with DHA 3 μM for malignant cells, dysplasias/pre-malignant and normal keratinocytes used. S.E.M=Standard error of means.

Figure 3-7. Overview of the effect of PUFA on keratinocyte cell growth

*Overview of the means± S.E.M of the MTT viability test with DHA 5 μM and 3 μM and 5μM of EPA for malignant cells, pre-malignant and normal keratinocytes averages. S.E.M=Standard error of means. * is significantly different from the mean value of normal keratinocytes (* = p<0.05 and ** = p <0.01 *** = <0.001 as measured by Mann-Whitney U rank test).*

A.

B.

Figure 3-8. Overview of the effect of PUFA on epidermal and oral keratinocyte cell growth

Overview of the means± S.E.M of the MTT viability test with DHA 5 μM and 3 μM and 5μM of EPA for malignant cells, pre-malignant and normal keratinocytes averages of (A) epidermal keratinocytes and (B) oral keratinocytes. S.E.M=Standard error of means. * is significantly different from the mean value of normal cells while red star * is significantly different from the mean value of the immortal normal oral cells (B) (* = p<0.05 and ** = p <0.01 *** = <0.001 as measured by Mann-Whitney U rank test).

3.2.2 Detection of Apoptosis

Initially, the flow cytometric annexin V/DAPI assay was performed in order to determine whether the cells were undergoing an apoptotic death after treatment with PUFA. This assay is very sensitive, because it is able to detect even the early apoptotic cells, as annexin V antibody binds with high affinity to PS (sub-chapter 2.4). The results are the averages of three independent experiments that were performed, analysing at least 10,000 cells. The cells were incubated in DHA or EPA at 5 μM or 10 μM for 48 h before analysis.

The assay showed a marked difference in the percentage of viable cells between untreated and treated cells after only 48 h treatment (figures 3-10 and 3-11). The viable cell percentages appear to decrease significantly (figures 3-10 and 3-11) while the early apoptotic cell percentage (annexin V +ve and DAPI –ve) as well as the late apoptotic and necrotic cell percentage (annexin V +ve and DAPI +ve) are increasing (figure 3-12). It is obvious that the effect of PUFA on the cells is cytotoxic, as they appear to cause cell death which appears to be apoptosis. A representative FACS analysis experiment is shown (figure 3-9).

In order to confirm the apoptotic cell death the apoptosis assay was followed by western blot analysis for detection of cleaved caspases. The SCC-25 cells were treated with 10 μM of DHA or EPA for 6, 17 and 24 hours and their lysates were obtained. A lysate of NHEK cells treated with cisplatin for 24 h was used as a positive control for high levels of cleaved caspase 3, while GAPDH was used as a loading control. Western blot analysis proved the cleavage of the apoptosis executioner caspase 3. Cleaved caspase 3 antibody detects the endogenous levels of the large fragments (17/19 kDa) of the activated caspase 3 resulting from cleavage. There is an up-regulation of cleaved caspase 3 after treatment of the SCC-25 cells with DHA for 5 h, and after treatment with EPA for 17 h. There was also an upregulation of cleaved caspase 8 (extrinsic pathway) and caspase 9 (intrinsic pathway) at the specific timepoints demonstrating the involvement of both apoptotic pathways (figure 3-13).

A.

B.

C.

D.

Figure 3-9: Apoptosis assay

Representative flow cytometry plots for annexin V-FITC/DAPI for cellular viability. The scatter plot (A) is shown for the untreated control where SSC=side scatter and FSC= forward scatter and the fluorescence plots are shown for each sample (B, C, D). SCC-25 cells were used in this example. Untreated control cells are shown in (B). Cells were treated with DHA 5 μM (C) and EPA 5 μM (D) for 48 h. Q3 shows the viable cell population (annexin V -ve, DAPI -ve), Q4 shows early apoptotic cells (annexin V +ve DAPI –ve), Q2 shows late apoptotic and necrotic cells (annexin V +ve, DAPI +ve) and Q1 shows only necrotic cells (annexin V –ve, DAPI +ve).

A.

B.

Figure 3-10. Means of viable cells percentages in the apoptosis assay

*Means± S.E.M of 3 independent annexin V/DAPI experiments. SCC-13, SCC-25, SVFHK and HEK-127 cells were incubated in 5 μM and 10 μM of DHA for 48 hours in KGM serum-free medium prior to trypsinisation. Then the cells were stained with annexin V and DAPI and analysed by flow cytometry. The percentages of viable cells (A) and the normalised with the control percentages (B) are shown. * is significantly different from the mean value of untreated control of each cell line (* = p<0.05 and ** = p <0.01 *** = <0.001 as measured by one-way ANOVA followed by post-hoc Bonferroni test).*

A.

B.

Figure 3-11. Means of viable cells percentages in the apoptosis assay

Means± S.E.M of 3 independent annexin V/DAPI experiments. SCC-13, SCC-25, SVFHK and HEK-127 cells were incubated in 5 µM and 10 µM of EPA for 48 hours in KGM serum-free medium prior to trypsinisation. Then the cells were stained with annexin V and DAPI and analysed by flow cytometry. The percentages of viable cells (A) and the normalised with the control percentages (B) are shown. * is significantly different from the mean value of untreated control of each cell line (* = p<0.05 and ** = p <0.01 *** = <0. as measured by one-way ANOVA followed by post-hoc Bonferroni test).

A.

B.

Figure 3-12. Means of early apoptotic and necrotic cells percentages of apoptosis assay

*Means± S.E.M of 3 independent annexin V/DAPI experiments. SCC-13, SCC-25, SVFHK and HEK-127 cells were incubated in 5 μM and 10 μM of DHA (A) and EPA (B) for 48 hours in KGM serum-free medium prior to trypsinisation. Then the cells were stained with annexin V and DAPI and analysed by flow cytometry. The percentages of early apoptotic and necrotic cells are shown. * is significantly different from the mean value of untreated control of each cell line (* = p<0.05 and ** = p <0.01 *** = <0.001 as measured by one-way ANOVA followed by post-hoc Bonferroni test).*

Figure 3-13. Cleavage of caspases on western blot

Representative western blots showing the cleavage of caspases 3, 9 and 8. Lysates are derived from SCC-25 cells treated with PUFA (10 μM). The experiment was repeated three times. The positive control is NHEK treated with cisplatin for 24h.

3.2.3 PUFA decrease proliferation of human oral cancer keratinocytes

To assess whether omega-3 PUFA alter tumour cell proliferation, we investigated the effects of EPA and DHA on ^{3}H-thymidine incorporation in SCC-25 cells. Treatments with 3 μM DHA and 3 μM EPA for 48 h significantly (p<0.001) decreased ^{3}H-thymidine incorporation compared to the untreated control (figure 3-14) which indicates a highly lower rate of DNA synthesis and proliferation following exposure to omega-3 PUFA .

Figure 3-14. ^{3}H-thymidine incorporation assay

Means± S.E.M of 6 independent ^{3}H-thymidine incorporation experiments. SCC-25 cells were incubated in 3 μM DHA and EPA for 48 hours in KGM serum-free medium and ^{3}H-thymidine was added 18 h prior to the incorporated thymidine measurement. The values were normalised to a percentage of the untreated control which was taken as 100%. * is significantly different from the mean value of the untreated control (*** = p<0.001 as measured by one-way ANOVA followed by post-hoc Bonferroni test).

3.3 Discussion

3.3.1 Effect of PUFA on malignant SCC cells

It is widely accepted that PUFA have a great anti-tumour effect and a potential to become successful prophylactic and therapeutic tools in cancer. As mentioned before, EPA and DHA have been observed to suppress cell growth and induce apoptosis in several types of human cancer cell lines like breast cancer (DeGraffenried *et al*, 2003; Schley *et al*, 2005) colon cancer (Chen & Istfan, 2000; Eitsuka *et al*, 2005; Habermann *et al*, 2009) lymphoma (Heimli *et al*, 2002), leukemia (Chiu & Wan, 1999; Finstad *et al*, 1998) neuroblastoma (Gleissman *et al*, 2010) hepatoma (Murata *et al*, 2001), pancreatic cancer (Merendino *et al*, 2005) prostate cancer (Edwards *et al*, 2008) and several others (Edwards *et al*, 2008; Merendino *et al*, 2005; Serini *et al*, 2008).

In this study, I examined whether PUFA specifically inhibit SCC growth at physiological concentrations, using the epidermal SCC-13 and the oral SCC-25 cell lines. DHA and EPA showed significant suppressive effects on the survival of malignant cells after only 4 days at concentrations in the physiological range (figure 3-2). PUFA were particularly effective on oral cancer keratinocytes SCC-25 as they eliminated most viable cells at concentrations $\geq 3 \mu M$. The epidermal squamous cell carcinoma cell line SCC-13 showed higher resistance compared to SCC-25; however, their growth seemed to be significantly decreased by the PUFA as well, at concentrations $>3 \mu M$. We showed, for the first time to our knowledge, that EPA and DHA affect oral and epidermal SCC cell growth.

Our study was extended in the pre-malignant SVFHK cell line and three dysplasias, D17, D19 and D20. DHA and EPA showed great inhibitory effects on the survival of pre- malignant SVFHK cells after only 4 days, eliminating the viable cells at concentrations $\geq 3 \mu M$ (figure 3-2). The dysplasias growth was also negatively affected by PUFA (figure 3-3). D20 was very sensitive to both DHA and EPA ≥ 3 μM. D17 was sensitive to DHA ≥ 3 μM and EPA ≥ 5 μM. However, 57% of the cells

were viable at 3 μM of EPA. D19 seemed to be the most resistant at 3 μM of the PUFA, especially for DHA (76% viability) but very sensitive at concentrations ≥5 μM. Generally, PUFA were potent in inhibiting oral premalignant cells; however, some dysplasias were more sensitive than others. D19 which is severe lateral tongue erythroleukoplakia seems to be more resistant than D20, which is a moderate lateral tongue leukoplakia and very sensitive to PUFA. D17 which is buccal mucosa mild/moderate leukoplakia was more sensitive than D20 but less sensitive than D19 to EPA. It seems that the degree of sensitivity of dysplasias to PUFA depends on the type (erythroplakia or leukoplakia) and stage of oral dysplasia (mild, moderate, severe), and the position of origin.

Several studies carried out on cultured cancer cells in vitro have demonstrated the pro-apoptotic effect of EPA or DHA (Serini *et al*, 2009). The available data indicate that apoptosis induced by EPA or DHA may follow both the extrinsic (involving caspase 8 and 3) and intrinsic (involving mitochondria and caspase 9 and 3) apoptotic pathways. Some studies demonstrated that these compounds caused apoptosis by triggering only the intrinsic pathway. Some examples are the studies where PUFA increased the activity of caspase-3 and -9 in lymphoma cells (Heimli *et al*, 2002) and in hepatocellular carcinoma lines (Lim *et al*, 2009). However, in many studies the activation of both pathways of apoptosis is observed which agree with our results. In human colon cancer cells DHA has been reported to increase the expression of both the caspases 8 and 9, and the activation of cytochrome c (Narayanan *et al*, 2001), and both EPA and DHA caused cleavage of caspase 8 with additional downregulation of Bid protein expression and Bcl-2 mRNA. Tsuzuki reported induction of several genes associated with both pathways of apoptosis by conjugated EPA, such as those who encode for Fas, Bid, Bas, Bak, caspase 3, 8, 7 and 9 by RT-PCR and microarrays (Tsuzuki *et al*, 2007). Which pathway of apoptosis is induced may be related to the different actions of these fatty acids in different cells, to the different omega-3 PUFA used (EPA, DHA or fish oil) and to the different ways of administration.

In the present study, the flow cytometric annexin V/DAPI apoptosis assay showed that the epidermal SCC-13, the oral SCC-25, and the pre-malignant SVFHK cell line cells were undergoing an apoptotic death after treatment with PUFA. As mentioned

before, annexin V detects the early apoptotic cells because it binds to PS which translocate from the interior side of the plasma membrane to the outer layer during the initial stages of apoptosis. DAPI detects the late apoptotic cells and the necrotic cells. Decrease of the viable cell population was detected after 48 h PUFA treatment (figures 3-10 and 3-11) and subsequent increase of the early apoptotic and late apoptotic/necrotic cell population (figure 3-12). This demonstrates that the growth inhibitory effect of PUFA on the cells is at least partially cytotoxic, as they appear to cause cell death via apoptosis. Western blot analysis confirmed that the PUFA cause apoptosis via the apoptosis cleavage of caspase 3. Caspase 3 is one of the key executioners of apoptosis as it is responsible for the engagement of the full caspase cascade necessary to destroy the cell (sub-chapter 3.1). The cleavage of caspase 8 and caspase 9 were also detected, demonstrating the involvement of extrinsic and the intrinsic apoptotic pathways, respectively (figure 3-13). This could mean that both EPA and DHA trigger both apoptotic pathways at the same time in order to destroy the cells. However, there is another potential explanation. At the extrinsic apoptotic pathway, caspase 8 is the initiator caspase and can subsequently cause the activation of execution caspase-3 which results in apoptosis. As mentioned before (subchapter 3.1), this can be done by two alternative pathways downstream of caspase 8 activation: In most cells, stimulation of death receptors causes enough activated caspase 8 to result in caspase 3 cleavage activating the full caspase cascade necessary for the apoptosis of the cell. However, some cell types don't manage to activate enough caspase 8, so the pro-apototic signal of caspase 8 causes the proteolysis of Bid, the engagement of cytocrome c/Apaf-1 and caspase 9 cleavage of the intrinsic mitochondrial pathway (Logue and Martin 2008). It is possible that the same applies to our cells as well.

In addition, it has been observed that PUFA inhibit cancer cell proliferation. Schley *et al*, showed that EPA and DHA significantly decreased breast cancer cell proliferation as estimated by decreased [methyl-3H]-thymidine uptake and expression of proliferation-associated proteins (proliferating cell nuclear antigen, PCNA, and proliferation-related kinase, PRK) as measured by western blotting (Schley *et al*, 2005). In addition, Schley *et al* showed an increase in DNA fragmentation, loss of mitochondrial membrane potential, and increased activity of caspase proteins supporting the hypothesis that omega-3 PUFA also induce apoptotic

cell death. The results of my study agree with these observations as it was observed that, in the oral cancer SCC-25 cell line, treatment with 3 μM of DHA and EPA for 48 h significantly (p<0.001) decreased ^{3}H-thymidine incorporation compared to the untreated control, indicating a highly lower rate of DNA synthesis and cell proliferation following exposure to omega-3 PUFA. The ^{3}H-thymidine incorporation after DHA treatment was just 2.5% and after EPA treatment was 27% of the untreated control which demonstrates a marked decrease for both of them but bigger for DHA. Similarly to Schley *et al*, I already have shown that PUFA also cause apoptosis by the annexin V assay and via caspase cleavage. This means that DHA and EPA are not only cytotoxic but also cytostatic as it was demonstrated that omega-3 PUFA both impair proliferation and induce apoptosis of oral cancer cells.

3.3.2 Effect of PUFA on normal keratinocytes

Different studies have investigated the ability of omega-3 PUFA to regulate the apoptotic process in normal cells. It appears that PUFA protect against chronic neurodegenerative diseases and atherosclerosis by inhibiting apoptosis. The beneficial effects of omega-3 PUFA have been documented in a variety of central nervous system disorders, including Zellweger's syndrome, schizophrenia, depression and Alzheimer's disease (Michael-Titus, 2007). Moreover, omega-3 PUFA appear to have significant neuroprotective potential in spinal cord trauma. In rats, when DHA was administered intravenously 30 min after spinal cord injury (King *et al*, 2006) it induced significant neuroprotection, reducing neuronal cell loss, oligodendrocyte loss, and decreasing the apoptosis 1 week after the injury. It has been also demonstrated that treatment of resting human umbilical vein endothelial cells with DHA reduces oxidative stress and apoptosis (Pfrommer *et al*, 2006), explaining the atheroprotective effects of omega-3 PUFA.

DHA is the most abundant fatty acid in the brain and generally the membrane of the neural cells are enriched in omega-3 fatty acids (Dyall & Michael-Titus, 2008). DHA

is an essential fatty acid during development and prevents neuronal cell death (Kim, 2007). Even though normal nervous tissue has high levels of omega-3 PUFA, neuronal tumours, such as gliomas, are deficient in DHA (Martin *et al*, 1996). This suggests that DHA depletion could be an adaptation survival mechanism of neuronal tumours. In normal nervous tissue, the active lipid mediators of DHA, resolvins and neuroprotectins, decrease inflammation partly through the inhibition of oxidative stress and apoptosis (Mukherjee *et al*, 2004). In more detail NPD1 appear to up-regulate the anti-apoptotic proteins Bcl-2 and Bcl-xL and down-regulate the expression of the pro-apoptotic proteins Bax and Bad in neurons and human retinal pigment epithelial cells (Bazan, 2005; Mukherjee *et al*, 2004). Gleissman showed that, in neuroblastoma cell lines, exogenous DHA is oxygenated to the monohydroxy fatty acids 17-HDHA, 14-HDHA, 7-HDHA, and 4-HDHA, but not to resolving RvD1 and protectin PD1 despite the presence of the enzymes required for this their production (Gleissman *et al*, 2010). Even though this mechanism might be tumour survival mechanism against resolution of inflammation, however makes the tumour cells more susceptible to DHA than the normal cells which actually convert it to resolvins and protectins. It is possible that something similar happens in our case where SCC cells more susceptible to PUFA induced apoptosis compared to the normal keratinocytes.

On the other hand, PUFA may cause apoptosis in normal cells. A number of *in vivo* studies have demonstrated that omega-3 PUFA affect normal colonic mucosa cells by inducing apoptosis. This has been viewed as a chemopreventive effect, since this specific cell population is highly exposed to carcinogenic agents from the diet, and is subject to high turnover. In rats treated with EPA or DHA (Calviello *et al*, 1999) cell proliferation was suppressed, and the number of apoptotic cells in colon mucosa was increased, without altering the homeostasis of normal colonic mucosa. The enhanced colonocyte deletion in normal mucosa was observed also in humans treated with a mixture of EPA and DHA for 2 years (Cheng *et al*, 2003a). These findings support the hypothesis that the dietary intake of omega-3 PUFA, at appropriate levels, may display a strong chemopreventive action against colon cancer, and this protective action is based on the induction of apoptosis in colonocytes. It was also observed that DHA induces apoptosis in cultured rat mesenteric vascular smooth muscle cells (Diep *et al*, 2000). Since the apoptosis of these cells may affect the structure of blood

vessels, it has been suggested that this effect explains the blood pressure-lowering effect caused by omega-3 PUFA in hypertension. It has been also demonstrated that DHA induces apoptosis in proliferating human umbilical vein endothelial cells, which has been related to the anti-angiogenic and anti-tumoural effects of omega-3 PUFA (Kim *et al*, 2005).

In the present study, normal keratinocytes were used as controls, to test the cancer specificity of PUFA. In the normal foreskin epidermal keratinocytes NHEK-131 and HEK-127 initially tested (figure 3-2), while PUFA eliminated the viable cells at 10 μM, they did not have a large effect on cell survival at smaller doses. Approximately 60% and 100% of the cells were viable at 3 μM DHA and EPA, respectively. However, while EPA had little effect on the survival of normal cells at 5 μM, DHA seemed to negatively affect normal cell growth at that concentration. The flow cytometric annexin V/DAPI apoptosis assay confirmed that the higher concentrations of PUFA inhibit normal HEK-127 cells, causing apoptosis. The higher percentage of death in the annexin V/DAPI assay for the EPA treatment in figures 3-11 and 3-12B, even at the control cells, compared to the MTT is probably due to the trypsinisation of the cells before the flow cytometry. Primary cells are, generally, more delicate and sensitive to trypsinisation than the malignant cells. Also, the annexin V/DAPI assay detects the early apoptotic cells that are not recognised by the MTT assay.

The study was later expanded in oral normal primary keratinocytes NHOK-810, NHOK-846 and NHOK-811 and also two normal immortal oral cell lines OKF4 and OKF6 (figure 3-4). MTT assays were performed using these cells. OKF6 was more resistant to PUFA inhibitory effect. There is even a growth stimulating effect at 1 μM. The growth is decreased very little at concentrations 3-5 μM of EPA and 3 μM of DHA. OKF4 is more sensitive, especially to DHA. However, a significant percentage of cells are viable at 3-5 μM of EPA (64% and 46%). The normal oral primary cells NHOK-810, NHOK-846 and NHOK-881 seemed more sensitive at PUFA higher concentrations >5 μM but more resistant at 3 μM of EPA.

Overall, I can conclude that PUFA, at least at the free fatty acid form that were used in the present study, can be cytotoxic for normal cells. The apoptosis observed in

normal cells could be explained by the hypothesis that omega-3 PUFA may display a chemopreventive action against oral cancer, like in colon cancer, by induction of apoptosis in keratinocytes, similar to colonocytes, since this the oral tissue is highly exposed to harmful chemicals and carcinogenic agents from the diet, as well.

However, PUFA are more tolerated by the normal cells than SCC cells, the premalignant cells and most of the dysplasias at doses 3-5 μM of EPA and 3μM of DHA (figures 3-5 and 3-6). Especially, EPA shows higher selectivity in killing malignant and premalignant more than normal cells. The selectivity of the most potent concentrations of DHA and EPA were demonstrated at figure 3-7 which summarises the means of the viable percentages of the cells comparing malignant, premalignant (SVFHK and dysplasias) and normal keratinocytes. The pattern observed is that PUFA inhibit malignant cells more, then premalignant and less of all the normal cells at 3 μM. At 5 μM of EPA premalignant cells are even more inhibited than the malignant ones and the normal cells are again the least suppressed. Figure 3-8 shows the selectivity of the same concentrations of PUFA separately for epidermal and oral keratinocytes, summarising the means of the viable percentages of the cells comparing malignant, premalignant (dysplasias) and normal keratinocytes. As regards the epidermal keratinocytes (figure 3-8A), EPA 3 μM and 5 μM seem to be more potent eliminating the pre-malignant SVFHK cells, than the malignant SCC-13 and affecting a little or not at all (105% and 83% respectively) the normal keratinocytes. As regards the oral keratinocytes (figure 3-8B), again EPA seems to be the most potent and selective especially at 3 μM where preferably inhibits the growth of the malignant SCC-25, then the dysplasias and a lot less the immortal normal cells and the primary oral cells (75% and 64% respectively).

In summary, EPA seemed to be better tolerated by the normal epidermal cells. For final conclusions, PUFA need to be tested on animal models in the future, as the normal cells proliferate more in culture than *in vivo*, so the differential effects on normal and SCC growth may be even greater. Also, the normal keratinocytes in culture are under stress compared to physiological conditions, making them more susceptible to death. It was obvious in our study, that the immortal normal cells OKF4 and OKF6, which seem to be under optimal conditions in culture, were less susceptible to PUFA death that the other oral primary keratinocytes. However, the

existing results are really promising as they demonstrate that EPA and DHA are able to eliminate SCC cells at a physiological doses (3 μM for both or 5 μM for EPA) without totally eliminating normal keratinocytes (P<0.05).

Chapter 4

EFFECT OF TELOMERASE ON PUFA ACTION

CHAPTER 4: Effect of telomerase on PUFA action

4.1 Background

Telomerase was originally discovered by Elizabeth Blackburn and Carol Greider who recently shared the 2009 Nobel Prize in Medicine. Telomerase is an enzyme that maintains the ends of mammalian chromosomes (telomeres) by adding TTAGGG repeats to them (figure 4-1). This is the telomerase canonical function. Telomerase consists of a catalytic subunit called *TERT* and an RNA component, *TERC*, which serves as a template for nucleotide addition. Telomerase function blocks telomere erosion and therefore suppresses cellular senescence and apoptosis and promotes tumour progression. It is not surprising that high levels of telomerase activity are detected in most tumours and specifically in 90% of cases of oral squamous-cell carcinoma (OSCC). Telomerase can also be detected at high levels in germ line cells, where the enzyme is active maintaining the lengths of telomeres, while in most somatic cells telomerase activity is very low (McCaul *et al*, 2002; Parkinson & Minty, 2007). It was observed that the correction of telomere dysfunction by the forced expression of *TERT* can reconstitute the function of telomerase activity (Weinrich *et al*, 1997) and lead to the extension of the lifespan of normal human cells (Bodnar *et al*, 1998).

Apart from the canonical function of telomerase, namely telomere lengthening, the catalytic subunit TERT has also been investigated for "non-canonical" functions, which can either require the presence of *TERC* or not. TERT appears to play an important role in promoting the formation of skin (Gonzalez-Suarez *et al*, 2001) and mammary tumours (Artandi *et al*, 2002) upon chemical carcinogenesis *in vitro,* independently from telomere lengthening function. More evidence that TERT can function independently of the telomerase lengthening derives from the use of the *TERT-HA* gene construct. *TERT-HA* is a variant of *TERT* that contains a hemaglutinin (HA) epitope inserted at the C-terminus. The TERT-HA protein was catalytically active but not able to elongate telomeres or extend cellular replicative life span in fibroblasts (Counter *et al*, 1998). In addition, the ectopic expression of

TERT as well as *TERT-HA* imparted a tumourigenic phenotype in normal human cells that maintained telomeres by a telomerase-independent mechanism called Alternative Lengthening of Telomeres (ALT) (Stewart *et al*, 2002). In summary, telomerase activity does not necessarily depend on its ability to maintain telomeres (Cong & Shay, 2008; Parkinson *et al*, 2008).

Eitsuka *et al* suggested that the omega-3 PUFA inhibit telomerase in cell free assays and cell culture experiments, using DLD-1 human colorectal adenocarcinoma cells. The athors also suggested that omega-3 PUFA inhibited the expression of the *hTERT* gene, in parallel with *CMYC* and protein kinase C (*PKC*) (Eitsuka *et al*, 2005). However, it is still unclear whether the growth inhibition by PUFA depends on telomerase (or TERT) inhibition, or the reverse.

A.

B.

Figure 4-1. Telomerase and telomeres

(A) The telomerase complex consists of the reverse transcriptase component (TERT), the RNA component (TERC), the protein dyskerin, and other associated proteins (NHP2, NOP10, and GAR1). Telomerase adds telomeric repeats (TTAGGG) to the 3' hydroxyl end of the leading strand of the telomere, with a sequence in TERC serving as the template for nucleotide addition (Buckingham & Klingelhutz, 2011).

(B) Schematic representation of the chromosomes and the telomeres (end of chromosomes) (Szostak, 2010).

4.2 Results

4.2.1 Investigation of the possible telomerase activity inhibition induced by PUFA

It has been suggested that the omega-3 PUFA inhibit telomerase in colon adenocarcinoma DLD-1 cells (Eitsuka *et al*, 2005), so I examined whether EPA and DHA inhibit immortal HaCaT keratinocyte growth and telomerase activity. Moreover, I investigated whether any growth inhibitory effects of PUFA are dependent on the transcriptional down regulation of *TERT* and/or its transcriptional activator *CMYC*. The role of *CMYC* was tested by using HaCaT over-expressing *CMYC (Cerezo et al, 2002)* or the same cell line over-expressing *hTERT*, or an oncogenic *hTERT* variant that cannot lengthen telomeres.

HaCaT and DLD-1 cells were incubated in different concentrations of DHA and EPA. After 5 days incubation, the MTT assay was used to determine the number of viable cells. The cells were incubated in RPMI 1640 medium containing 3% v/v FBS (Fetal Clone II serum) for 5 days. DHA and EPA, at concentrations >10 μM, had a marked effect on the cell viability of HaCaTs and DLD-1 (figure 4-2). The higher dose of PUFA (50 μM) showed 80-95% cell growth inhibition. According to the results, this effect does not seem to be related to TERT, TERT-HA and c-MYC over-expression, as there is no significant difference between the viable cells of HaCaT-Puro and HaCaT-TERT, -TERT-HA and –c-MYC (P>0.05) at 30 μM and 50 μM of PUFA.

Surprisingly, when the cells were incubated in RPMI containing 10% FBS (figure 4-3), PUFA did not appear to have any major effect on cell viability. This phenomenon was investigated further in chapter 7.

A.

B.

Figure 4-2: The effect of PUFA on HaCaT and DLD-1 cell growth in 3% FBS medium
Means± S.E.M of 3 MTT experiments. Each experiment was conducted in duplicate. HaCaT-PURO, HaCaT-TERT, HaCaT-TERT-HA, HaCaT-c-MYC, and DLD-1 cells were incubated in 3% FBS medium with different (A) DHA and (B) EPA concentrations.

A.

B.

Figure 4-3: The effect of PUFA on HaCaT and DLD-1 cell growth in 10% FBS medium

Means± S.E.M of 3 MTT experiments. Each experiment was conducted in duplicate. HaCaT-PURO, HaCaT-TERT, HaCaT-TERT-HA, HaCaT-c-MYC, and DLD-1 cells were incubated in 10% FBS medium with different (A) DHA and (B) EPA concentrations.

4.3 Discussion

4.3.1 Investigation of the possible role of telomerase activity on PUFA effect

It has been suggested that the omega-3 PUFA inhibit telomerase in cell free assays and cell culture experiments with DLD-1 colorectal adenocarcinoma cells. Moreover, omega-3 PUFA appeared to inhibit the expression of the *hTERT* gene, in parallel with *CMYC* and protein kinase C (*PKC)* (Eitsuka *et al*, 2005). Acute inhibition of telomerase by short hairpin RNAs causes an immediate effect on cancer growth and survival in a telomerase-specific manner (Li *et al*, 2005). *CMYC* is a positive regulator of *TERT* transcription (Wu *et al*, 1999) but it is unclear whether *CMYC* mediates the repression of *TERT* by PUFA. Furthermore, it is still unclear whether the growth inhibition by PUFA is dependent on telomerase (or TERT) down-regulation and inhibition, or the reverse.

In this study, I examined whether EPA and DHA inhibit immortal HaCaT keratinocyte growth, and whether any growth inhibitory effects are modified by over-expression of *CMYC*, *TERT* or *TERT-HA* which is an oncogenic *TERT* variant that cannot lengthen telomeres. In order to test this, we used HaCaT over-expressing *CMYC* (Cerezo *et al*, 2002) or the same cell line over-expressing *TERT*, or *TERT-HA*. DLD-1 cells were also used as a control, because the published experiments were performed using this cell line (Eitsuka *et al*, 2005). The hypothesis that *TERT* down-regulation and hence *CMYC*, as it regulates *TERT*, caused cytostasis and/or cell death would be supported if ectopic *MYC* or *TERT* expression blocked or reduced growth inhibition. DHA and EPA had a marked effect on cell viability of HaCaTs and DLD-1 after 5 days incubation in a 3% v/v FBS containing medium, however, the growth inhibition was not affected by *TERT*, *TERT-HA* and *CMYC* over-expression. This means that overexpression of *TERT*, *TERT-HA* and *CMYC* does not protect the cells from the growth inhibitory effect of PUFA and as a result, the down-regulation of *TERT* and *CMYC*, reported by others (Eitsuka *et al*, 2005), is most likely to be only a consequence and not the cause of PUFA-induced death at these doses. Further investigation is essential in order to determine whether the

inhibition of telomerase is the cause or the result of PUFA-induced cell death, examining the telomerase activity at doses of DHA and EPA that do not cause extensive cell death. However, due to time limitations, this possibility was not investigated further during this project.

Interestingly, when the cells were incubated in RPMI containing 10% v/v FBS (figure 4-3), PUFA did not showed any marked cell growth inhibition. This observation indicates that one or more components of the serum seem to provide protection of the cells against DHA and EPA. This was investigated further and is discussed in chapter 7.

Chapter 5

REACTIVE OXYGEN SPECIES

CHAPTER 5: Reactive oxygen species

5.1. Background

Oxygen is necessary for life but can be poisonous, so aerobic organisms survive its presence by antioxidant defence mechanisms (Halliwell, 2007a). Small amounts of potentially toxic reactive oxygen species (ROS) are generated in eukaryotic cells by oxidase and during electron transport in the mitochondria or the endoplasmic reticulum. Leaks of electrons can produce superoxide radicals (O_2^-), which may be reduced, giving rise to hydrogen peroxide (H_2O_2) (Girotti, 1998).

Oxidative stress refers to a serious imbalance between ROS production and antioxidant defences, and it can cause damage to cells, often called 'oxidative damage' (Halliwell, 2007a). Halliwell and Whiteman defined oxidative damage as "the biomolecular damage caused by attack of ROS upon the constituents of living organisms" (Halliwell & Whiteman, 2004). Oxidative damage can result either from oxidative stress or from defects in the repair systems (Halliwell, 2007a; Halliwell & Whiteman, 2004)

PUFA and the long-chain omega-3 PUFA in particular, are susceptible to free radical attack that leads to lipid peroxidation. The formation of lipid hydroperoxides begins with the removal of hydrogen from the unsaturated fatty acids by ROS. The lipid radical which is produced then reacts with oxygen and forms a fatty acid peroxyl radical. This product is able to attack fatty acids in cell membranes, propagating lipid peroxidation (Biondo *et al*, 2008). Lipid peroxidation can injure the mitochondria, which results in further ROS generation (Catala, 2009). The major effects of the products of lipid peroxidation are inhibition of DNA synthesis, cell division and tumour growth, and induction of tumour cell death (Girotti, 1998). However, peroxidation of lipids can disturb the membrane assembly, changing the fluidity and permeability and inhibiting metabolic processes (Catala, 2009). Chemotherapy drugs belonging to the anthracycline family appear to induce tumour cell death partly by ROS formation, which can cause irreversible damage of cancer cells (Malhotra & Perry, 2003). The long-chain omega-3 PUFA appear to increase the potency of these

drugs by elevating the production of oxygen free radicals (Biondo *et al*, 2008). Also, several studies suggest that the increase in ROS generation might be one of the mechanisms of omega-3 PUFA anti-tumour action (Arita *et al*, 2001; Gleissman *et al*, 2010; Maziere *et al*, 1999; Tsuzuki *et al*, 2007). It seems as though the right amount of ROS is needed to promote cell proliferation and cancer; beyond a threshold, too many ROS can trigger apoptosis (Halliwell, 2007b).

5.2 Results

5.2.1 ROS detection after treatment with omega-3 PUFA

In order to investigate if the omega-3 PUFA increase the levels of oxidation in the cells, we used several assays to determine the levels of ROS and oxidative damage in the cells before and after PUFA treatment.

First, the DCF assay was used. This assay contains a cell-permeable fluorogenic probe that detects the reactive oxygen species (ROS) and nitric oxide (NO) in cells and it was used to determine the overall oxidative stress in cells. Firstly, the assay was used to determine the general oxidative levels in the different cell lines that had been used for MTT and apoptosis assays until then. So, I examined the oxidation levels of the oral and epidermal malignant cell lines, respectively, SCC-25 and SCC-13, the pre-malignant epidermal cell line SVFHK and the normal epidermal keratinocytes HEK-127 and NHEK-131. The human fetal skin fibroblast cell line HFF, which has low ROS levels, was used as a negative control. The malignant and pre-malignant cells showed high levels of ROS and, surprisingly, the normal keratinocytes showed even higher levels but the difference is not significant ($p > 0.05$ as measured by the non-parametric Mann-Whitney U rank test) (figure 5-1).

Then, the DCF assay was used to assess the oxidation in the SCC-25 cell line after treatment with DHA and EPA for 16 hours. The hydroethidine (HE) assay was also used for superoxide detection. Cytosolic HE exhibits blue fluorescence but once it is oxidised by superoxide to ethidium, it intercalates within DNA and stains the cell nucleus a bright fluorescent red (Carter *et al*, 1994). Again, the HFF cell line was used as a negative control and also the SCC-25 cells were treated with 100 μM of the oxidation inducer *tert*-butyl hydroperoxide (TBHP), for 2 hours at 37^0C, and were used as positive controls. The DCF assay showed an increase in the oxidation levels of SCC-25 after treatment with EPA and DHA for 16 hours at the higher concentrations (figure 5-2). The ROS levels showed a 2.5-fold increase after EPA 5 μM and a 3-fold increase after DHA treatment. However, as regards 3 μM, there was

only a slight increase during DHA treatment, but no increase when EPA is used. The HE assay showed a 10-20% increase in superoxide after treatment with both omega-3 PUFA (figure 5-3).

Figure 5-1. DCF oxidation levels assay for different cell lines

The oxidation levels of the malignant cell lines SCC-25 and SCC-13, the pre-malignant cell line SVFHK and the normal epidermal keratinocytes HEK-127 and NHEK-131 were assessed by DCF staining and FACs analysis. The human fetal skin fibroblast cell line HFF, which has low ROS levels, was used as a negative control. Means of median DCF fluorescence ± STDEV of 5 independent experiments are shown. The difference between malignant/pre-malignant and normal cells is not significant (p>0.05 as measured by Mann-Whitney U rank test).

A.

B.

(1)   *(2)*   *(3)*

Figure 5-2. Oxidation levels measured by DCF assay in SCC-25 cells after treatment with PUFA

(A) The oxidation levels of the oral malignant cell line SCC-25 after treatment with omega-3 PUFA were determined by DCF staining and FACs analysis which measured the fluorescent intensity of DCF. Means of median DCF fluorescence ± STDEV of 3 independent experiments are shown. SCC-25 cells were incubated in different DHA and EPA concentrations for 16 hours in KGM serum-free medium. SCC-25 cells treated with the ROS inducer TBHP (for 2 hours) were used as a positive control, while human fetal fibroblasts (HFF) were used as a negative control. (B) Representative picture of DCF positive stained cells showing DCF staining (1), nuclear staining with Hoechst 33258 (2) and an overlay of the two (3).

Figure 5-3. Oxidation levels measured by HE assay in SCC-25 cells after treatment with PUFA

The oxidation levels of the oral malignant cell line SCC-25 the pre-malignant cell line after treatment with omega-3 PUFA were determined by HE staining and FACs analysis which measured the fluorescent intensity of HE. Means of median HE fluorescence ± STDEV of 3 independent experiments are shown. SCC-25 cells were incubated in different DHA and EPA concentrations for 16 hours in KGM serum-free medium. SCC-25 cells treated with the ROS inducer TBHP (for 2 hours) were used as a positive control, while human fetal fibroblasts (HFF) were used as a negative control.

The oxidative damage levels were additionally assessed by the 8-oxo-dG assay. 8-hydroxy-2'-deoxyguanosine (8-oxo-dG) is a modified nucleoside, which is a very commonly detected by-product of DNA damage (Halliwell, BJ 2007), caused by oxidative radicals. 8-oxo-dG can serve as a sensitive indicator of physiological and environmental damage to DNA. A mouse monoclonal anti-8-oxo-dG antibody was used for the detection of 8-oxo-dG by immunocytochemistry (figure 5-4). After the staining the cells were visualised by fluorescence microscopy and the pictures were analysed with the Metamorph imaging software (Sunnyvale, CA). HFF cells were again used as the negative control. A sample with no primary antibody and another with no secondary antibody were also used as negative controls. SCC-25 cells were treated with 100 µM of the oxidation inducer *tert*-butyl hydroperoxide (TBHP), for 2 hours at 37°C and were again used as positive controls. This assay measures the mean fluorescent intensity of the 8-oxo-dG antibody signal in the nucleus. It appears that SCC-25 cells already have oxidative damage in their nucleus without any treatment compared to the HFF negative control (figure 5-5). After treatment with EPA and DHA for 16 hours, the oxidative damage increased for both treatments. As regards DHA, we can see an approximate 30% increase in 8-oxo-dG intensity at both 3 µM and 5 µM concentrations. As regards EPA, there was a 45% increase in 8-oxo-dG intensity at 3 µM and a 1.5-fold increase at 5µM concentration. At the higher concentration of 10 µM of EPA there was a 4-fold increase in 8-oxo-dG intensity.

A.

C.

B.

Figure 5-4. Images of 8-oxo-dG staining after treatment with EPA

Representative images of SCC-25 cells staining, strongly positive with the 8-oxo-dG antibody. The SCC-25 cells were incubated with EPA 10 µM for 16 hours prior to staining. (A) shows the nuclear staining with Hoechst 33258, (b) shows the 8-oxo-dG staining, and (C) an overlay of the two.

A.

B.

Figure 5-5. Oxidation damage measured by 8-oxo-dG staining after treatment with PUFA

The oxidation damage of the oral malignant cell line SCC-25 after treatment with omega-3 PUFA were determined by 8-oxo-dG staining. Means of 8-oxo-dG antibody fluorescence intensity ± STDEV of 3 independent experiments are shown. SCC-25 cells were incubated in different DHA and EPA concentrations for 16 hours in KGM serum-free medium. SCC-25 cells treated with the ROS inducer TBHP (for 2 hours) were used as a positive control while human fetal fibroblasts (HFF) were used as a negative control.

5.2.2 Treatment with antioxidants

Since PUFA appear to have oxidative properties, I examined if PUFA inhibited cell growth through oxidation. To examine this possibility, I tested the effect of PUFA in the presence of the well-known anti-oxidant, α-tocopherol (α-TOC), added to the medium. MTT assays were performed using 40 μM of α-TOC in the incubation medium added just before the addition of the PUFA. In the presence of α-TOC, DHA and EPA showed an increase in the growth inhibition effect on the malignant cells at concentrations between 0-0.1 μM after 4 days (figures 5-6 and 5-7). So instead of protection, α-TOC showed an inhibitory effect. At higher doses, α-TOC offered a slight protective effect in some cases, but it was not high enough to fully protect from the effect of PUFA and rescue the cells.

Then, the effect of another anti-oxidant on SCC-25 cells was also examined to confirm the results with α-TOC. I tested the effect of the antioxidant n-tert-butyl-α-phenylnitrone (PBN) (Nakao *et al*, 1996). Two concentrations of PBN were used, 600 μM and 800 μM. PBN anti-oxidant activity was tested and the concentration of 800 μM was shown to reduce the frequency of oxidative damage and senescence in human fibroblast cultures, showing that PBN is effective at this concentration (Pitiyage *et al*, 2011). PBN was added to the KGM medium just before the lipids. However, at no dose did we see a protective effect of PBN against the omega-3 PUFA (figure 5-8). Rather than protection, PBN at 600 μM concentration showed a stimulatory effect on SCC-25 cell growth in the untreated control but was unable to rescue the cells treated with PUFA.

A.

B.

C.

Figure 5-6. The effect of a-tocopherol on the cell growth inhibitory effect of DHA

Means± S.E.M of 6 independent MTT experiments without and 3 experiments with the addition of 40 μM of the antioxidant a-tocopherol (a-TOC) in SCC-25 (A), SCC-13 (B), SVFHK (C). Each experiment was conducted in duplicate. Cells were incubated in different DHA concentrations for 4 days in KGM serum-free medium. S.E.M=Standard error of the mean.

A.

B.

C.

Figure 5-7. The effect of a-tocopherol on the cell growth inhibitory effect of EPA

Means± S.E.M of 6 independent MTT experiments without and 3 experiments with the addition of 40 μM of the antioxidant a-tocopherol (a-TOC) for SCC-25 (A), SCC-13 (B), SVFHK (C). Each experiment was conducted in duplicate. Cells were incubated in different EPA concentrations for 4 days in KGM serum-free medium. S.E.M=Standard error of the mean.

A.

B.

Figure 5-8. The effect of PBN on the SCC-25 cell growth inhibitory effect of PUFA

Means± S.E.M of 3 independent MTT experiments for SCC-25 with the addition of none, 600 µM and 800 µM of the antioxidant PBN in KGM serum-free medium. Each experiment was conducted in duplicate. Cells were incubated in different DHA (A) and EPA (B) concentrations (3, 5 and 10 µM) for 4 days S.E.M=Standard error of the mean.

5.3 Discussion

5.3.1 ROS production by treatment with PUFA and its role in PUFA mechanism of action

ROS have a great range of potential actions on cells. On the one hand, ROS can be considered pro-cancer and cancer-promoting as they appear to promote proliferation, invasiveness, angiogenesis and metastasis, and suppress apoptosis (Halliwell, 2007b). In 1984, it was shown that when mouse fibroblasts are exposed to ROS they can be transformed (Zimmerman & Cerutti, 1984). The radiation induces carcinogenesis mainly via ROS production which causes DNA damage by the formation of the highly reactive hydroxyl radical (OH$^{\bullet}$) which attacks the DNA leading to the formation of 8-oxo-dG and other products (Halliwell, 2007b). However, on the other hand, too much ROS production can damage or kill the cell. It can also be considered anti-cancer by promoting cell-cycle arrest, senescence, cell death such as apoptosis and necrosis, and inhibiting angiogenesis. The same can be said about other types of reactive species, such as RNS (reactive nitrogen species) for example NO (nitric oxide) (Halliwell, 2007b).

Generally speaking, moderate levels of reactive species (RS) tend to promote apoptosis in most normal cells (Chandra *et al*, 2000). However, in some malignant cells, RS can have the opposite effect, inhibiting apoptosis. For example, in the melanoma cell line M14, the decrease in superoxide radicals (O$_2^{\bullet -}$) levels by CuZnSOD (a major cellular scavenger of O$_2^{\bullet -}$) over-expression led to apoptosis, while the decrease in CuZnSOD levels inhibited apoptosis (Pervaiz *et al*, 1999). These 'pro-survival' effects in malignant cells can be achieved by the oxidative inactivation of caspases and might sometimes occur for example via higher cytosolic pH that prevents caspase activation (Akram *et al*, 2006). In contrast, H$_2$O$_2$ promotes apoptosis by attacking and damaging the DNA and the mitochondria, but, additionally, by lowering the cytosolic pH (Akram *et al*, 2006).

It has been argued that some cancer cells use ROS as a survival mechanism to suppress apoptosis, induce proliferation, metastasis and angiogenesis, and promote genetic instability caused by the increased oxidative DNA damage (Halliwell, 2007b; Mori *et al*, 2004; Radisky *et al*, 2005). Many malignant cells produce high levels of ROS in culture (Szatrowski & Nathan, 1991). As regards the present study, the DCF assay also showed that the malignant (SCC-13 and SCC-25) and pre-malignant cells (SVHFK) used, had high levels of ROS compared to the human fetal fibroblast cell line (HFF). Do malignant cells have high ROS levels *in vivo* or just *in vitro*? Malignant cells from chronic lymphocytic leukaemia (CLL) patients showed increased ROS production compared to normal lymphocytes (Zhou *et al*, 2003) and many such studies have shown increased levels of 8-oxo-dG in human and other animal tumours (Halliwell, 2007b; Neeley & Essigmann, 2006; Sanchez *et al*, 2006). Halliwell proposes that increased oxidative damage levels in malignant cells could either result from: higher RS formation with no changes in the antioxidant defence mechanisms, unchanged RS formation with decreased antioxidant defences, or failure to repair oxidative damage, which leads to rise in levels, or any combination of the above (Halliwell, 2007b).

However, we need to point out that cells in culture are in an abnormal state, as culture media are often deficient in antioxidants and antioxidant precursors. Also, most cells in the human body are, normally in an environment with low O_2 levels (<10 mmHg O_2). Most adherent cells are cultured as a monolayer under 95% air and 5% CO_2 which corresponds to a hyperoxic environment of around 152 mmHg O_2. This is likely to increase ROS production in the cancer cells (Halliwell, 2007a; Halliwell, 2007b). In the present study, surprisingly high levels of ROS were observed in the normal epidermal keratinocytes NHEK-131 and HEK-127. A trend of higher ROS in the normal cells than the malignant and pre-malignant cell lines was observed but the difference was not significant ($p > 0.05$). This, possibly, is a result of their presence in culture, because normal cells are expected to have lower levels of ROS and high anti-oxidant defences. If the increase in ROS by PUFA is one of the mechanisms that promotes cell death, maybe the effect *in vivo* is even less toxic to the normal cells, if their ROS levels are lower.

Several studies have suggested that PUFA can cause cell death in malignant cells via oxidation, in combination with chemotherapy drugs. Germain *et al* reported that doxorubicin efficacy toward MDA-MB-231 human breast cancer cells in culture were enhanced by the addition of DHA (29 µM). This effect was decreased by the addition of the anti-oxidant α-tocopherol (Germain *et al*, 1998). Also, in rats fed with DHA (approximately 0.7 g/day), for several weeks prior to and 6 weeks during chemotherapy, epirubicin was observed to have increased cytotoxicity toward mammary tumours, while this effect was decreased by the addition of α-tocopherol to the diet (Colas *et al*, 2006). Arsenic trioxide is an anti-cancer agent that has been used in the treatment of acute promyelocytic leukemia induced apoptosis by a ROS-dependent pathway. The addition of DHA (25 µM) enhanced its cytotoxic effect and increased ROS production in leukemia cells *in vitro* and again this effect was reduced by the anti-oxidant vitamin E (Sturlan *et al*, 2003).

Moreover, PUFA on their own have been reported to induce oxidation. Arita *et al* reported an increase in ROS generation in HL-60 leukaemia cells after treatment with EPA and other PUFA, hypothesising that this might be the mechanism that triggers apoptosis (Arita *et al*, 2001). Maziere *et al* showed that PUFA led to stimulation of intracellular ROS production and lipid peroxidation products in human fibroblasts, which was followed by activation of NFκB and was prevented by α-tocopherol (Maziere *et al*, 1999). Elevation of ROS after DHA treatment has been also reported in MCF-7 breast cancer cells *in vitro* and *in vivo* (Kang *et al*, 2010). Three anti-oxidants, including α-tocopherol (5 µM), protected the cells from DHA-induced death. Tsuzuki suggested that conjugated EPA induces apoptosis of DLD-1 colorectal adenocarcinoma cells via lipid peroxidation (Tsuzuki *et al*, 2007) while the increase in ROS was suggested as one of the possible mechanisms of PUFA-induced growth inhibition in neuroblastoma (Gleissman *et al*, 2010). However, Finstad *et al* showed that in Ramos lymphoma cells vitamin E could not reverse the EPA-induced apoptosis, while Raji lymphoma cells were protected from EPA by vitamin E (Finstad *et al*, 1998). This shows that, depending on the cell type, lipid peroxidation may not be the only mechanism of PUFA induced cell death.

In the present study, our cells appear to already have increased ROS before any treatment with PUFA. After treatment with PUFA, the SCC-25 oral malignant cells

showed an increase in ROS after 16 hours, by DCF and HE flow-cytometric assays, especially at the higher concentrations of EPA and DHA used. Increased oxidative damage was also observed for the untreated cells by 8-oxo-dG staining. This damage appeared to increase after treatment with EPA and DHA. So, is this the mechanism or one of the mechanisms that EPA and DHA use to induce growth inhibition and apoptosis? When the two well-known anti-oxidants, α-tocopherol (α-TOC) and PBN were used, a protective effect was not observed in the MTT assay after 4 days. Both anti-oxidants failed to rescue the cells from the omega-3 PUFA inhibitory effect. Similar results were observed when different doses of PBN (50-1000 μM) were used on HaCaT cells (chapter 7), as PBN did not provide any protection against PUFA. This can have different explanations: First, it may mean that oxidation is not the mechanism that causes the PUFA-induced cytotoxicity. Second, it may mean that it is not the only or the main mechanism of PUFA action. Third, it could mean that the oxidation levels after the addition of PUFA are so high that the anti-oxidants fail to reverse the induction of apoptosis.

Chapter 6

MOLECULAR MECHANISM OF PUFA INDUCED GROWTH INHIBITION

Chapter 6: Molecular mechanism of PUFA-induced growth inhibition

6.1 Background: Molecular mechanisms involved in omega-3 PUFA growth inhibitory effect

A number of signalling pathways have been reported as being differentially affected by omega-3 PUFA. The molecular mechanism of EPA and DHA tumour suppressive action is not fully understood and defined and it is becoming increasingly clear that they are actually pleiotropic (Berquin *et al*, 2008; Chapkin *et al*, 2008). The pleiotropic transcriptional alterations induced by omega-3 lipids were demonstrated by microarray analysis *in vitro* (Edwards *et al*, 2004) and *in vivo* (Berger *et al*, 2006; Habermann *et al*, 2009).

6.1.1 PI3K pathway

The phosphatidylinositol 3-kinase (PI3K) affects a number of cellular functions including proliferation, apoptosis and differentiation. Specifically, activated PI3K promotes cell growth and survival and inhibits apoptosis. The activity of PI3K is elevated in many cancers where it results in a disturbance of the cell growth and survival control, giving a growth advantage and metastatic competence (Hennessy *et al*, 2005). There are three classes of PI3Ks. Class I_A PI3Ks are implicated in cancer and consist of a regulatory sub-unit (p85) and a catalytic sub-unit (p110). There are three classes of p110 (p110α, p110β and p110γ) (Courtney *et al*, 2010). When PI3K is activated, it phosphorylates phosphatidylinositol-4,5-bisphoshate (PtdIns(4,5)P_2 or PIP_2) to produce PtdIns(3,4,5)P_3 (PIP_3). The tumour suppressor phosphatase and tensin homolog deleted on chromosome 10 (PTEN) dephosphorylates PIP3 to PIP2 terminating PI3K-dependent signalling. Akt is a primary downstream mediator of the PI3K signalling pathway. Akt is a serine/threonine kinase which functions directly to

promote cell survival and protect cells from apoptotic death, by phosphorylating and inactivating several downstream targets (figure 6-1). The Pleckstrin Homology (PH) domain of Akt binds to PIP3 and it is recruited to the membrane, which results in conformational change allowing its phosphorylation. Akt is activated when it is phosphorylated on threonine 308 by phosphoinositide dependent kinase 1 (PDK1) and on serine 473 by PDK2 and has several downstream targets. It promotes cell survival by inhibiting the proapoptotic members of the Bcl-2 family Bad and Bax, and by negative regulation of the transcription factor NF-κB and forkhead transcription factors. Akt also phosphorylates Mdm2, which antagonises p53-mediated apoptosis. Akt is also involved in the activation of mTOR, which leads to increased p70 S6 kinase activity (Courtney *et al*, 2010; Hennessy *et al*, 2005).

There are reports of differential effects of omega-3 PUFA on the PI3K pathway. Some studies reported the decrease of phosphorylation and activity after EPA and DHA treatment (Lee *et al*, 2003; Schley *et al*, 2005), while others showed upregulation of Akt kinase activity via inactivation of its downstream target GSK3β after EPA treatment (Murata *et al*, 2001). Finally, no effect of DHA on p-Akt levels in cancer cells were also reported (Lim *et al*, 2009; Lim *et al*, 2008).

6.1.2 COX pathway

Prostaglandins (PG) are a group of autocrine and paracrine hormones that mediate many cellular and physiological processes. Prostaglandin H2 (PGH2) is an intermediate in the formation of the prostaglandins. Cyclooxygenase (COX) is a prostaglandin synthase that catalyses the formation of PGH2 from AA (Bakhle, 2001). There are two COX enzymes; COX-1 and COX-2. COX-1 is normally present in most types of cells and is a housekeeping enzyme, while COX-2 is normally absent from most cells. COX-2 levels increase rapidly and in large amounts in pathological situations like cancer or inflammation (Bakhle, 2001; Greenhough *et al*, 2009). COX-2 is overexpressed in cancer including colon cancer and head and

neck cancer (Chan *et al*, 1999; Greenhough *et al*, 2009; Mendes *et al*, 2009) but also in other types of tumours, such as lung, breast and prostate cancer (Bakhle, 2001), which is why there is a marked interest in studying the biology of COX-2 in relation to tumourigenesis.

Initially, the COX enzymes catalyse the formation of the unstable intermediate PG2 from AA, which is then converted into PGH2 by the peroxidase activity of COX. PGH2 is the precursor for several prostaglandins, which are formed by specialised prostaglandin synthases (Cha *et al*, 2006; Greenhough *et al*, 2009). The prostaglandins of series 2 which are synthesised by this pathway include the PGE2, PGD2, PGF2a, PGI2 (also known as prostacyclin) and thromboxane-A2 (TXA2) (Greenhough *et al*, 2009). However, as mentioned in chapter 1 (1.3.6), the COX pathway is involved in the metabolism of EPA to series-3 prostanoids (prostaglandins, prostacyclins and thromboxanes), which seem to be anti-inflammatory or less inflammatory than the series 2 prostanoids produced from AA (figure 1-11) (Molendi-Coste *et al*, 2011). The anti-inflammatory omega-3 PUFA mediators, the resolvins, can also be produced by a COX-2-dependent pathway, in the presence of aspirin by generating their aspirin, triggered form (Serhan, 2009; Serhan & Chiang, 2008). So, the role of the COX pathway is more complicated than initially thought.

Several studies support a critical role for COX-2 during colorectal tumourigenesis. The administration of the COX-2-selective nonsteroidal anti-inflammatory drugs (NSAID) celecoxib significantly suppressed the growth of existing adenomas and prevented the formation of new ones (Arber *et al*, 2006; Greenhough *et al*, 2009; Steinbach *et al*, 2000). The pro-tumourigenic effects of COX-2 in the colon largely depend on its role in producing high levels of PGE2 in human colorectal adenomas and carcinomas (Pugh & Thomas, 1994). *In vivo* studies have revealed that, when tissue prostaglandin levels were reduced through NSAID treatment, prevention of adenoma development in familial adenomatous polyposis patients was more effective (Giardiello *et al*, 2004). Removal of PGE2 by a PGE2 monoclonal antibody in mice inhibited the growth of transplantable tumours *in vivo* (Stolina *et al*, 2000). Treatment with a COX-2 inhibitor has been reported to reduce COX-2 mRNA and protein expression and synthesis of PGE2 in mammary and oral epithelial cells

(Mendes *et al*, 2009; Subbaramaiah *et al*, 2000). The inhibition of COX-2 in an oral SCC cell line (NS-398) actually caused inhibition of proliferation of the cancer cells which were expressing COX-2 mRNA, via suppression of PGE2 production (Sumitani *et al*, 2001). Also inhibition of COX-1 and COX-2 caused increased terminal differentiation in mouse skin keratinocytes *in vivo* (Akunda *et al*, 2004). However, COX-2/PGE2 signalling is likely to act in combination with other signalling pathways that become deregulated in cancer, which need to be targeted in conjunction with the COX-2/PGE2 pathway for efficient cancer prevention and therapy.

While good evidence suggests that the COX-2/PGE2 pathway is tumour-promoting and that its inhibition is useful in the prevention of different types of cancer, this does not appear to be the case under all circumstances (Greenhough *et al*, 2009). Most NSAIDs inhibit the COX-2/PGE2 pathway. However, some NSAIDs exert their antitumour effects *in vitro* independently of their ability to inhibit COX-2 or to decrease PGE2 (Elder *et al*, 1997; Hanif *et al*, 1996), and some other NSAIDs actually induce COX-2 expression (Paik *et al*, 2000; Pang *et al*, 2003). In addition, studies showed that the COX-2/PGE2 pathway may even act in a tumour suppressive manner under some circumstances (Bol *et al*, 2002; Murata *et al*, 2004; Patsos *et al*, 2010; Wilson & Potten, 2000) and this issue is discussed later in more detail (sub-chapter 6.3). Generally, the role of COX-2 and/or prostaglandins in cancer might not be as straightforward as initially proposed (Greenhough *et al*, 2009).

Several studies have supported the inhibition of COX-2 by omega-3 PUFA in different types of cancer such as colon cancer (Narayanan *et al*, 2004), breast cancer (Horia & Watkins, 2007), cholangiocarcinoma cells (Lim *et al*, 2008) and hepatocellular carcinoma cells (Lim *et al*, 2009). Gleissman reported that DHA decreased PGE2 production in neuroblastoma cells (Gleissman *et al*, 2010).

6.1.3 MAPK pathway

Mitogen-activated protein kinases (MAPK) are a widely conserved family of serine/threonine protein kinases involved in many cellular programs such as cell proliferation, differentiation, migration, and death (Dhillon *et al*, 2007). In mammalian cells, there are three well-defined MAPK pathways: the extracellular-signal-regulated kinase (ERK) pathway, the JUN N-terminal kinase (JNK) pathway and the p38 pathway (figure 6-1). The signals that cause MAPK activation are usually initiated on the cell surface, primarily by membrane-bound receptors, such as EGFR. So, extracellular stimuli activate the MAPK pathways through mechanisms mediated by GTPases, including Ras, Rac, Cdc42 (cell-division cycle 42) and Rho (Ras homologue). MAPK pathways are activated via sequential phosphorylation events. Firstly, MAPK kinases (MAPKKs) are phosphorylated at two serine residues by MAPK kinase kinases (MAPKKKs), such as Raf, MEKK (MAPK/ERK kinase kinase) and TAK (TGFβ-activated kinase). Activated MAPKKs then phosphorylate MAPKs ERK (extracellular-signal-regulated kinase), JNK (JUN N-terminal kinase) and p38, on both threonine and tyrosine residues, which results in the catalytic activation of these MAPKs. The phosphorylation of the threonine and tyrosine residues on MAPKs results in a substantial conformational change of the protein that increases substrate accessibility and enhances catalysis. Activated MAPKs can translocate to the nucleus to phosphorylate a wide variety of downstream targets, including protein kinases and transcription factors, which facilitate the transcription of MAPK-regulated genes (Liu *et al*, 2007a).

ERK signalling is deregulated in approximately one-third of all human cancers. The ERK1/2 (p44/42 MAPK) signalling pathway can be activated in response to a diverse range of extracellular stimuli including mitogens, growth factors, and cytokines, and it is associated historically with proliferation. However, it is now clear that deregulation of this pathway is linked to many other aspects of the tumour phenotype, so the precise molecular mechanism of ERK1/2 is still controversial (Dhillon *et al*, 2007). In cancer, where growth factors and mitogens activate ERK, ligand-mediated activation of receptor tyrosine kinases triggers guanosine triphosphate (GTP) loading of the Ras GTPase, which can then recruit Raf kinases to

the plasma membrane for activation. MEK1 and MEK2 are activated when phosphorylated by Raf (Raf-1, B-Raf and A-Raf) and then activate ERK1 and ERK2 through phosphorylation of activation loop residues Thr202/ Tyr204 and Thr185/Tyr187, respectively (Dhillon *et al*, 2007).

JNK and p38 pathways are activated preferentially by stress, DNA-damaging agents, oxidation and inflammatory cytokines. There are three genes – *Jnk1, Jnk2 and Jnk3*. Alternative splicing of these genes creates a total of 10 JNK isoforms. JNK activation requires dual phosphorylation (on tyrosine and threonine residues) by MEK4 and MEK7 (Dhillon *et al*, 2007). In response to stresses such as UVB radiation, oxidative stress and DNA-damage, JNK binds to and phosphorylates p53. This can result in an increase of p53 transcriptional activity and p53 stabilization (Cheng *et al*, 2003b; She *et al*, 2001). JNK seems to have a tumour suppressive function linked to its ability to promote apoptosis (Kennedy *et al*, 2003). In mammals, p38 isoforms are also strongly activated by environmental stresses and inflammatory cytokines. p38 is required for expression of TNFa and interleukin-1 during inflammatory responses. There are four isoforms of p38 (α, β, γ and δ) which are phosphorylated by MEK3 and MEK6 (Dhillon *et al*, 2007). The phenotype of mice disrupted in the MEK3 and MEK6 genes or the p38a gene supported the idea that p38 functions as a tumour suppressor (Bulavin & Fornace, 2004).

Traditionally, the activation of ERK is linked to cell survival and proliferation (Mansour *et al*, 1994; Xia *et al*, 1995). However, there are some studies which show that when cell death is triggered, activation of ERK is observed, which is as a survival mechanism and a final attempt of the cells to reverse their fate (Haase *et al*, 2001; Persons *et al*, 1999; Wilson *et al*, 1999). However, more and more studies show that this is not always the case and activation of ERK could actually cause apoptosis or cycle arrest (Elder *et al*, 2002; Galve-Roperh *et al*, 2000; Pumiglia & Decker, 1997; Stanciu *et al*, 2000). In summary, ERK activation effect is not as straightforward as previously thought and it depends on the type, strength and duration of the stimulus and on the cell type (Elder *et al*, 2002).

Some studies link the tumour suppressive action of PUFA with the MAPK pathway. Serini showed that PUFA can promote apoptosis through the up-regulation of

MAPK phosphatase-1 (MKP-1) and by reducing the levels of p-ERK1/2 and p-p38 in lung cancer cells (Serini *et al*, 2008). Also, it was reported that DHA lowered the activation of *Ras* oncogenes and ERK activation in mouse colon cells (Collett *et al*, 2001) and EPA decreased MAPK activity, inhibiting cell proliferation in hepatoma cells (Murata *et al*, 2001).

6.1.4 Ca^{2+} release

Ca^{2+} is one of the most versatile signalling mediators in cells and is required for the activation of many cellular processes (Chapkin *et al*, 2008). Increasing evidence indicates that changes in the intracellular homeostasis and compartmentalization of Ca^{2+} can result into cell death either through apoptosis or necrosis (Berridge *et al*, 2000). Eukaryotic cells are able to increase their cytosolic Ca^{2+} levels via release from intracellular stores or influx via plasma membrane channels. The endoplasmic reticulum (ER) is the major storage organelle. However, functional compartmentalization of Ca^{2+} exists within the various cellular organelles such as mitochondria (Chapkin *et al*, 2008). In fact, it is now recognised that mitochondria play a key role in both apoptosis and necrosis by regulating energy metabolism, intracellular Ca^{2+} homeostasis, activation of caspases and the release of reactive oxygen species (ROS) (Jacobson & Duchen, 2002; Ott *et al*, 2007). It was recently shown that DHA and butyrate synergistically enhance both mitochondrial Ca^{2+} accumulation and lipid peroxidation, which serve as triggers for apoptosis in a p53-independent manner (Kolar *et al*, 2007). EPA has also been reported to affect intracellular homeostasis (Berquin *et al*, 2008). EPA induced a Ca^{2+} release from the intracellular Ca^{2+} stores and simultaneously inhibited Ca^{2+} influx via Ca^{2+} channels in the plasma membrane, resulting in a depletion of the intracellular Ca^{2+} stores which caused inhibition of translation initiation, and preferentially down-regulated oncogenes and G1 cyclins (Aktas & Halperin, 2004).

Figure 6-1. MAPK, PI3K and STAT signalling pathways

MAPK, PI3K and STAT signalling pathways activated by epidermal growth factor receptors (EGFR) Akt is activated by PIP3, after PI3K activation, and phosphorylates several downstream targets. EGFR also triggers activation of GTPases, such as RAS which activates MAPKKKS. These MAPKKKs phosphorylate MAPKKs, which then activate the MAPKs, ERK1/2 p38 and JNK. Activated MAPKs translocate to the nucleus to phosphorylate a wide variety of downstream targets (protein kinases and transcription factors) which facilitate the transcription of MAPK-regulated genes.

6.1.5 NFκB pathway

Another mechanism involved in the long-chain omega-3 PUFA action is NFκB activity. PUFA have been reported to decrease NFκB activity and expression (Narayanan *et al*, 2003; Schley *et al*, 2005). Active NFκB promotes cellular survival and inhibits apoptosis, promoting tumourigenesis. NFκB exists as dimers usually located in the cytoplasm, associated with an inhibitor protein IκB which maintains NFκB in the cytoplasm by preventing the display of the nuclear localization sequence. After the right stimulus, IκB kinase-α phosphorylates IκB allowing dissociation from NFκB. Then, phosphorylated IκB is targeted for degradation through the ubiquitin- 26S proteosome pathway and NFκB is then free to localise to the nucleus. There, it initiates and regulates the transcription of various genes involved in cell growth control and inflammatory responces, for example pro-inflammatory cytokine genes, such as TNF-α (Novak *et al*, 2003). It has been suggested that omega-3 PUFA modulate signalling involved in the activation of NFκB, such as Akt activation (Lee *et al*, 2003; Schley *et al*, 2005), TNF-α signalling (Novak *et al*, 2003; Weber *et al*, 1995), and IκBα phosphorylation (Novak *et al*, 2003).

6.1.6 PPAR activation

The peroxisome proliferator-activated receptor (PPAR) family of nuclear receptors consists of three proteins PPAR*α*, *β*/*δ*, and *γ*, with different ligand specificity, tissue distribution, and developmental expression. PPARs ligands are primarily long chain unsaturated and polyunsaturated fatty acids and their metabolites (Edwards & O'Flaherty, 2008). PPAR*β*/*δ* plays a key role in lipid metabolism of peripheral tissues. PPAR*β*/*δ* is highly expressed in colon and promotes colon cancer (Michalik *et al*, 2004), after stimulation by arachidonic acid, upregulation of COX-2 leading to overproduction of prostaglandin PGE2 which causes growth of colon cancer cells. PPAR*γ* has various effects on cancer. It controls fat metabolism by regulating genes

involved in lipogenesis, insulin sensitivity, and adipocyte differentiation (Edwards & O'Flaherty, 2008; Michalik *et al*, 2006). Although PPARγ activators have been widely shown to inhibit cancer growth *in vitro,* the effects are more complicated *in vivo*: they inhibit but sometimes promote cancer growth*,* depending on cellular conditions (Edwards & O'Flaherty, 2008). It was demonstrated (Sun *et al*, 2008) that DHA induced apoptosis in MCF-7 human breast cancer cells by the activation of PPARγ and consequent up-regulation of syndecan-1 (SDC-1) which is the major proteoglycan of plasma membrane produced by epithelial cells, which regulates growth factor signalling and cell–cell and cell–matrix interactions. In Ramos cells, irradiation and DHA were shown to synergise to induce apoptosis mediated at least in part via activation of PPARγ and suppression of NF-κB activation induced by Gamma-IR (Zand *et al*, 2007).

6.1.7 Membrane structure and function and lipid rafts

Omega−3 PUFA are rapidly incorporated into cells, primarily into membrane phospholipids (Chapkin *et al*, 1991; Stillwell & Wassall, 2003). Increasing evidence suggests that DHA is able to alter basic properties of cell membranes, such as acyl chain order and fluidity, phase behaviour, elastic compressibility, ion permeability, fusion, rapid flip-flop and resident protein function (Chapkin *et al*, 2008; Stillwell & Wassall, 2003). These DHA-induced alterations in membrane structure and function have been proposed to underlie its pleiotropic effects (Chapkin *et al*, 2008). Moreover, the plasma membranes of all eukaryotic cells contain specific regions in which key signal transduction proteins are localised. These regions are called "lipid rafts" and are composed mostly of cholesterol and sphingolipids, therefore do not integrate well into the fluid phospholipid bilayers and as a result form microdomains (Hancock, 2006). DHA and possibly EPA, being polyunsaturated, are incompatible with sphingolipid and cholesterol so they appear to alter lipid raft behaviour and protein function (Chen *et al*, 2007).

In summary, the omega-3 PUFA mechanism of action is very complicated and not clear yet as it is obvious that several different signalling pathways (some of them analysed in this sub-chapter) can be affected in different circumstances and in different ways.

6.2 Results

6.2.1 Investigation of signalling pathways involved in the effect of PUFA on cancer cell growth

In order to investigate the molecular mechanisms underlying the effect of PUFA on keratinocyte growth, some signalling pathways previously mentioned to be involved in EPA and DHA anti-cancer action were examined. The expression of different proteins was analysed by western blotting. The SCC-25 cells were firstly treated with 10 μM of DHA or EPA for 5, 17 and 24 hours. The higher dose of PUFA was chosen in order to observe the protein expression levels more easily taking into consideration that the effect would be longer and more visible. Lysates were obtained from the different conditions, as described in Chapter 2 (Materials and Methods). GAPDH was used as a loading control. Moreover, the protein expression of total proteins, such as total Akt and p44/42 MAPK (Erk1/2), is shown. Protein expressions were normalised with GAPDH or total protein expression when densitometry was used.

In order to test if the PI3K pathway was involved, the expression of phosphorylated Akt was examined. As mentioned before, Akt is a serine/threonine kinase, which promotes cell survival by inhibiting apoptosis when activated by phosporylation. Specifically Akt is phosphorylated on threonine 308 by PDK1 and on serine 473 by PDK2. Phosphorylation at serine 473 is required for full activation of Akt. Phospho-Akt (Ser473) rabbit mAb detects endogenous levels of Akt only when phosphorylated at Ser473. Total Akt was also examined. The results demonstrated that there was not a notable change of phospho-Akt (Ser473) after treatment with DHA and EPA after 5-17 hours (figure 6-2).

Next, as COX-2 was previously mentioned to be affected by PUFA, I examined its expression in the SCC-25 cells. Surprisingly, treatment with EPA and DHA appeared to cause a 2-fold up-regulation in the expression of COX-2 after 5 hours. The up-regulation was reduced after 17 h for EPA. As regards the DHA treatment, 1.5-fold up-regulation was observed after 17 h (figure 6-3).

Next, I examined the MAPK pathway. Upon stimulation, MEK1 and MEK2 activate ERK1/p44 and ERK2/p42 through phosphorylation of activation loop residues Thr202/ Tyr204 and Thr185/Tyr187, respectively. Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) rabbit mAb detects endogenous levels of p44 and p42 MAP Kinase (Erk1 and Erk2) when dually phosphorylated at Thr202 and Tyr204 of Erk1 (Thr185 and Tyr187 of Erk2), and singly phosphorylated at Thr202. The result was unexpected but striking. The expression levels of phospho-p44/42 MAPK (Erk1/2) appeared to be highly up-regulated after treatment with DHA or EPA for 5 h. The up-regulation was sustained until 24 h (figure 6-4).

As the change in phospho-ERK1/2 expression was the most striking and sustained for a long period of time, I concentrated on this specific protein. Next, I investigated if there was the same result in the lower, more selective, concentrations of EPA and DHA and earlier time-points, in order to find out when the up-regulation starts. The results showed that the up-regulation of phospho-ERK1/2 starts as early as only 30 min after treatment with the lower doses of EPA and DHA (figure 6-5). The increase in phospho-ERK1/2 is also obvious after 2 hours. I chose the 2 hour time point to continue my further analysis, as it is an early time point where the upregulation is obvious.

I also examined if phospho-JNK was up-regulated after treatment with PUFA for 2 h, as it belongs to the stress related part of the MAK pathway (figure 6-6). We can see that JNK is highly phosphorylated after treatment with both DHA and EPA at the higher doses (10 μM). At the lower dose of 5 μM, phospho-JNK levels are a lot lower but still visible (more after DHA treatment).

Figure 6-2: P-Akt protein expression levels

Representative image of western blot assay showing the expression levels of P-Akt after treatment of SCC-25 cells with 10 μM of DHA or EPA for 5 and 17 hours. The protein levels of total Akt and GAPDH housekeeping gene are also shown to prove equal loading.

A.

B.

Figure 6-3: COX-2 protein expression levels

A. Representative image of western blot assay showing the expression levels of COX-2 after treatment of SCC-25 cells with 10 μM of DHA or EPA for 5 and 17 hours. The protein levels of GAPDH housekeeping gene are also shown to examine if the loading is equal.

B. Graph showing the quantification of the results by densitometry. The expression levels of COX-2 were normalised with GAPDH expression levels. The means± SEM of three independent experiments are shown.

A.

B.

Figure 6-4: Phospho-ERK1/2 protein expression levels

A. Representative image of western blot assay showing the expression levels of phospho-ERK1/2 (P-ERK1/2) after treatment of SCC-25 cells with 10 μM of DHA or EPA for 5, 17 and 24 hours. The protein levels of total ERK1/2 and GAPDH housekeeping gene are also shown to examine if the loading is equal.

B. Graphs showing the quantification of the results by densitometry. The expression levels of phospho-ERK1/2 were normalised with the total ERK1/2 expression levels. The means± SEM of three independent experiments are shown.

A.

B.

Figure 6-5: Phospho-ERK1/2 protein expression levels at earlier time-points at lower PUFA concentrations

Representative image of western blot assay showing the expression levels of phospho-ERK1/2 (P-ERK1/2) after treatment of SCC-25 cells with 3 μM and 5 μM of DHA or EPA for 30 min and 2 hours. The protein levels of total ERK1/2 and the GAPDH housekeeping gene are also shown to examine if the loading is equal.

2 h

Figure 6-6: Phospho-JNK protein expression levels

Representative image of western blot assay showing the expression levels of phospho-JNK after treatment of SCC-25 cells with 5 μM and 10 μM of DHA or EPA for 2 hours. The protein levels of total ERK1/2 and GAPDH housekeeping gene are also shown to examine if the loading is equal.

6.2.2 Investigation of the involvement of the MAPK pathway in PUFA induced apoptosis

As the elevation of phospho-ERK1/2 was the most striking and early event after PUFA treatment, I hypothesised that it is involved in the PUFA induced growth inhibitory effect, which can include apoptosis or growth inhibition or both. In order to investigate this further, the use of several inhibitors was needed in order to see if the inhibition of ERK1/2 phosphorylation can block the PUFA-induced cell growth inhibition. First, the MEK inhibitor U0126 was used to block the ERK1/2 phosphorylation mediated by MEK. I hypothesised that EGFR activation might be an earlier event which causes phospho-ERK1/2 elevation. In order to investigate this, the EGFR inhibitor AG1478 was used. Finally, the caspase inhibitor QVD-Oph was also included.

Before the proliferation assays with the specific inhibitors, western blot assays were necessary to prove the inhibitors actually worked and to investigate the correct concentrations and timing.

In the pilot experiment, AG1478 950 μM and QVD-Oph 10 μM were used to treat the SCC-25 cells (figure 6-7). The inhibitors were added in the culture for 1.5 hours before the addition of the EPA and DHA. Two hours after the addition of PUFA, the lysates were obtained. The first experiment with 10 μM EPA and DHA demonstrated that the EGFR inhibitor AG1478 successfully inhibited ERK1/2 phosphorylation without affecting the endogenous levels of the untreated control. The caspase inhibitor QVD-Oph did not affect the levels of phospho-ERK1/2, confirming that ERK1/2 phosphorylation is not a consequence of apoptosis. This was expected as the cleavage of caspases was observed at later time-points so apoptosis occurs much later.

Figure 6-7: Phospho-ERK1/2 protein expression levels after treatment with inhibitors and 10 μM of PUFA in a pilot experiment

Western blot image of pilot experiment showing the expression levels of phospho-ERK1/2 (P-ERK1/2) after treatment of SCC-25 cells with 950 μM AG1478 EGFR inhibitor and 10 μM QVD-Oph caspase inhibitor for 1.5 hours and then addition of 10 μM of DHA or EPA for 2 hours. The protein levels of total ERK1/2 are also shown to examine if the loading is equal.

In the subsequent experiment, the MEK inhibitor U0126 (5 μM or 2.5 μM) and a lower concentration of AG1478 (950 nM) were used (figures 6-8 and 6-9). Two concentrations of PUFA, 5 μM and 10 μM were used this time. Again, the inhibitors were added to the culture for 1.5 hours before the addition of the EPA and DHA. Two hours after the PUFA were added, the lysates were obtained. The EGFR inhibitor, AG1478 was very potent in knocking down p-ERK1/2 at this lower concentration, without affecting the endogenous levels of the untreated control. Again, the caspase inhibitor QVD-Oph did not affect the levels of phospho-ERK1/2. The MEK inhibitor U0126, at 5 μM for DHA and 2.5 μM for EPA treatment respectively, inhibited p-ERK in DHA treated cells but also reduced the endogenous levels of the control.

Figure 6-8: Phospho-ERK1/2 protein expression levels after treatment with inhibitors and DHA

Representative image of western blot assay showing the expression levels of phospho-ERK1/2 (P-ERK1/2) after treatment of SCC-25 cells with 950 nM AG1478 EGFR inhibitor, 5 μM U0126 MEK inhibitor and 10 μM QVD-Oph caspase inhibitor for 1.5 hours and then addition of 5 μM or 10 μM of DHA for 2 hours. The protein levels of total ERK1/2 and GAPDH are also shown to examine if the loading is equal.

Figure 6-9: Phospho-ERK1/2 protein expression levels after treatment with inhibitors and EPA

Representative image of western blot assay showing the expression levels of phospho-ERK1/2 (P-ERK1/2) after treatment of SCC-25 cells with 950 nM AG1478 EGFR inhibitor, 2.5 μM U0126 MEK inhibitor and 10 μM QVD-Oph caspase inhibitor for 1.5 hours and then addition of 5 μM or 10 μM of EPA for 2 hours. The protein levels of total ERK1/2 and GAPDH are also shown to examine if the loading is equal.

After the first assessments proved that the inhibitors used were potent, one more extended experiment was done, with different concentrations of the inhibitors, in order to decide which concentration to use in the cell assays. AG1478 (at 100 nM and 1 μM) and U0126 (at 2.5 μM, 5 μM and 10 μM) were used again. Two more inhibitors were added to our panel. Firstly, the EGFR blocking antibody (EGFR Mouse anti-Human Monoclonal (Azide-free) (225) Ab) (at 0.5 μg/ml, 1 μg/ml and 10 μg/ml) was used, in order to confirm that EGFR inhibition reduces phospho-ERK1/2 levels induced by PUFA. Secondly, another MEK inhibitor called AZD6244 (30 nM, 100 nM and 300 nM) was also used. The inhibitors were added in the culture for 1.5 hours before the addition of PUFA. This time, SCC-25 cells were treated with the most selective PUFA concentration of 3 μM. Two hours after the PUFA were added, the lysates were obtained.

During this experiment, apart from phospho-ERK1/2 and total ERK1/2, the protein expression levels of the phospho-ERK downstream target phospho-p90RSK and the total protein RSK1/2/3 were also examined. As expected, phospho-p90RSK was up-regulated after DHA and EPA treatment following ERK1/2 phosphorylation.

The EGFR blocking antibody (figure 6-10) was very effective in knocking down the PUFA induced phospho-ERK1/2 elevated levels, without affecting the endogenous basal levels of the control. Subsequently, phospho-p90RSK was also knocked down. EGFR blocking antibody seemed to be effective at even the lower concentration (0.5 μg/ml), which appears to be appropriate to use for the cell assays. The EGFR inhibitor AG1478 (figure 6-11) was also very potent in knocking down phospho-ERK1/2 and phospho-p90RSK levels, without affecting the endogenous levels of the control. Both concentrations used were successful, so the lower one (100 nM) appeared to be the most appropriate to use.

The MEK inhibitor U0126 (figure 6-12) appeared to reduce ERK1/2 and p90RSK phosphorylation but it also affected the endogenous levels of the control. It appeared that the middle dose of 5 μM was the most appropriate. The second MEK inhibitor AZD6244 appeared to be more potent than U0126. AZD6244 (figure 6-13) knocked down phospho-ERK1/2 and phospho-p90RSK levels without affecting the levels of the control, as much. The lower (3 nM) and the middle dose (30 nM) were probably

the most potent ones to use as they do not markedly affect the basal levels of phospho-ERK1/2.

The next step would be to test whether the use of the inhibitors would reduce apoptosis after PUFA treatment. MTT or apoptosis assays could be used for this purpose. Unfortunately, due to time limitations, I was not able to perform these experiments, which will be the first future step of the project.

Figure 6-10: Phospho-ERK1/2 and target phospho-p90RSK protein expression levels after treatment with EGFR blocking antibody and 3 μM of PUFA

Representative image of western blot assay showing the expression levels of phospho-ERK1/2 and target phospho-p90RSK after treatment of SCC-25 cells with 0, 0.5 μg/ml, 1 μg/ml and 10 μg/ml of the EGFR blocking antibody for 1.5 hours and then addition of 3 μM of DHA or EPA for 2 hours. The protein levels of total ERK1/2, total RSK1/2/3 and GAPDH are also shown, to examine if the loading is equal.

Figure 6-11: Phospho-ERK1/2 and target phospho-p90RSK protein expression levels after treatment with AG1478 EGFR inhibitor and 3 μM of PUFA

Representative image of western blot assay showing the expression levels of phospho-ERK1/2 and target phospho-p90RSK after treatment of SCC-25 cells with 0, 10 nM and 100 nM of AG1478 EGFR inhibitor for 1.5 hours and then addition of 3 μM of DHA or EPA for 2 hours. The protein levels of total ERK1/2, total RSK1/2/3 and GAPDH are also shown to examine if the loading is equal.

Figure 6-12: Phospho-ERK1/2 and target phospho-p90RSK protein expression levels after treatment with U0126 MEK inhibitor and 3 μM of PUFA

Representative image of western blot assay showing the expression levels of phospho-ERK1/2 and target phospho-p90RSK after treatment of SCC-25 cells with 0, 2.5 μM, 5 μM, 10 μM of U0126 MEK inhibitor for 1.5 hours and then addition of 3 μM of DHA or EPA for 2 hours. The protein levels of total ERK1/2, total RSK1/2/3 and GAPDH are also shown to examine if the loading is equal.

Figure 6-13: Phospho-ERK1/2 and target phospho-p90RSK protein expression levels after treatment with AZD6244 MEK inhibitor and 3 μM of PUFA

Representative image of western blot assay showing the expression levels of phospho-ERK1/2 and target phospho-p90RSK after treatment of SCC-25 cells with 0, 30 nM, 100 nM, 300 nM of AZD6244 MEK inhibitor for 1.5 hours and then addition of 3 μM of DHA or EPA for 2 hours. The protein levels of total ERK1/2, total RSK1/2/3 and GAPDH are also shown to examine if the loading is equal.

6.3 Discussion

As mentioned before, the tumour suppressive action of EPA and DHA is pleiotropic and not clearly defined yet. Several reports involve different signalling pathways in omega-3 PUFA function. I attempted to investigate the involvement of some of these pathways in the growth inhibitory action of omega-3 PUFA in malignant oral keratinocytes.

First, the PI3K pathway was examined. There are different reports regarding the effect of EPA on the PI3K activity. EPA and/or DHA decreased Akt phosphorylation and activity in breast cancer cells and the murine monocytes RAW264.7 (Lee *et al*, 2003; Schley *et al*, 2005). DeGraffenried *et al* showed that EPA (0.2-200 μM) caused a dose-dependent decrease in p-Akt in higher doses. However, the lower doses of EPA resulted in an increase in phospho-Akt. Murata *et al* has also reported upregulation of Akt kinase activity via increased phosphorylation, and subsequent inactivation, of the downstream target GSK3β after EPA treatment of hepatoma cells (Murata *et al*, 2001). Finally, Lim *et al* showed no effect of DHA on p-Akt levels in the hepatocellular carcinoma cell line HCC and cholangiocarcinoma cells, while GSK3β levels of phosphorylation were decreased (possibly linked to β-catenin degradation) (Lim *et al*, 2009; Lim *et al*, 2008). My results agree with Lim *et al*, as there was no change in p-Akt levels observed after treatment of SCC-25 cells with EPA and DHA.

Secondly, the COX-2 expression was investigated, as it is overexpressed in many types of cancer, including head and neck cancer (Bakhle, 2001; Greenhough *et al*, 2009; Mendes *et al*, 2009). Several studies reported the inhibition of COX-2 by omega-3 PUFA. PUFA have been reported to have an apoptotic effect in colon cancer by down-regulating COX-2 (Narayanan *et al*, 2004). Moreover, omega-3 PUFA reduced COX-2 and NFκB expression in MDA-MB-231 breast cancer cells (Horia & Watkins, 2007). DHA reduced the COX-2 promoter activity and the COX-2 protein expression in cholangiocarcinoma cells (Lim *et al*, 2008) and hepatocellular carcinoma cells (Lim *et al*, 2009). Gleissman reported DHA decreased PGE2 production in neuroblastoma, and hence inhibited COX-2 activity (as COX-2 produces PGE2) (Gleissman *et al*, 2010).

In contrast to those studies, my results demonstrated a 2-fold increase in COX-2 protein expression after 5 h of DHA and EPA treatment of oral SCC-25 cells. After 17 h the levels declined for EPA, while for DHA there was a sustained over-expression (1.5-fold) but the levels were also decreased. These results agree with one study that examined the effects of PUFA on HaCaT epidermal keratinocytes. They showed that EPA actually induced an almost 4-fold increase in COX-2 levels (Chene *et al*, 2007), mediated by PPARγ activation. They proposed that, despite the extensive literature documenting a deleterious role of COX-2 activity in various pathophysiological conditions, the induction of this enzyme by omega-3 PUFA may actually have an anti-inflammatory effect.

The consequences of increased COX-2 expression in inflammation are complicated. They can be protective or deleterious depending on the balance between the pro-inflammatory and the anti-inflammatory prostanoids produced by various cell types under different circumstances. On the one hand COX-2 produces series 2 pro-inflammatory prostanoids from AA (Greenhough *et al*, 2009). However, it also produces series-3 prostanoids from EPA metabolism which are anti-inflammatory or less inflammatory than the series 2 prostanoids produced from AA (figure 1-11) (Molendi-Coste *et al*, 2010). Also, the anti-inflammatory omega-3 PUFA metabolites, the resolvins, can also be produced by a COX-2-dependent pathway, in the presence of aspirin (Serhan, 2009; Serhan & Chiang, 2008). Also, it has recently been shown that COX-2 is induced during the resolution of an inflammatory response, and leads to the production of anti-inflammatory but not pro-inflammatory prostaglandins, while inhibition of COX-2 at that point results in the persistence of the inflammation (Gilroy *et al*, 1999). Finally, the recently engineered *fat-1* mice, can convert omega-6 to omega-3 fatty acids and have a lower ratio of omega-6/omega-3 fatty acids their tissues compared to wild type (WT) mice. *Fat-1* mice showed a dramatic reduction in melanoma formation and growth. The level of omega-3 fatty acids and their metabolite PGE3 (which is produced via the COX pathway) were much higher than in the WT mice (Xia *et al*, 2006). Thus, COX-2 appeared to be a bifunctional regulator of inflammatory processes. As inflammation is cancer-promoting, COX-2 can be tumour- promoting or tumour-preventing depending on different circumstances.

Moreover, while several studies demonstrate an important role of COX-2 expression during tumourigenesis, others support the involvement of COX-2 in apoptosis. As discussed before (subchapter 6-1), COX-2- selective NSAIDS appear to significantly suppress the growth of existing colorectal adenomas and prevent the formation of new ones (Arber *et al*, 2006; Greenhough *et al*, 2009; Steinbach *et al*, 2000), while inhibition of COX-2 in a oral SCC cell line (NS-398) actually caused inhibition of proliferation of the cancer cells via suppression of PGE2 production (Sumitani *et al*, 2001) and caused increased terminal differentiation in mouse skin keratinocytes *in vivo* (Akunda *et al*, 2004). While, most NSAIDs exert their antitumour effects by inhibiting the COX-2/PGE2 pathway, some NSAIDs exert their antitumour effects *in vitro* independently of their ability to inhibit COX-2 or to decrease PGE2 (Greenhough *et al*, 2009). It was shown that NS-398, a COX-2 selective inhibitor, can cause apoptosis in colon carcinoma cells independently of COX-2, as it had similar effects in colon cancer cells that expressed and colon cancer cells that did not express COX-2 (Elder *et al*, 1997). Also, NSAIDs caused changes in proliferation and apoptosis in a colon cancer cell line that lacks COX transcripts and does not produce PGs, and in another one that does produce PGs (Hanif *et al*, 1996). Furthermore, some other NSAIDs actually induce COX-2 expression. The NSAID flufenamic acid induced COX-2 expression in colon cancer cells and murine macrophages (Paik *et al*, 2000), while three other NSAIDs induced COX-2 expression in human airway smooth muscle cells independently of PGE2 production (Pang *et al*, 2003).

In addition, several studies have shown that the COX-2/PGE2 pathway may even be tumour suppressive under specific circumstances. Wilson *et al* demonstrated a reduction in the number and size of intestinal tumours *in vivo* after the exogenous administration of the synthetic PGE2 analogue 16, 16-dimethyl-PGE2 (Wilson & Potten, 2000). A very recent study showed that the cannabinoid anandamide can induce cell death in the apoptosis-resistant HCT116 Bax-/- colorectal cell line in a COX-2 –dependent manner. Also, elevated COX-2 expression sensitised the SW480 colorectal cancer cells (low endogenous COX-2) to anandamide-induced death, while COX-2 suppression via RNAi inhibited anandamide-induced cell death in the HCA7 colorectal cancer cells (high endogenous COX-2 expression) (Patsos *et al*,

2010). Finally, transgenic mice over-expressing COX-2 via the keratin 14 promoter, which causes COX-2 over-expression and elevated PGE2 levels in the skin, were more resistant to the development of induced skin tumours induced instead of being more sensitive, as it was initially expected (Bol *et al*, 2002).

Generally, the role of COX-2 and/or prostaglandins in cancer might not be as straightforward as initially proposed (Greenhough *et al*, 2009) and could be tumour suppressive or tumour promoting under different circumstances. The elevation of the expression of COX-2 in SCC-25 cells in the present study after DHA and EPA treatment could be tumour suppressive, by causing apoptosis or growth arrest. Also, COX-2 can participate in the metabolism of EPA and DHA into anti-inflammatory and tumour suppresive prostanoids such as the series 3 prostanoids or resolvins or even new undiscovered metabolites of omega-3 PUFA. This remains to be investigated by the use of COX-2 inhibitors and their effects on keratinocyte growth and apoptosis.

Some studies link the PUFA tumour suppressive action with the MAPK pathway. Serini showed that PUFA can promote apoptosis through the up-regulation of MAPK phosphatase-1 (MKP-1), which de-phopshorylates the two residues on activated MAPKs, and reduces the levels of p-ERK1/2 and p-p38 in lung cancer cells (Serini *et al*, 2008). Also, it was reported that DHA lowered the activation of Ras oncogenes and ERK activation in mouse colon cells (Collett *et al*, 2001), and EPA decreased MAPK activity and inhibited cell proliferation in hepatoma cells (Murata *et al*, 2001). Interestingly and unexpectedly, in our study the expression levels of phospho-ERK1/2) (phospho-p44/42 MAPK) were up-regulated after treatment with DHA and EPA and not down-regulated. The activity of ERK1/2 increases within 5 h of treatment with PUFA, continues to increase after 17 h and reaches maximal levels at 24 h. So there is a sustained and increasing ERK1/2 activity. The elevation of ERK1/2 phosphorylation was confirmed at the lower more selective concentrations of EPA and DHA, and started as early as 30 min after PUFA treatment. The activation of the downstream target of p-ERK, p-p90RSK was also demonstrated.

Typically, the activation of ERK is supposed to promote cell survival and proliferation (Mansour *et al*, 1994; Xia *et al*, 1995). Activation of MAPK was

observed in basal and suprabasal keratinocytes of human and transgenic mouse psoriatic lesions and healing mouse skin wounds. The activation of MAPK stimulated proliferation and delayed terminal differentiation in keratinocytes expressing MAPKK-1, without exhibiting key properties of transformed cells (Haase *et al*, 2001). However, in some cases ERK is activated after the cell death stimulus, as a survival mechanism of the cells to reverse their fate (Haase *et al*, 2001; Persons *et al*, 1999; Wilson *et al*, 1999). A recent study demonstrated that treatment of adenocarcinoma HeLa cells with TRAIL induces cell death and in addition causes up-regulation of p-ERK1/2 (Lee do *et al*, 2006). They suggested that ERK1/2 activation plays a protective role, as a cellular defence mechanism to survive, via the regulation of the Bcl-2/Bax ratio and several mitochondrial events during TRAIL-induced apoptosis. Furthermore, the use of cisplatin in ovarian cancer cells caused elevation of p-ERK which, when inhibited, let to cisplatin-induced cytotoxicity enhancement (Persons *et al*, 1999). Other studies have also reported that ERK1/2 activate cytoprotective mechanisms against Fas-induced cytotoxicity in Jurkat cells (Wilson *et al*, 1999) and against apoptosis induced by serum deprivation in PC12 cells (Kim *et al*, 2000).

Recent investigations, also, increasingly implicate ERK1/2 activation in cell death, depending on the type and duration of the stimulus and cellular context. Long-term ERK1 activation as a result of anchorage deprivation resulted in cell cycle arrest and telomerase inhibition in human stratified squamous epithelial cells (Crowe *et al*, 2005). Similarly, increased MAPK activation was observed in lung cancer cell lines anchorage deprivation (Wei *et al*, 2001). Dose and time-dependent ERK activation was proved to be necessary for mediating cisplatin-induced apoptosis of human cervical carcinoma HeLa cells, as MEK inhibitors PD98059 and U0126 and suramin (a growth factor receptor antagonist) prevented apoptosis, while pre-treatment of cells with TPA (activator of the ERK pathway) enhanced their sensitivity to cisplatin (Wang *et al*, 2000). Cannabinoids were shown to signal apoptosis by a pathway involving Raf1/extracellular signal-regulated kinase activation in glioma cells, prevented by PD98059 (Galve-Roperh *et al*, 2000). An inducible, activated form of the Raf-1 proto-oncogene resulted in a prolonged increase in MAPK activity and growth arrest of neuronal cells, with inhibition of CDK2 activity, while all these effects of were reversed by treatment of cells with PD98059 (Pumiglia & Decker,

1997). All these studies demonstrate that the early and sustained ERK activation I observe after omega-3 PUFA treatment in oral SCC cells would possibly be the mechanism of PUFA-induced apoptosis or cycle arrest or both. This remains to be proven by MTT and apoptosis assays after the inhibition of p-ERK. If ERK activation drives the growth inhibitory and apoptotic effect of PUFA, the inhibition of its activation should reduce these effects.

Recently, Elder *et al* demonstrated that sustained ERK1/2 activation mediates apoptosis caused by the NSAID NS-398 in colon cancer cells, while the UO126 MEK inhibitor protected the cells from these anti-proliferative effects and also reduced COX-2 protein levels (Elder *et al*, 2002). As the authors had previously shown that NS-398 caused apoptosis via COX-2 level elevation in colon cancer cells (Elder *et al*, 2000) they suggested that NS-398 cause ERK1/2 activation, which results in COX-2 elevation and the induction of apoptosis (Elder *et al*, 2002). As there is COX-2 activation 5 h after PUFA addition in our study, it is likely that COX-2 is linked to the ERK-induced growth inhibitory effect in this case as well. In order to investigate this, a COX-2 inhibitor should be used and the possible block of growth inhibition needs to be examined.

Some studies have associated ERK activation and concomitant cell death to ROS and DNA damage induction. Tang *et al* showed that DNA damage stimuli activated ERK1/2 in various cell lines, which contributed to either cell cycle arrest or apoptosis in response to low or high intensity DNA insults, respectively. Inhibition of ERK activation by PD98059 or U0126 resulted in partial release of the cell cycle and strongly attenuated apoptosis (Tang *et al*, 2002). Stanciu *et al* showed that delayed and persistent activation of ERKs is associated with glutamate-induced oxidative toxicity in HT22 neuronal cells and immature primary cortical neurons, while U0126 protects the cells from glutamate toxicity. They suggest that glutamate-induced ERK activation is downstream of a burst of reactive oxygen species (ROS) accumulation (Stanciu *et al*, 2000). In our case, DHA and EPA appeared to increase ROS production and DNA damage after 16 hours treatment (chapter 5). However, the use of two different anti-oxidants (α-tocopherol and PBN) failed to reverse the growth inhibitory effects of the omega-3 PUFA. Also, ERK activation happens very early after PUFA treatment, in contrast to the study of Stanciu (Stanciu *et al*, 2000).

So ROS induction might contribute to the ERK activation and growth inhibitory effect I observe. However, this is not supported by my current evidence.

In conclusion, it is unclear yet if the up-regulation of p-ERK1/2 after treatment with PUFA in the present study is a survival mechanism or if it is involved in the apoptosis machinery. The first steps in investigating these possibilities were the use of two MEK inhibitors in order to examine their effects on ERK and p90RSK activation by western blot and to enable us to decide which are the best concentrations for the cell assays. U0126 reduced ERK1/2 and p90RSK phosphorylation, but it also affected the endogenous levels of the control. AZD6244 seemed more potent than U0126, as it inhibited phospho-ERK1/2 and phospho-p90RSK levels without reducing the basal levels of the control as much. I also used the pan-caspase inhibitor QVD-Oph which did not affect the levels of phospho-ERK1/2, demonstrating that ERK1/2 phosphorylation is not a consequence of apoptosis, reducing the possibility that it is just a survival mechanism.

As the MAPK pathway can be triggered by epidermal growth factor receptor (EGFR) activation, I investigated whether blocking of EGFR activity can inhibit p-ERK and p-p90RSK. An EGFR blocking antibody (EGFR Mouse anti-Human Monoclonal 225) and the EGFR inhibitor AG1478 were used. Interestingly, the EGFR blocking antibody inhibited the PUFA induced p-ERK1/2 and p-p90RSK very effectively, without affecting the endogenous levels of the control. The EGFR inhibitor AG1478 was also potent in reducing p-ERK1/2 and p-p90RSK levels, without affecting the basal levels of the control. Both were very effective even at the lower doses used.

In 1978, EGFR was identified as a protein that showed increased phosphorylation when bound to EGF in the A431 squamous cell carcinoma cell line. The EGFR gene is located on chromosome 7p12-13 and codes for a 170 kDa receptor tyrosine kinase. This family consists of EGFR (also known as ERBB1/HER1), ERBB2 (HER2/NEU), ERBB3 (HER3) and ERBB4 (HER4). All proteins have four functional domains: an extracellular ligand-binding domain, a transmembrane domain, an intracellular tyrosine kinase domain and a C-terminal regulatory domain (Burgess *et al*, 2003; Mitsudomi & Yatabe, 2010). The extracellular domain consists of four domains. The

tyrosine kinase domain consists of an N-lobe and a C-lobe, and ATP binds to the cleft formed between these two lobes. The C-terminal regulatory domain has several tyrosine residues that are phosphorylated upon ligand binding (figure 6-14A). Eleven different ligands are known to bind to the ERBB family of receptors (Mitsudomi & Yatabe, 2010). The binding of ligands to the extracellular domain of EGFR generally leads to the formation of homodimers and heterodimers. The process is mediated by rotation of domains I and II, which results in the exposure of the dimerization domain (figure 6-14B) (Burgess *et al*, 2003). In the cytoplasm, the kinase domain dimerises asymmetrically in a tail-to-head orientation (figure 6-14C). Dimerization consequently stimulates the intrinsic tyrosine kinase activity of the receptors and triggers autophosphorylation of specific tyrosine residues within the cytoplasmic regulatory domain (Mitsudomi & Yatabe, 2010). These phosphorylated tyrosines serve as binding sites of adaptor proteins. Signal transducers then bind to these adaptors to initiate several signalling pathways such as the MAPK and PI3K/AKT pathways (figure 6-1) as well as the signal transducer and activator of transcription (STAT) 3 and STAT5 pathways (Mitsudomi & Yatabe, 2010).

EGFR is expressed in a variety of human tumours, including those in the lung, head and neck, colon, pancreas, breast, ovary, bladder and kidney, and in gliomas (Mitsudomi & Yatabe, 2010; Ratushny *et al*, 2009). The fact that the EGFR antibody and the EGFR inhibitor block ERK1/2 phosphorylation and function in our SCC-25 cells, as determined by the inhibition of the ERK1/2 target phospho-p90RSK, suggests that the PUFA may be causing apoptosis by increasing the secretion of an unknown EGFR ligand. As most SCCs possess increased levels of EGFR (Stanton *et al*, 1994) and are known to be inhibited from growing by levels of EGF that are optimal for normal keratinocytes (Gulli *et al*, 1996). I hypothesise that the release of EGFR ligand(s) by PUFA may lead to a suprastimulation of ERK1/2 and as a result trigger apoptosis.

Omega-3 PUFA can also alter the lipid composition of the plasma membrane, which may affect the membrane fluidity and structure and the way growth factors, cytokines, and hormones interact with their receptors, and the resulting signal transduction. The microdomains, thought to play important roles in signalling, called 'lipid rafts' are particularly interesting (Berquin *et al*, 2008; Chapkin *et al*, 2008). A

recent study indicated that EPA and DHA changed the lipid raft composition in MDA-MB-231 breast cancer cells, led to sustained phosphorylation of the EGFR receptor and downstream p38 MAPK pathway (even though it caused a marked decrease in EGFR levels in lipid rafts). The EGFR activation paradoxically caused reduction of cell growth (Schley *et al*, 2007). Thus, there is a possibility that the EGFR activation that causes ERK activation in our case is triggered by the alterations caused to the membrane lipid rafts by EPA and DHA.

Furthermore, JNK phosphorylation was also observed, especially at the higher concentration of EPA and DHA. As mentioned in the introduction of this chapter, the JNK and p38 pathways (MAPK sub-pathways) (figure 6-1) are activated preferentially by stress, DNA-damaging agents, oxidation and inflammatory cytokines (Dhillon *et al*, 2007). JNK is tumour suppressive as when activated can promote apoptosis (Kennedy *et al*, 2003). So probably, JNK activation can also participate in the PUFA induced apoptosis and could also be a result of EGFR over-activation as well, or it could be triggered by ROS production. However, JNK activation was observed very early (after 2 hours treatment) so it is not clear if ROS was involved. p38 activation has not been investigated yet.

It is also worth mentioning that the activation of ERK, JNK and p38 could be triggered after endoplasmic reticulum (ER) stress. Thiazolidinediones stimulate cells to activate p38, ERK1/2 and JNK (Edwards & O'Flaherty, 2008; Gardner *et al*, 2005) by discharging Ca^{2+} from the ER to evoke an ER stress response; this activates Ca^{2+}/calmodulin kinase II, proline-rich tyrosine kinase 2, protein kinases C, *c-Src*, EGFR, the ERK1/2 and JNK pathways, the double stranded RNA-activated protein kinase, and p38. Double stranded RNA-activated protein kinase inactivates eukaryotic initiation factor-2 (EIF-2), blocking protein translation (Gardner *et al*, 2005; Palakurthi *et al*, 2001). Also, as mentioned in the subchapter 6.1, EPA induced a Ca^{2+} release from the intracellular Ca^{2+} stores and simultaneously inhibited Ca^{2+} influx via Ca^{2+} channels in the plasma membrane, resulting in a depletion of the intracellular Ca^{2+} stores which caused inhibition of translation initiation, and also activated eIF2a kinase, which are signs of ER stress (Aktas & Halperin, 2004; Berquin *et al*, 2008). Other studies have also recently shown that EPA has similar

Figure 6-14: The EGFR protein

(A) The structure of the EGFR protein is shown with the four functional domains; the extracellular ligand-binding domain, the transmembrane domain, the intracellular tyrosine kinase domain (with the N-lobe and a C-lobe) and the C-terminal regulatory domain. (B) The activation by rotation of the extracellular ligand-binding sub-domains and (C) the dimerization by ligand binding are shown (Mitsudomi & Yatabe, 2010).

effects on ER calcium discharge (Abedin *et al*, 2006; Palakurthi *et al*, 2000). Jackobsen *et al* found that, as an early response, in SW620 colon carcinoma cells, DHA induced the expression of different factors involved in ER stress response, such as XBP1, PERK, ATF4, ATF6 and phosphorylated EIF2a. However, it failed to induce apoptosis in their model (Jakobsen *et al*, 2008). My study showed activation of JNK and ERK pathways caused by EGFR activation, so it could be related to ER stress. However, no other features of the ER stress were investigated. The possible effect of omega-3 PUFA on ER-stress and ER-stress induced apoptosis is a quite unexplored issue, worth further investigation.

Finally, some studies showed that EPA and DHA can actually trigger PPAR activation as mentioned in subchapter 6.1. DHA induced apoptosis in MCF-7 human (Sun *et al*, 2008) and in Ramos cells (combined with irradiation) (Zand *et al*, 2007) by the activation of PPARγ. Chene et al, also reported that EPA induced an increase on COX-2 expression (Chene *et al*, 2007) mediated by PPARγ activation proposing an anti-inflammatory effect. Moreover, PPARγ has been implicated in the activation of MAPK pathway and ER stress (Gardner *et al*, 2005). Taking all these together, it is possible that PPAR activation is also participating in the PUFA-induced growth inhibitory effect and apoptosis. This has not been investigated yet.

In summary, my study showed that the omega-3 PUFA, EPA and DHA, cause high, early and sustained activation of ERK1/2. The activation of the ERK substrate, p-90RSK, was also confirmed. EGFR appears to cause ERK1/2 activation. We hypothesise that EGFR which is over-expressed in SCC cells, is suprastimulated by a ligand that omega-3 PUFA release, resulting in ERK pathway activation which leads to apoptosis and/or growth arrest. EGFR activation can be also triggered by lipid raft alterations or ER stress. As COX-2 expression was also increased, COX-2 could be also implicated in the cell growth inhibitory effect of PUFA being a downstream target of ERK pathway during ERK-induced apoptosis, or could produce a tumour-suppressive EPA and DHA derivative. The stress-related and tumour-suppresive JNK protein is also expressed, possibly participating in PUFA- induced apoptosis. These hypotheses remain to be proven in the near future by inhibition of the over-expressed molecules and their effect on SCC cell growth and apoptosis.

Chapter 7

FATTY ACID INTERACTION WITH SERUM AND MEMBRANE INCORPORATION

Chapter 7: Fatty acid interaction with serum and membrane incorporation

7.1 Background: Fatty acid transport in the keratinocytes

Lipid molecules have limited solubility in aqueous solutions. In order to overcome this limitation and be available for utilization by the various cells and tissues, lipids are either bound by specific carrier proteins, or are part of larger lipid-protein complexes called lipoproteins. Therefore, the majority of the long-chain fatty acids are bound to plasma albumin in the circulation and to cytosolic proteins in the cell cytoplasm (Glatz *et al*, 2002). As mentioned in chapter 1, the uptake of fatty acids from the circulation into cells includes the sequence: adsorption, transmembrane movement and desorption. The albumin-bound fatty acids (FA) need to dissociate from albumin and either bind to plasma membrane proteins or integrate into the lipid bilayer. Following transport across the plasma membrane, the FA bind to other proteins in the cytoplasm (Doege & Stahl, 2006) (figure 7-1). The FA uptake process is still controversial and unclear. FA can be taken up by the cells by passive diffusion through membrane lipid via flip-flop mechanisms. The diffusion is thought to become more significant at high ratios of FA: albumin (> 2:1) as the concentration of unbound FA (FA dissociated from albumin) increases (Coburn *et al*, 2001). Studies with model membrane systems and intact cells (adipocytes), and using techniques such as dual fluorescence approaches, provided evidence that fatty acids diffuse very rapidly across the plasma membrane (Hamilton *et al*, 2002). However, under physiological conditions, the passive diffusional uptake of FA does not mean that a protein-facilitated FA uptake does not take place. Passive diffusion and protein-mediated transmembrane transfer of FA seem to co-exist as separate ways of FA transport. These two routes seem to contribute to the overall rate of FA transport, which could depend on the nature of the membrane (plasma membrane organelle or vesicular membrane), cell type and functional state of the cell, FA availability and FA type, and different hormonal environment (Glatz *et al*, 2002; Khnykin *et al*, 2011). Although the occurrence of both long chain FA uptake processes are now widely accepted, in recent years many studies have demonstrated that protein-

mediated transport accounts for the majority of fatty acid uptake by tissues with high long chain PUFA metabolism and storage (Doege & Stahl, 2006). The candidate proteins that have been proposed to be involved in the uptake process of PUFA include FA translocase (FAT/CD36) (Coburn *et al*, 2001), FA binding proteins (FABPs) (Storch & McDermott, 2009; Storch & Thumser, 2010), long chain fatty acyl-CoA synthetases (ACSL) (Gargiulo *et al*, 1999), long chain fatty acyl-CoA binding protein (ACBP) (Knudsen *et al*, 2000) and FA transport proteins (FATPs) (Doege & Stahl, 2006) (figure 7-1).

The three major lipids in the stratum corneum of the epidermis are 15% free fatty acids, 25% cholesterol and 50% ceramides. Some FA (with carbon chains of up to C16) can be synthesised by keratinocytes *de novo* and, in addition, need to be taken up from the circulation, which is protein mediated. Several fatty acid transporters are expressed in skin. A significant amount of the FAs produced or taken up from diet, are further elongated into very long chain FAs (C ≥18) (Jakobsson *et al*, 2006). During keratinisation, long-chain, highly saturated species (14-28 carbons) replace the short-chain FAs. The majority are ≥20 carbons, with 22–24 carbon lengths being the most abundant (Khnykin *et al*, 2011).

Essential fatty acid deficiency leads to abnormalities in skin function, resulting in scaly dermatosis, permeability of skin to water and hair loss (Burr & Burr, 1929; Khnykin *et al*, 2011). As mentioned before, the human body cannot produce LA (C18:2, n-6) and LNA (C18:3, n-3). Also, AA (C20:4 n-6) of the epidermis, must be synthesised in the liver and transported into keratinocytes, as well as other LCFA and VLCFA that also have to be translocated across the keratinocyte plasma membrane. EPA and DHA can be obtained from dietary sources and are incorporated into the epidermal lipids (Ziboh *et al*, 1986). FAs have multiple roles in the epidermis. They can be part of triglycerides, phospholipids, glycosylceramides and ceramides, forming the epidermal permeability barrier. In addition FAs in keratinocytes play a role in energy generation and storage and can be potent signalling molecules (Khnykin *et al*, 2011). Inflammatory skin diseases, such as atopic dermatitis and psoriasis, are characterised by changes in FA composition in

Figure 7-1. Free fatty acid uptake and action in mammalian cells

Albumin-free fatty acids (FFAs) are generated from lipoproteins by the action of endothelial lipoprotein lipase (LpL). At physiological conditions, the majority of FFAs are bound to albumin, whereas the concentration of unbound FFAs in this equilibrium is low. FFAs are transferred via the plasma membrane into the cell mainly by a protein-mediated mechanism, either by interaction of the FFAs directly with FA translocase (FATP) complexes, or by binding to cell-surface proteins, such as CD36, which introduces them to the FATPs. On the cytosolic side, FFAs are quickly activated and coupled to CoA by the catalysis of ACSL or the by FATPs. Then, FA binding proteins (FABPs) or long chain fatty acyl-CoA binding protein (ACBP) facilitate the intracellular unloading of the transporters and the synthetases and function as an intracellular fatty acid buffer. Intracellularly, FFAs can have functions in energy generation and storage, membrane synthesis, protein modification, and activation of nuclear transcription factors. FFAs can also signal extracellularly, e.g., by stimulating GPR40 in β-cells to induce insulin secretion or by activating TLRs to initiate the innate immune response (Doege & Stahl, 2006).

keratinocytes, indicating their possible involvement in the modulation of inflammation (Sala-Vila *et al*, 2008; Ziboh *et al*, 1986). Moreover, free FA contribute to the acidic pH of the stratum corneum, regulating permeability and the antimicrobial barrier, inflammation and desquamation (Hachem *et al*, 2003).

Studies have shown active uptake of FAs by keratinocytes, which preferably transport essential FAs, i.e., LA and AA than non-essential FA, such as oleic acid (C18:1, n9) (Schurer *et al*, 1994). It was demonstrated that FATP1, -3, -4 and -6, along with CD36/FAT, are expressed in adult murine epidermis and specifically FATP1 and -3 were expressed predominantly by keratinocytes (Schmuth *et al*, 2005). FATPs expression in humans was comparable to that in mice and experiments with primary human keratinocytes showed that FATP4 was the most abundant FATP expressed in culture. The induction of differentiation resulted in approximately a 50% reduction in the level of FATP protein. In contrast to human and mouse epidermis, neither FATP1, -3 nor -6 were expressed in primary cultures of differentiated and un-differentiated human keratinocytes (Schmuth *et al*, 2005). Moreover, CD36/FAT, plasma-membrane FABPs and ACSL are expressed at different levels in undifferentiated and differentiated human keratinocyte cultures (Harris *et al*, 1998).

The essential role of protein FA uptake mediators was highlighted by a number of mouse models. CD36 knockout mice did not show any apparent skin phenotype, whereas muscles and adipose tissues had a defective LCFA uptake and utilization (Coburn *et al*, 2000; Febbraio *et al*, 2002). CD36-mediated uptake of FAs in keratinocytes can be compensated by other FA transporter proteins.

FABP5 (KFABP) is predominantly expressed in keratinocytes, where it has been proposed to act as a cellular lipid chaperone in keratinocyte homeostasis (Krieg *et al*, 1993). It has also been detected in tongue and thymus epithelia. It seems that FABP5 is the only FABP detected in the epidermis (Krieg *et al*, 1993; Ogawa *et al*, 2011; Owada *et al*, 2002). Compared with other tissues, human keratinocytes contain lower levels of FABP5, while these levels increase with keratinocyte differentiation (Siegenthaler *et al*, 1994). Overexpression of FABP5 is associated with hyper-proliferative skin diseases, such as psoriasis, atopic dermatitis and basal and

squamous cell carcinomas (Masouye *et al*, 1996). It is hypothesised that FABP5 expression is increased in response to increased lipid traffic related to abnormal proliferation and differentiation of keratinocytes in these diseases. The skin of FABP5 knockout mice appears normal, but the water-barrier function of the epidermis is altered in these mice (Owada *et al*, 2002). It was also shown that FABP5 deletion disrupts keratinocyte migration, suggesting a role in cell motility (Kusakari *et al*, 2006). The absence of obvious skin phenotype could mean that other FABP members or other FA-transport proteins compensate for FABP5 deficiency. Ogawa *et al* showed that FABP5 deletion affects keratinocyte differentiation, but not proliferation (Ogawa *et al*, 2011). Total FA content in FABP5-deficient epidermis was decreased, including decreased saturated, monounsaturated and polyunsaturated FAs. Specifically, LA was significantly decreased, while AA and LNA levels were the same (Ogawa *et al*, 2011). In summary, the high expression of FABP5 in hyperproliferative skin conditions suggests that FABP5 may have a role in the pathogenesis of such diseases disrupting metabolism of FAs and their derivates.

Several FATPs, including FATP4, are expressed in the epidermis (Schmuth *et al*, 2005) and animal models suggest that FATP4 plays an important role in skin homeostasis. Recently, mutations in the *FATP4* gene were found responsible for Ichthyosis Prematurity Syndrome (IPS) (Klar *et al*, 2009) which is a rare disorder of keratinization, characterised by premature birth, scaly erythroderma and neonatal respiratory complications. FATP4 knockout mice die either *in utero* (Gimeno *et al*, 2003) or shortly after birth (Moulson *et al*, 2003) showing a disturbed epidermal barrier and hyperkeratosis, leading to very tight, thick skin (wrinkle free phenotype). It is very interesting that it was possible to rescue the FATP4 knockout mice from death by keratinocyte-specific transgenic expression of FATP4, leading to viable and fertile mice with only mild skin and hair abnormalities, demonstrating the importance of FATP4 in the epidermis (Moulson *et al*, 2007). However keratinocyte-specific expression of FATP4, mutated in the acyl-CoA synthetase domain, did not rescue the skin phenotype, indicating the ability of FATP4 to activate fatty acids is crucial for its function (Moulson *et al*, 2007). Herrmann et al. created mice with conditional FATP4 deficiency in the epidermis which displayed structural changes in the epidermis and an impaired barrier function, but the phenotype was not nearly as severe as that seen in mutant neonates (Herrmann *et al*, 2005). The explanation for

this apparent difference could be that in adult skin FATP4 deficiency is compensated by other FATPs, thus FATP4 is more critical for the generation of the epidermal barrier and less important for its maintenance (Khnykin *et al*, 2011).

Although the exact mechanism by which FAs are transported into keratinocytes is still unclear, all these studies show that different FA transporters play important roles in the epidermis and keratinocyte homeostasis.

7.2 Results

7.2.1 The omega-3 PUFA inhibit growth and induce apoptosis in pre-malignant keratinocytes and colon carcinoma cells but their action is inhibited by FBS

Although it had been reported that the omega-3 PUFA inhibited the growth of DLD-1 (Eitsuka *et al*, 2005) we were unable to confirm these observations or extend them to HaCaT cells in medium containing 10% v/v FBS. Figure 7-2A shows the results of the MTT assays conducted on the pre-malignant cell line HaCaT and the colon carcinoma line DLD-1 after treatment of the cells for 4 days with DHA and EPA in medium containing 10% v/v FBS. As FBS batches are variable, we hypothesised that our FBS might be rich in survival factors or antagonists of the omega-3 PUFA, and so re-tested the compounds in medium with reduced (3%) concentrations of FBS. Figure 7-2B shows that under these conditions both HaCaT and DLD-1 were strongly inhibited by both omega-3 PUFA at 30 μM and 50 μM, supporting our hypothesis.

Additionally, the SCC-25 oral cancer cell line was tested to examine the effect of PUFA on cell growth in 10% FBS medium (figure 7-3). Instead of the KGM serum free medium where SCC-25 are usually cultured in during our experiments(chapters 3,5,6) in this case SCC-25 were cultured in FAD medium containing 10% FBS. The MTT assay was performed using the same conditions as the MTT assays in KGM medium. DHA and EPA were added at concentrations 3-100 μM, which is a much higher range than the one used in serum free medium (0.1-10 μM). It is obvious that the FBS also antagonised the effect of PUFA on SCC-25 growth inhibition. SCC-25 cells were almost entirely dead at PUFA concentrations of >3 μM in the KGM serum free medium (figure 3-1), while only a small percentage of cells were dead at the medium containing 10% FBS (figure 7-3). Surprisingly, only EPA 5 μM seemed to be more inhibitory, but overall the serum protected the cells from the inhibitory effect of the PUFA.

A.

Medium with 10% FBS

B.

Medium with 3% FBS

Figure 7-2: Effect of PUFA on HaCaT and DLD-1 growth in 10% and 3% FBS medium

*HaCaT and DLD-1 cultures were plated on day 1 and allowed to attach in either RPMI containing 10% v/v FBS (A) or 3% v/v FBS (B). On day 2, DHA or EPA was added in increasing concentration 0.1-50 µM. 0.1% v/v ethanol was used as a control. Ethanol alone had a negligible effect. Fresh lipids were added on day 5 and the MTT assay conducted on day 7. The bars represent the mean values ± SEM of 3 independent MTT experiments, conducted in duplicate. The red bars represent the 50 µM DHA and the green bars 50 µM EPA. * is significantly different from the mean value of 3% v/v FBS medium untreated control (* = p<0.05 and ** = p <0.01 *** = <0.001 as measured by one way ANOVA followed by post-hoc Bonferroni test).*

Medium with 10% FBS

SCC-25

Figure 7-3: Effect of PUFA on SCC-25 growth in 10% FBS medium

SCC-25 cultures in 10% FBS FAD medium were plated on day 1 and allowed to attach for 3 days. On day 4, DHA or EPA was added in increasing concentration 3-100 μM. 0.1% v/v ethanol was used as a control. Fresh lipids were added on day 6 and the MTT assay conducted on day 8 (after 4 days of PUFA treatment). The bars represent the mean values +/- SEM of 3 independent MTT experiments, conducted in duplicate. The red bars represent the DHA and the green bars the EPA.

7.2.2 The protective effect of FBS against omega-3 PUFA is not related to serum growth/survival factors

In medium containing 3% v/v FBS, 50 μM EPA and DHA each lowered the number of viable cells in the controls by approximately 60-80% (figure 7-4). However, at concentrations which encompassed the physiological range of these growth factors in serum and plasma, IGF-1 (1-30 ng/ml; figure 7-4A) (Seto *et al*, 2001), EGF (0.3-30

ng/ml; figure 7-4B) (Pietrzak *et al*, 1999), FGF-a (0.1–3.0 ng/ml; figure 7-4C) (Rizzino *et al*, 1988) FGF-b (0.1-3.0 ng /ml; figure 7-4D) (Pardo *et al*, 2002), TGF-α (1-40 ng/ml; figure 7-4E) (Pehlivan *et al*, 2001) and TGF-β$_1$ (3-300 pg/ml; figure 7-4F) (Wenisch *et al*, 1995) did not prevent the EPA or DHA from inhibiting the cellular proliferation FBS (M.Hassan and Z.Nikolakopoulou). As before, when the PUFA were added to medium containing 10% v/v FBS at the same concentration (50 μM), they failed to show a significant growth inhibitory effect (figure 7-4G). EGF was slightly inhibitory in the HaCaT controls at a concentration of 30 ng/ml, but this was not statistically significant (p = 0.08). FGF-a and FGF-b showed a slight stimulation of HaCaT growth at higher concentrations, but again this was not statistically significant in either case. TGF-α showed a slight relative protective effect but this was largely due to the inhibitory effect of TGF-α in the controls, at concentrations of 5 ng/ml and above. TGF-α caused a significant growth inhibition of 30-50% at 10-40 ng/ml (p < 0.05). TGF-β$_1$ concentrations of 3-10 pg/ml showed a slight growth stimulatory effect in the HaCaT controls but at 30 pg/ml and above was progressively inhibitory and at 0.3 ng/ml significantly inhibited growth by 45-50% (p < 0.05).

7.2.3 The anti-oxidant n-tert-butyl-α-phenylnitrone (PBN) does not protect against the growth inhibitory effects of omega-3 PUFA

Finally, as serum contains anti-oxidants, the effect of the known antioxidant n-tert-butyl-α-phenylnitrone (PBN) was tested (Nakao *et al*, 1996). As omega-3 PUFA can produce oxidative damage in keratinocytes especially at higher doses (Chapter 5), we used different concentrations of PBN ranging from 50 μM to 1000 μM to test the effect of PBN on PUFA activity in a medium with 3% v/v FBS (M.Hassan and Z.Nikolakopoulou). However, there was no protective effect of PBN against the omega-3 PUFA (figure 7-5). We tried 2 different dose ranges; the first one with 5 different concentrations (50 μM, 100 μM, 200 μM, 400 μM and 600 μM- figure 7-5A) and the second one with 800 μM, 900 μM and 1000 μM (figure 7-5B). However, at no dose did we see a protective effect. Rather than protection, PBN at

50 µM to 400 µM showed a slight stimulatory effect in HaCaT cell growth (figure 7-5A). However, from 600 µM to 1000 µM PBN showed a gradual inhibition of cellular proliferation in HaCaT (figure 7-5B). PBN at 800 µM reduced the frequency of senescent cells and oxidative damage in human fibroblast cultures, showing that PBN is effective at this concentration (Pitiyage *et al*, 2011). Therefore, our data argue against an antioxidant effect being responsible for the antagonistic effects of FBS against omega-3 PUFA-induced HaCaT growth inhibition, in contrast to the recent report of others (Kanno *et al*, 2011).

Figure 7-4. Effect of growth factors on PUFA induced growth inhibition in 3% FBS medium

Means± S.E.M of 3 independent MTT experiments in duplicate. HaCaTs were plated on day 1 and allowed to attach in either RPMI containing 3% v/v FBS or 10% v/v FBS as a negative control. On day 2 50 μM the omega-3 PUFA were added. 0.1% v/v ethanol was used as a control. Fresh lipids were added on day 5 and the MTT assay conducted on day 7. (A) –(F) show the effect of the lipids in different concentrations of the following growth factors in 3% v/v FBS; (A) IGF-1, 1-30 ng/ml, (B) EGF, 0.3 – 30 ng/ml, (C) FGF-a, 0.1 – 3 ng/ml, (D) FGF-b, 0.1-3 ng/ml, (E) TGF-α, 1-40 ng/ml (F) TGF-β, 3-300 pg/ml, (G) 10% v/v FBS Control.

A.

B.

Figure 7-5. Effect of an anti-oxidant on PUFA induced HaCaT growth inhibition in 3% FBS medium

The figure shows the effect of the omega-3 PUFA in two sets of different concentrations of PBN in RPMI with 3% v/v FBS. The protocol was the same as for figure 7-4. The blue bars represent the untreated controls, the red bars the 50 μM DHA and the green bars the 50 μM EPA. The bars represent the means of 3 experiments performed in duplicate +/- SEM.

7.2.4 Albumin is the major serum antagonist of DHA- and EPA-induced growth inhibition

The concentration of albumin in human blood and bovine serum is in the range of 3-5% w/v (Corti *et al*, 1994) and so would likely be 0.3-0.5% w/v in medium containing 10% FBS but only 0.09-0.15% in medium containing 3% v/v FBS. Figure 7-6A shows that BSA at an added concentration of 0.1-0.3% w/v in medium containing 3% v/v FBS (total concentration range 0.2-0.45% w/v BSA) was able to partially protect HaCaT cells from the growth inhibitory effects of both DHA and EPA. However, BSA was only able to protect to 50% of the level of 10% FBS (see figures 7-2A, 7-4G and 7-6C). At concentrations of 1-3% w/v, BSA alone caused considerable growth inhibition.

Figure 7-6B shows that broadly similar results were obtained when BSA was substituted with human serum albumin, except that the optimum concentration was 0.3-1.0% w/v and that the level of protection was 75% of that of 10% FBS (see figures 7-2A, 7-4G and 7-6C). This data supports the hypothesis that the interaction of dietary omega-3 PUFA with albumin in humans might mute their anti-cancer effects. At their optimum protective doses, both BSA and human albumin stimulated HaCaT cell growth in the control cultures at 10-20% and 20-40%, respectively. However, this did not account for their much greater effect on the omega-3 PUFA-treated cells.

Annexin V staining followed by FACS analysis showed that human albumin antagonises the growth inhibitory effect of 50 μM EPA or DHA by inhibiting HaCaT apoptosis (figures 7-7 and 7-8). There is a clear decrease in the early, late and total apoptotic populations after addition of 0.3% or 1% w/v human albumin in the 3% v/v FBS medium after only 72h (figures 7-7 and 7-8A) and a concomitant increase in viable cells (figures 7-7 and 7-8B). It is apparent that human albumin protects HaCaT cells from DHA- and EPA-induced apoptosis and that this partially accounts for its protective effect on HaCaT growth (figure 7-6).

A.

B.

C.

Figure 7-6. Effect of human and bovine serum albumin on PUFA induced growth inhibition

*The figure shows the effect of the omega-3 PUFA on HaCaTs in different concentrations of (A) bovine (BA), and (B) human serum albumin (HA) in RPMI plus 3% v/v FBS. The effect of DHA and EPA in RPMI plus 10% v/v FBS is also shown (C).The protocol was the same as for figure 7-3. The blue bars represent the untreated controls, the red bars 50 μM DHA and the green bars 50 μM EPA. The bars represent the means of 3 experiments performed in duplicate +/- SEM. * is significantly different from the mean value of the 3% v/v FBS, DHA or EPA treated control, respectively, with no added albumin (* = p<0.05, ** = p <0.01 and *** = p<0.001 as measured by one way ANOVA followed by post-hoc Bonferroni test).*

Figure 7-7. Representative apoptosis assay experiment that shows albumin protective effect against PUFA growth inhibition

The figure shows the results of the annexin V apoptosis assay. The HaCaT cells were incubated for 72 hours, either untreated in 10% FBS RPMI or untreated, 50 μM DHA treated or 50 μM EPA treated in 3% FBS RPMI with 0%, 0.3% or 1% w/v human serum albumin. The cells were incubated with annexin V-FLUOS and DAPI and analysed by flow cytometry. A representative annexin V/ DAPI experiment is shown. Viable HaCaT cells are negative for both annexin V and DAPI; early apoptotic cells are positive for annexin V while being negative for DAPI; late apoptotic cells are positive for both annexin V and DAPI and necrotic cells are positive for DAPI while being negative for annexin V.

A.

B.

Figure 7-8. Mean percentages of apoptotic HaCaT cells in 10% medium or 3% medium supplemented or not with human albumin

The bars represent the mean percentages of (A) the early apoptotic (blue bars) and the late apoptotic cells (red bars) combined together and (B) the live cells in 10% FBS RPMI, 3% FBS RPMI and 3% FBS RPMI with 0.3% or 1% w/v human albumin. The cells were either untreated, or treated with 50 µM DHA or EPA. The bars represent the means of 3 independent experiments +/- SEM. * is significantly different from the mean value of 3% v/v FBS treated control with no added albumin (*=p<0.05 and ** = p <0.01 as measured by one way ANOVA followed by post-hoc Bonferroni test).

7.2.5 Lipid analysis after PUFA treatment

In order to determine the fatty acid composition of malignant and normal keratinocytes and investigate any changes that occur after PUFA treatment in their membrane, lipid analysis by gas chromatography was performed. Specifically the fatty acid composition of SCC-25 and NHEK-131 phospholipids was analysed. The malignant oral cells SCC-25 (8.7×10^4 cells) and the primary oral cells NHEK-131 ($2 \times 8.7 \times 10^4$ cells) were plated in 60 mm culture dishes (VWR). After 3 days, the cells were treated with DHA 3 μM. After 48 h treatment, the lipids were extracted using the Folch method. Then, the phospholipids were isolated from the total lipid extract by TLC, and finally, the fatty acids in the phospholipids were transesterified to produce fatty acid methyl esters (FAME) which were further analysed by gas chromatography coupled with mass spectrometry. The values of the detected fatty acids were normalised to 100% and expressed as mol% which were calculated by normalising the measured fatty acids values to 100% and then dividing by their molecular weights.

Table 7-1 shows the fatty acid composition of the malignant SCC-25 and the table 7-2 the fatty acid composition of the primary epidermal keratinocytes NHEK-131. Also figure 7-9 shows the omega-3 and omega-6 fatty acid profile of the oral cancer cell line SCC-25 and the normal keratinocytes NHEK-131 (a). The changes in these profiles after DHA 3 μM treatment for 48 h are shownd in figure 7-10. Fatty acids are expressed in mol% and include LA, AA and adrenic acid/DPA (docosapentaenoic acid) omega-6 combined, EPA and DHA/DPA omega-3 combined.

These are preliminary data from one experiment, after the optimisation of the method. More repeats are needed for final conclusions. However, we can observe differences between malignant and normal keratinocytes (figure 7-9). The normal keratinocytes appear to have significantly higher amounts of omega-6 and omega-3 fatty acids compared to the malignant ones. Specifically, as regards the omega-6 fatty acids, the NHEK-131 have approximately 50% more AA and 3-fold higher

Fatty Acids	SCC-25 Control mol%	SCC-25 in DHA 3 μM mol%
14:0	1.71	1.50
16:0	38.48	36.93
18:0	37.19	37.98
18:1	6.60	5.66
20:0	0.00	0.00
20:1	0.00	0.00
18:2n-6 (LA)	2.26	1.81
20:4n-6 (AA)	4.86	4.10
22:4/5n-6 (Adrenic acid/DPA n-6)	1.27	1.38
20:5n-3 (EPA)	1.11	1.96
22:5/6n-3 (DPA/DHA n-3)	6.51	8.69

Table 7-1. Fatty acid composition of SCC-25

The fatty acid composition of SCC-25 untreated and treated with DHA 3 μM for 48 h. The fatty acids are expressed in mol%.

Fatty Acids	NHEK-131 Control mol%	NHEK-131 in DHA 3 μM mol%
14:0	0.20	0.23
16:0	28.58	29.79
18:0	33.08	31.71
18:1	4.55	3.77
20:0	0.49	0.29
20:1	0.40	0.27
18:2n-6 (LA)	1.17	0.97
20:4n-6 (AA)	7.28	8.75
22:4/5n-6 (Adrenic acid/DPA n-6)	3.89	3.15
20:5n-3 (EPA)	1.50	1.84
22:5/6n-3 (DPA/DHA n-3)	18.87	19.24

Table 7-2. Fatty acid composition of NHEK-131

The fatty acid composition of normal oral keratinocytes NHEK-131, untreated and treated with DHA 3 μM for 48 h. The fatty acids are expressed in mol%.

Figure 7-9. Omega-3 and omega-6 fatty acid composition of keratinocytes

The fatty acid profiles of the oral cancer cell line SCC-25 and the normal keratinocytes NHEK-131 are shown. The fatty acids are expressed in mol% and include LA (linoleic acid), AA (arachidonic acid) and adrenic acid+DPA (docosapentaenoic acid) omega-6 combined, EPA and DHA+DPA omega-3 combined.

amount of adrenic acid/DPA (n-6) combined than SCC-25. However the normal cells have almost 50% less LA. As regards the omega-3 fatty acids, NHEK-131 have approximately 30% more EPA and almost 3-fold higher amount of DPA (n-3)/ DHA combination than SCC-25.

After treatment with DHA for 48 h (figure 7-10A), SCC-25 showed a 30% increase in DPA n-3/DHA and 70% increase in EPA, and also a notable 15% decrease in AA. Also LA slightly decreased, while the combination of adrenic acid/DPA (n-6) stayed almost the same. As regards the normal NHEK-131 cells (figure 7-10B), DHA treatment for 48 h resulted in a slight (2%) increase in DPA n-3/DHA and a 22% increase in EPA, while AA had a 20% increase. The combination of adrenic acid/DPA (n-6) showed a 20% increase and also LA slightly decreased.

A.

B.

Figure 7-10. Omega-3 and omega-6 fatty acid composition of keratinocytes after DHA treatment

(A) The fatty acid profiles of the oral cancer cell line SCC-25 and (B) the normal keratinocytes NHEK-131, after DHA 3 μM treatment for 48 h, are shown. The fatty acids are expressed in mol% and include LA (linoleic acid), AA (arachidonic acid) and adrenic acid+DPA (docosapentaenoic acid) omega-6 combined, EPA and DHA+DPA omega-3 combined.

7.3 Discussion

7.3.1 Serum albumin antagonises DHA- and EPA-induced growth inhibition

EPA, DHA have been observed to induce apoptosis in many human cancer cell lines (Chen & Istfan, 2000; Edwards & O'Flaherty, 2008; Eitsuka *et al*, 2005; Finstad *et al*, 1998; Heimli *et al*, 2002; Merendino *et al*, 2005; Schley *et al*, 2005; Serini *et al*, 2008) and we extended these observations to the pre-malignant keratinocyte line HaCaT. However, when we investigated the effect of DHA and EPA on the epidermal pre-malignant keratinocyte line HaCaT as well as the colon adenocarcinoma line DLD-1, we observed that the concentration of FBS in the growth medium greatly antagonised the growth inhibitory effect of both PUFA in both cell lines, even though Eitsuka *et al* had reported an inhibitory effect of the same concentrations of PUFA in DLD-1 cells cultured in 10% FBS medium (Eitsuka *et al*, 2005).

Our first hypothesis was that anti-apoptotic survival factors such as IGF-1 in serum might be responsible for this observation, but extensive testing of IGF-1, EGF, FGF-a, FGF-b, TGF-α and TGF-β1 did not support this hypothesis.

Our second hypothesis was that anti-oxidants present in FBS, such as vitamin E and its derivatives, might be the antagonists because DHA and EPA do cause an increase in reactive oxygen species (ROS) production and increase the level of oxidative damage in keratinocytes (chapter 5). However, the antioxidant PBN showed no protective effect against DHA- or EPA-induced HaCaT growth inhibition, thus arguing against this hypothesis.

Finally, as serum proteins such as albumin are known to be lipid-binding (Huang *et al*, 2005) and specifically albumin has three high–affinity binding sites for fatty acids (Hamilton & Kamp, 1999), we hypothesised that such proteins would sequester free omega-3 PUFA like DHA and EPA and reduce their effective concentration in FBS and hence *in vivo*. This hypothesis was supported by the observations that both

bovine and human serum albumin at concentrations of 0.3-1.0% w/v were able to inhibit the effects of high doses of DHA and EPA without being toxic to the HaCaT cells and at least some of these effects were due to the inhibitory effect of albumin on omega-3 PUFA-induced apoptosis. However, the protection afforded to omega-3 PUFA-treated HaCaT cells by albumin was only 50-75% of that produced by 10% FBS, suggesting that other inhibitors of the apoptotic action of omega-3 PUFA are present in FBS. It should also be noted that albumin increased the growth of control cultures slightly and although this was insufficient to explain the protective effects on omega-3 PUFA-treated cultures, these data may indicate that FBS contains some free fatty acids normally unbound to albumin that inhibit HaCaT growth.

The concentrations of albumin we observed to protect against omega-3 PUFA-induced apoptosis and growth inhibition are consistent with those found in human plasma and so are likely to be physiological. Fatty acids are thought to bind to albumin which transports them to the cell plasma membrane. As discussed in subchapter 7.1, fatty acids have to be dissociated from albumin in order to integrate into the plasma membrane or bind to its proteins and then be transferred across the membrane by either diffusion (passive flip-flop) (Hamilton & Kamp, 1999) or by a protein-carrier (Doege & Stahl, 2006). Albumin binding may delay the process of adsorption into the cell membrane (Hamilton & Kamp, 1999) but it is not considered to be an inhibitor of fatty acid transfer into the cells but a carrier of them. Rather, there is the general perception that PUFA need albumin to enter the cells. That is why many scientists in the literature use fatty acids bound to albumin in their *in vitro* and *in vivo* experiments. In our study we identified albumin to be a major antagonist of omega-3 PUFA anti-cancer action, which has implications for the ability to achieve pharmacologically active doses of DHA and EPA in the target tissue. Our results agree with another recent study which showed that albumin lowered DHA cytotoxicity towards hepatocellular carcinoma HepG2 cells (Kanno *et al*, 2011). They showed that albumin can reduce the ROS production caused by DHA treatment. However, DHA did not affect the growth of two other hepatocellular carcinoma cell lines. If oxidation was the only mechanism of the DHA-induced cytotoxicity, we would expect DHA to inhibit the other two cancer cell lines, as well. As regards our keratinocytes, the addition of the antioxidant PBN (50-1000 μM) did not protect the cells from the DHA growth inhibitory effect indicating that the

oxidative stress does not appear to be the driving mechanism of DHA-induced cytotoxicity.

According to our results, the free fatty acid fraction of DHA and EPA appears to be associated with efficacy in cancer and not the albumin bound fraction. So we hypothesise that when free PUFA are bound to albumin their anti-cancer effect is inhibited. This might mean that albumin binding inhibits the uptake of DHA and EPA into the cells. The design of new modified fatty acids that have a reduced interaction with albumin may significantly increase their therapeutic potential.

7.3.2 Lipid profile of keratinocytes prior and after PUFA treatment

The available preliminary data from one experiment are not sufficient for final conclusions. However, there were some clear differences between malignant and normal keratinocytes. The normal keratinocytes have significantly higher amounts of omega-6 and omega-3 fatty acids in their membrane compared to the malignant ones. As regards the omega-6 fatty acids, the NHEK-131 show higher levels of AA and adrenic acid/DPA than SCC-25 while the levels of LA are a lot lower. As regards the omega-3 fatty acids, NHEK-131 have 30% higher levels of EPA and almost 3 times more DPA (n-3)/DHA than SCC-25. This could explain, at least partially, the selectivity of PUFA induced inhibition towards malignant that normal keratinocytes. The high amount of omega-3 fatty acids incorporated in the normal keratinocyte membranes may mean that PUFA are not particularly toxic to the normal keratinocytes, at least at specific concentrations. However, the malignant cells have lower levels of PUFA which could be a survival mechanism that enables them to escape from death, as omega-3 PUFA are highly toxic to the cancer cells.

The preliminary data which were collected after treatment with 3 μM DHA for 48 h showed some changes in the omega-3 and omega-6 levels. As regards the omega-3 fraction, after the DHA treatment, SCC-25 showed an expected 30% increase in

DPA n-3/DHA levels and also a 70% increase in EPA. However, normal NHEK-131 keratinocytes showed only a slight (2%) increase in DPA n-3/DHA and a 22% increase in EPA. The small DPA/DHA increase might mean that there is enough DHA/DPA in the membrane and that is why they were not incorporated in the membrane. It could mean that no or low amounts of DHA entered the membrane, that DHA was converted to EPA or that at least some of the DHA was just transferred in the cytoplasm before the 48 h time point. In summary, more experiments are needed to have a clear picture of the fatty acid profile of these cell lines and the changes that PUFA treatment causes, but if reproducible, this may offer a partial explanation of the differential sensitivity of SCC cells to PUFA.

Chapter 8

GENERAL DISCUSSION

AND

FUTURE PLANS

Chapter 8. General discussion and future plans

8.1 General discussion

Head and neck cancer, including oral cancer, is the sixth most common malignancy in the United Kingdom and worldwide, with squamous-cell carcinoma (SCC) being the most frequent type of head and neck cancer. The main risk factors for HNSCC are tobacco usage and alcohol consumption (Hunter *et al*, 2005; Morita *et al*, 2010). Despite a reduction in the number of people smoking, the number of cases is predicted to nearly double in certain parts of the UK by 2020, placing a significant burden on the health service. Each oral cancer patient costs £54,000 to treat and survival rates have barely improved in 40 years. Therefore, there is a strong health economic benefit for reducing the number of advanced cases in the UK but also worldwide, particularly in high-risk groups (Speight *et al*, 2006).

At the moment, the main treatments available in oral cancer are surgery, chemotherapy and radiotherapy, or a combination of them (Haddad & Shin, 2008) and all are aggressive types of treatment. The surgery can cause facial dysmorphia of the patient and generally these treatments could even create permanent functional impairments in eating, drinking, taste and speech. Chemotherapy also kills the normal cells along with the cancer cells and causes many side effects (e.g. hair loss, pain, weight loss). The biggest problem in SCCs is that they very often recur because of incomplete excision or the appearance of secondary cancers. SCCs arise from a generalised field of abnormal mucosa that can involve in widely separated areas of the aerodigestive tract. Second field or second primary cancers are a common cause of relapse (Braakhuis *et al*, 2002; Hunter *et al*, 2005; Slaughter *et al*, 1953; Tabor *et al*, 2002), suggesting that the cost-effective prevention of secondary tumours is an important priority.

Cancers are directly linked to nutrition (Weisburger & Horn, 1982) so several studies are trying to identify natural products from nutritional sources for chemoprevention and therapy. Recent evidence suggests that natural dietary products, such as omega-3

fatty acids have anti-cancer activities. The most important dietary omega-3 fatty acids are the cis-5,8,11,14,17-eicosapentaenoic acid (EPA) (20:5n-3) and the cis-4,7,10,13,16,19-docosahexaenoic acid (DHA) (22:6n-3). These lipids are contained in oily cold-water fish such as salmon, trout, mackerel, anchovies and sardines, while fish take them from phytoplankton and zooplankton (Larsson *et al*, 2004). Humans are able to produce EPA and DHA from the omega-3 fatty acid α-linolenic acid (LNA) but only 5–10% of LNA is converted to EPA (Jump, 2002), so EPA and DHA are mainly acquired through the diet.

Long chain PUFA are essential components of the diet and they play a significant role in several physiological processes such as plasma membrane synthesis, metabolic energy production and storage, gene expression (Doege & Stahl, 2006; Jump & Clarke, 1999), innate immune responses (Li, 2004), insulin release via GPCR activation (Steneberg *et al*, 2005) and foetus development, especially of the brain and retina (Cunningham & McDermott, 2009; Herrera, 2002). It is widely accepted that EPA and DHA could play an important role in decreasing the incidence and development of a wide variety of diseases, including cardiovascular, inflammatory, neurodegenerative and immune disorders, and cancer (Calder, 2004; Dyall & Michael-Titus, 2008; Serini *et al*, 2009).

EPA and DHA have been observed to induce apoptosis in various human cancer cell lines such as breast (Schley *et al*, 2005) and colon cancer (Chen & Istfan, 2000; Eitsuka *et al*, 2005), lymphoma and leukaemia (Heimli *et al*, 2002) (Finstad *et al*, 1998), lung, pancreatic and prostate cancer (Edwards *et al*, 2008; Merendino *et al*, 2005; Serini *et al*, 2008). Animal studies have also demonstrated that there is a negative relationship between diets rich in omega-3 PUFA and breast, prostate, lung, and colon cancer (Bartsch *et al*, 1999; Edwards & O'Flaherty, 2008; Rose *et al*, 1996; Tsubura *et al*, 2009; Yuri *et al*, 2003). Furthermore, several human studies have shown that consumption of a diet enriched in omega-3 PUFA can protect against a variety of malignancies, including breast (Sasaki *et al*, 1993), prostate (Terry *et al*, 2004) and colon cancer (Yang *et al*, 2003).

Several studies have demonstrated that omega-3 PUFA are able to sensitise tumour cells to anticancer drugs *in vitro* (Biondo *et al*, 2008; Lindskog *et al*, 2006; Maheo *et*

al, 2005) and *in vivo* using cancer animal models (Cha *et al*, 2002; Colas *et al*, 2006; Hardman *et al*, 2002). Human clinical trials which have examined the effects of omega-3 PUFA on tumour response to chemotherapy have shown promising results, such as an increase in body weight and energy levels of advanced colorectal cancer patients during chemotherapy, after EPA and DHA enriched supplements (Read *et al*, 2007) and improved response to chemotherapy of breast cancer patients with higher concentrations of DHA in breast adipose tissue whereas low DHA levels were associated with no response or even tumour growth (Bougnoux *et al*, 1999). The phase II clinical trial showed that the use of DHA during chemotherapy was devoid of adverse side effects and could improve the outcome of chemotherapy when it was highly incorporated into plasma phospholipids (Bougnoux *et al*, 2009).

Very few studies have investigated the effect of omega-3 PUFA on oral or skin squamous cell carcinoma. Ellatar and Lin reported a decrease in the synthesis of the pro-inflammatory and tumour promoting series 2 prostaglandins, PGE2 and PGF2, after treatment of SCC-25 cells with EPA, DHA and other PUFA (Elattar & Lin, 1989). Ramesh and Das examined the effect of topical omega-3 and omega-6 free fatty acids on two stage skin carcinogenesis in mice. EPA inhibited both the initiation and promotion stages of skin carcinogenesis whereas DHA inhibited the number of papillomas only in the initiation stage. No direct correlation between lipid peroxidation and papilloma formation was observed but a trend towards an increase in the formation of lipid peroxides in parallel with the inhibitory effect of fatty acids on papilloma development was noticed (Ramesh & Das, 1996). When the authors used fish oil as a treatment, in the same mouse model, papilloma formation was only inhibited during the promotion stage, and was associated with an increase in lipid peroxidation but no effect on cell proliferation (Ramesh & Das, 1998). Injected omega-3 PUFA decreased the mucosal/epidermal response to irradiation in mice. In the HEP-2 HNSCC xenograft transplanted in nude mice, a decrease of tumour growth was observed when treated with omega-3 PUFA or EPA alone but this effect was maximal when combined with irradiation. The effects were associated with inhibition of angiogenesis and tumour proliferation, and decreased expression of COX-2 (Wen *et al*, 2003). One clinical trial in head and neck cancer post-surgical patients was reported, during which omega-3 enhanced supplements were consumed for 3 months in order to explore any improvement in body weight. Only serum

protein levels improved, with good tolerance of the supplements (de Luis *et al*, 2008).

In my PhD project, I attempted to take this research further. I tested the potential of the omega-3 fatty acids DHA and EPA to act as selective chemopreventative and therapeutic agents against oral cancer and investigated the mechanism of action. In particular, I tested the ability of DHA and EPA to specifically inhibit pre-malignant and malignant keratinocyte growth, including epidermal and oral malignant keratinocytes. Additionally, I tested the effect of omega-3 PUFA on normal keratinocytes.

Firstly, I examined whether PUFA specifically inhibit SCC growth at physiological concentrations, using the epidermal SCC-13 and the oral SCC-25 cell lines. Epidermal cells were added to this study because epidermal and oral keratinocytes are very similar, and also to gain insight regarding the effect of omega-3 PUFA on epidermal SCC. DHA and EPA suppressed the growth of the malignant cells after only 4 days, at concentrations within the physiological range. PUFA were very potent on the oral cancer keratinocytes SCC-25, as they eliminated most viable cells at concentrations $\geq 3\mu M$, while the epidermal SCC-13 growth was also significantly decreased by PUFA, at concentrations $> 3\mu M$, even though they were more resistant than SCC-25.

DHA and EPA also proved to be effective in inhibiting the pre-malignant epidermal and three oral dysplasias. Generally, PUFA were potent in inhibiting premalignant keratinocytes; however some oral dysplasias were more sensitive than others. The degree of sensitivity of dysplasias to PUFA is likely to depend on the type, stage of oral dysplasia and the location of origin.

Normal oral and epidermal keratinocytes were generally less sensitive than their premalignant and malignant counterparts, but somewhat more sensitive than the normal immortalised cells, possibly because senescence may make them more sensitive.

In summary PUFA, at least as the free fatty acid form, can be cytotoxic to normal cells, at higher doses. However, PUFA display selectivity towards neoplastic cells. It is also true that cell culture is not the optimal environment for normal cells as they are under stress and dividing rapidly, which may contribute to their susceptibility to PUFA treatment, so that *in vivo*, the selectivity may be greater. For example, EPA has been shown before to inhibit tumour promotion in mouse skin (Ramesh & Das, 1996). In summary, my results are very promising as I showed that both the omega-3 fatty acids DHA and EPA can eliminate oral cancer and pre-malignant cells at concentrations that do not kill normal healthy cells. Specifically, EPA seems to be a potent and a selective therapeutic tool in inhibiting SCCs and pre-malignant cells in both oral and epidermal cancer. The sensitivity of premalignant cells is important because it is these cells that would comprise the clinically relevant part of a cancer field.

Several studies have provided evidence of the pro-apoptotic effect of EPA or DHA in different cancer cells *in vitro* (Serini *et al*, 2009). EPA or DHA were shown to trigger either the intrinsic (Heimli *et al*, 2002; Lim *et al*, 2009) or both the extrinsic and the intrinsic apoptotic pathways (Narayanan *et al*, 2001; Tsuzuki *et al*, 2007), probably depending on different cells, different omega-3 PUFA used (EPA, DHA or fish oil) and different regimes of administration. In my study, it was demonstrated that EPA and DHA caused an apoptotic cell death, by the flow cytometric annexin V/DAPI apoptosis assay and cleavage of caspase 3 by western blot analysis. The cleavage of caspase 8 and caspase 9 were also detected by western blot, demonstrating the involvement of the extrinsic and the intrinsic apoptotic pathways, respectively. This could mean that both EPA and DHA trigger both apoptotic pathways at the same time in order to destroy the cells, or that the extrinsic pathway is triggered but does not manage to activate enough caspase 8, so the pro-apoptotic signal of caspase 8 leads to the engagement of cytocrome c/Apaf-1 and caspase 9 cleavage of the intrinsic mitochondrial pathway (Logue & Martin, 2008).

In addition, it has been observed that PUFA inhibit cancer cell proliferation, in accordance to Schley *et al* who showed that EPA and DHA treatment caused decreased breast cancer cell proliferation and apoptotic cell death (Schley *et al*, 2005). In the present study, treatment of SCC-25 with 3 µM of DHA and EPA for 48

hours significantly decreased ^{3}H-thymidine incorporation, which indicates decreased DNA synthesis and cell proliferation following exposure to omega-3 PUFA. A marked decrease in ^{3}H-thymidine incorporation was observed for both PUFA but bigger for DHA. As it was demonstrated that omega-3 PUFA both impair proliferation and induce apoptosis of oral cancer cells, I can conclude that DHA and EPA are not only cytotoxic but also cytostatic.

The mechanism of EPA and DHA anti-tumour action is not fully understood, and many different possibilities have been reported. It is becoming increasingly clear that their action is actually pleiotropic (Berquin *et al*, 2008; Chapkin *et al*, 2008). I attempted to investigate some of the proposed mechanisms reported to be involved in EPA and DHA action.

I investigated the fatty acid profile of the malignant oral SCC-25 and the normal epidermal NHEK-131 via gas chromatography (GC). It was observed that the normal keratinocytes have significantly higher amounts of omega-6 and omega-3 fatty acids in their membrane compared to the malignant ones. This difference could explain, at least partially, the selectivity of PUFA induced inhibition towards malignant that normal keratinocytes. The high levels of omega-3 fatty acids in the normal keratinocyte membranes may mean that PUFA are not particularly toxic to normal keratinocytes at specific concentrations while the malignant cells lower levels of PUFA could be a survival mechanism, as omega-3 PUFA are highly toxic to the cancer cells. After exposure to DHA for 48 h, the malignant cells showed a marked increase in DPA n-3/DHA levels (30%) and EPA (70%), while normal keratinocytes showed only a slight (2%) increase in DPA n-3/DHA and smaller increase in EPA (22%). These are only preliminary data so more experiments are needed to have a clear picture of the fatty acid profile of these cell lines and the changes that PUFA treatment causes. However it could provide a partial explanation of the differential sensitivity of SCC cells to PUFA.

Another important issue was the discovery that albumin was an important antagonist of PUFA-induced apoptosis. This may lead to the design of modified lipids that do not bind albumin or to the substitution of bioactive PUFA metabolites for the PUFA themselves in order to increase their therapeutic potential.

Continuing the investigation of the PUFA anti-tumour mechanism of action, I examined the role of ROS. PUFA are susceptible to free radical attack that leads to lipid peroxidation (Biondo *et al*, 2008), which can injure the mitochondria and result in further ROS generation (Catala, 2009). This can cause inhibition of DNA synthesis, cell division and tumour growth, as well as induction of tumour cell death (Girotti, 1998). Some chemotherapy drugs induce tumour cell death partly by ROS formation (Malhotra & Perry, 2003). The long-chain omega-3 PUFA appear to increase the potency of these chemotherapy drugs, by elevating the production of ROS (Biondo *et al*, 2008; Colas *et al*, 2006; Germain *et al*, 1998; Sturlan *et al*, 2003). Also, several studies suggest that the increase in ROS generation might be one of the mechanisms of omega-3 PUFA anti-tumour action (Arita *et al*, 2001; Gleissman *et al*, 2010; Kang *et al*, 2010; Maziere *et al*, 1999; Tsuzuki *et al*, 2007).

Generally cancer cells seem to have high levels of ROS. Studies indicated that malignant cells from chronic lymphocytic leukaemia patients showed increased ROS production compared to normal lymphocytes (Zhou *et al*, 2003) and increased levels of 8-oxo-dG in human and animal tumours (Halliwell, 2007b; Neeley & Essigmann, 2006; Sanchez *et al*, 2006). In my study, I showed that all the malignant epidermal and oral cell lines, the pre-malignant epidermal line and the normal epidermal keratinocytes, had high levels of ROS compared to the human foetal fibroblast cell line (HFF), which were used as a negative control.

So, the cells already had increased ROS before any treatment with PUFA. After treatment with PUFA, SCC-25 cells were tested and an increase in ROS was observed after 16 hours by DCF and HE flow-cytometric assays, especially for the higher concentrations of EPA and DHA used. Oxidative damage was also observed for the untreated SCC-25 cells by 8-oxo-dG staining which increased after exposure to EPA and DHA. However, the two well-known anti-oxidants, α-tocopherol (α-TOC) and PBN, failed to rescue the cells from the omega-3 PUFA inhibitory effect at the MTT assay. A protective effect was not observed when PBN was used on HaCaT cells. This may mean that oxidation is not the mechanism that causes the PUFA-induced cytotoxicity or that it is not the only or the main mechanism of PUFA action in SCC. However, ROS production may be responsible for the toxicity to normal keratinocytes at higher doses. Generally, normal cells are under stress in

culture and this might contribute to the fact that they showed high levels of ROS *in vitro*. However, it could also mean that the oxidation levels after the addition of PUFA were so high that the anti-oxidants failed to reverse the induction of apoptosis.

It has also been reported that EPA and DHA can downregulate telomerase activity by inhibiting the expression of *CMYC,* and this was accompanied by slowed growth (Eitsuka *et al*, 2005), but in this study there was no distinction between cause and effect. I showed that HaCaT cells that were ectopically expressing *CMYC* or *TERT* under the control of a heterologous promoter were just as sensitive to growth inhibition by PUFA as the controls, strongly indicating that the inhibition of telomerase activity was an indirect result of growth arrest or death.

A number of signalling pathways are reported being differentially affected by omega-3 PUFA. PUFA have been reported to inhibit (Lee *et al*, 2003; Schley *et al*, 2005) or upregulate the PI3K pathway, inhibit COX-2 (Horia & Watkins, 2007; Lim *et al*, 2008; Narayanan *et al*, 2004) and decrease the levels of its product, PGE2 (Gleissman *et al*, 2010), modulate MAPK pathway by inhibiting p-ERK1/2 and p-p38 (Serini *et al*, 2008) or inhibit Ras and ERK activation (Collett *et al*, 2001). It has been suggested that omega-3 PUFA modulate signalling by decreasing the activation of NFκB, (Novak *et al*, 2003; Weber *et al*, 1995) and promoting the activation of PPARγ (Sun *et al*, 2008) which can induce apoptosis (Zand *et al*, 2007). Furthermore, it has been suggested that omega-3 PUFA affect intracellular Ca^{2+} homeostasis, triggering apoptosis (Aktas & Halperin, 2004; Kolar *et al*, 2007) and also alter basic properties of cell membranes, such as fluidity, phase behaviour, rapid flip-flop and resident protein function (Stillwell & Wassall, 2003) especially affecting lipid rafts by altering their behaviour and proteins function (Chen *et al*, 2007).

I investigated the involvement of some of the above signalling pathways in the PUFA growth inhibitory mechanism of action on SCC-25 cells.

In contrast to other studies, my results demonstrated a 2-fold increase in the COX-2 protein expression after 5 h of DHA and EPA treatment in oral SCC-25 cells. After 17 h the levels declined for EPA, while for DHA there was a sustained over-

expression (1.5-fold) but the levels also decreased. My results agree with one study that examined the effects of PUFA on HaCaT epidermal keratinocytes and reported an increase in COX-2 levels. While several studies demonstrate an important role of COX-2 expression during tumourigenesis and its tumourigenic role (Arber *et al*, 2006; Giardiello *et al*, 2004; Greenhough *et al*, 2009; Mendes *et al*, 2009), others support a tumour-suppressive role under some circumstances (Arber *et al*, 2006; Bol *et al*, 2002; Giardiello *et al*, 2004; Greenhough *et al*, 2009; Mendes *et al*, 2009; Murata *et al*, 2004; Patsos *et al*, 2010; Wilson & Potten, 2000). I hypothesise that COX-2 is involved in the cell inhibitory effect of PUFA. COX-2 could be causing apoptosis or growth arrest or producing anti-inflammatory and tumour suppressive EPA and DHA metabolite.

Unexpectedly, the phosphorylation of ERK1/2 was rapidly up-regulated (within 30 mins) after treatment with different doses of DHA and EPA and was sustained and increased for 24 h. The activation of the downstream target of p-ERK, p-p90RSK was also observed. Typically, the activation of ERK is expected to promote cell survival and proliferation (Mansour *et al*, 1994; Xia *et al*, 1995). Increased ERK phosphorylation is sometimes reported after a cell death stimulus as a survival mechanism (Lee do *et al*, 2006; Persons *et al*, 1999). Recent studies implicate the ERK pathway activation in cell death or cycle arrest depending on the type and duration of the stimulus and cellular context (Elder *et al*, 2002; Galve-Roperh *et al*, 2000; Persons *et al*, 1999; Pumiglia & Decker, 1997; Stanciu *et al*, 2000; Wang *et al*, 2000). As in the present study the omega-3 PUFA-induced ERK1/2 activation happened early and it was very strong and sustained, I hypothesise that it actually triggered apoptosis. The use of the pan-caspase inhibitor QVD-Oph did not affect the increased levels of p-ERK1/2 demonstrating that ERK1/2 phosphorylation is not a consequence of apoptosis, and that it is not just a survival mechanism.

In order to investigate whether the PUFA-induced up-regulation of p-ERK1/2 causes apoptosis, I needed to test if the inhibition of p-ERK blocks the apoptosis caused by PUFA. First, two MEK inhibitors were used in order to examine their effects on ERK and p90RSK activation by western blot and determine the optimum concentrations for the cell assays. They both reduced p-ERK1/2 and p-p90RSK levels, however AZD6244 seemed more potent than U0126, as it inhibited phospho-

ERK1/2 and phospho-P90RSK levels without reducing the basal levels of the control as much. The use of an EGFR blocking antibody and the EGFR inhibitor, AG1478 also inhibited the PUFA induced p-ERK1/2 and p-p90RSK very effectively without affecting the endogenous levels of the control, demonstrating that EGFR is the activator of the ERK pathway. I hypothesise that the omega-3 PUFA are causing apoptosis by increasing the secretion of an unknown EGFR ligand. Most SCCs have increased levels of EGFR (Stanton *et al*, 1994) and are inhibited from growing by levels of EGF that are optimal for normal keratinocytes (Gulli *et al*, 1996), so the release of EGFR ligand(s) by PUFA may lead to a suprastimulation of ERK1/2 and as a result trigger apoptosis (figure 8-1).

There was no change in p-Akt levels observed after treatment of SCC-25 cells with EPA and DHA, and this coupled with the sustained and early activation of ERK1/2 could indicate the type of signalling imbalance known to cause apoptosis or senescence.

Furthermore, JNK phosphorylation was also observed, especially at the higher concentration of EPA and DHA, which is activated preferentially by stress and can promote apoptosis (Kennedy *et al*, 2003). JNK phosphorylation was higher during DHA than EPA use. So, JNK activation can also participate in the PUFA induced apoptosis and could also be a result of EGFR over-activation as well, or it could be triggered by ROS production.

The beneficial effects of omega-3 PUFA in the treatment of cancer may involve a multitude of mechanisms but the accessibility of the aerodigestive tract cancer to potential therapeutic aerosols or gels makes it an attractive site to test the therapeutic and prophylactic potential of omega-3 PUFA. The safety and tolerability of these compounds has already been documented in other clinical indications. I propose that the manufacturing of an omega-3 containing spray or gel will be greatly beneficial, used on its own or in combination with the current treatments to improve the therapeutic outcome. More importantly, such a preparation could also be used by former oral cancer patients as a chemopreventive strategy in order to prevent relapse. This type of therapy would be inexpensive and easily accessible, so any patient could be treated at a very moderate cost. In conclusion, the present study demonstrated the

great potential of the omega-3 fatty acids, EPA and DHA, for oral (and epidermal) SCC treatment and provided information about the PUFA mechanism of action.

Figure 8-1. Hypothesis of PUFA-induced SCC inhibition by secretion of an EGFR ligand
Schematic representation of my hypothesis that omega-3 PUFA induce secretion of an EGFR ligand which leads to over-activation of EGFR (which is overexpressed in HNSCC). EGFR suprastimulation leads to MAPK activation, such as ERK and JNK pathways, and subsequent apoptosis and/or growth arrest. The image also shows where the different inhibitors used in the study act, blocking their targets.

8.2 Short term future plan

8.2.1 Test the link between the EGFR ligand, the EGFR/MAPK/ERK pathway and omega-3 PUFA-induced apoptosis

I will continue the experiments with the EGFR blocking antibody, the inhibitors A1478, AZD6244 and U0126 and a COX-2 inhibitor, to test the extent to which the induction of ERK1/2 phosphorylation through stimulation of the EGFR is linked to omega-3 PUFA-induced apoptosis. Apoptosis and MTT assays with the use of the optimal concentrations of the MEK and the EGFR inhibitors (determined by the western blot assays) and additional omega-3 PUFA treatment will assess the effect of EGFR and ERK activation on the PUFA growth inhibitory effect and apoptosis.

I will transfect small interfering RNAs (siRNAs) and establish the time interval over which the candidate molecules are knocked down. Alternatively, short hairpin RNAs (shRNAs) against the candidate EGFR ligand(s), ERK1, ERK2, COX-2 will be used and checked by western blotting to reduce expression by 70%. The knocked down SCC-25 cells will be treated with omega-3 PUFA and tested for the levels of total target protein, induced ERK1/2 phosphorylation and apoptosis. These experiments will establish the extent to which any potential secreted candidate ligand and ERK1/2 phosphorylation are required for omega-3 PUFA-induced apoptosis. If the pharmaceuticals that block ERK phosphorylation do not inhibit omega-3 PUFA-induced apoptosis in the pilot experiments, I will continue to survey candidate pro-apoptotic pathways and design experiments to inhibit ERK1/2 and these pathways in concert, to establish the key molecular events required for omega-3 PUFA-induced apoptosis and hence their chemopreventative and therapeutic action

8.2.2 Identification of the EGR ligand(s) induced by omega-3 PUFA in OSCC cells

I will treat the SCC-25 OSCC line with EPA, DHA and the ethanol vehicle control in serum-free medium, containing EGF or not and collect conditioned medium from the cells after 24 hours. The conditioned medium from radioactively-labelled SCC-25

cells treated with either the vehicle (ethanol) or EPA or DHA will then be sent for custom-made antibody arrays assay (Coppe *et al*, 2008) that carry antibodies against all the known EGFR ligands, with the purpose of identifying which ligands are up-regulated by omega-3 PUFA, and are candidates for inducing apoptosis or if these are not available, screen for them by using qPCR.

8.2.3 Lipid profile of keratinocytes prior and after PUFA treatment

I have already optimised the technique and have obtained preliminary data of the lipid analysis of the malignant SCC-25 and normal keratinocytes NHEK-131 after DHA treatment using gas chromatography (GC). The available preliminary data demonstrated clear differences between malignant and normal keratinocytes. The normal keratinocytes have significantly higher amounts of omega-6 and omega-3 fatty acids in their membrane compared to the malignant ones. These experiments need to be repeated with cells untreated and also treated with EPA and DHA, cells for final conclusions about the lipid profile of the cells and also the alterations after exposure to the omega-3 PUFA.

8.3 Long term future plans

8.3.1 Test the chemopreventative and chemotherapeutic potential of omega-3 PUFA *in vivo*

8.3.1.1 Test the ability of omega-3 PUFA to prevent squamous tumour formation

EPA seemed to be the more selective of the two omega-3 PUFA, so it is possible that the *in vivo* experiments will continue with just EPA. I will test the potential of EPA *in vivo* using the mouse two-stage epidermal tumourigenesis system which is a well characterised animal model where the genetics, cell and molecular biology of the initiation event and tumour progression events are known (Kemp, 2005; Payne & Kemp, 2005). EPA will be applied topically prior to each twice weekly application of the tumour promoter 12-O-tetradecanoylphorbol, 13-acetate (TPA) for a total of 12 weeks, as described before (Ramesh & Das, 1996). In other experiments EPA will be added to the animal feed. The pre-malignant (papillomas) and malignant (carcinomas) lesions will be counted and measured prior to sacrifice. Then, the tumours will be snap frozen in liquid nitrogen for histological confirmation, immunohistochemical analysis and lipid analysis. The experiments will test whether omega-3 PUFA are chemo-preventative for squamous neoplasia *in vivo* when orally administered or topically applied, and also will provide material to study the effect of EPA on apoptosis and ERK1/2 phosphorylation on normal and neoplastic epidermal tissue.

8.3.1.2 Test the ability of omega-3 PUFA to inhibit squamous tumour growth

I will then perform experiments on pre-formed papillomas. The size of papillomas and their rate of progression to carcinomas will be assessed as above. These experiments will test the effectiveness of EPA in inhibiting tumour growth and also its effect on tumour progression. The experiments will also test the potential of EPA as chemotherapeutic agent for squamous neoplasia. In these experiments mice will be sacrificed before the administration of EPA and at various times thereafter. The

tumours will be used for histological, immunohistochemical analysis, lipid analysis and also for protein extraction prior to western blot analysis. The samples will be subject to standard histological analysis to confirm the benign or malignant status, and also to examine the tumours for signs of angiogenesis and other requirements for tumour growth and invasion. The tumours will be checked for apoptosis, proliferation and ERK phosphorylation in frozen sections by immunofluorescence Commercial antibodies against cleaved caspase 3 will be used to measure apoptosis, and Ki67 to measure cell proliferation, as well as phospho- and total ERK1/2. Double labelling will be performed to determine whether ERK phosphorylation is associated with apoptosis and growth arrest by directly labelling the phospho-ERK1/2 antibody with a different fluorochrome to Ki67 or cleaved caspase 3.

8.3.1.3 Do endogenously produced omega-3 PUFA inhibit squamous neoplasia?

I will also test the role of endogenously generated omega-3 PUFA in suppressing squamous neoplasia by comparing the frequency of papillomas and carcinomas forming in a 12 week period in the *fat-1* transgenic mouse which converts omega-6 to omega-3 fatty acids endogenously and has high tissue levels of the latter (Kang *et al*, 2004). We will test heterozygotes and wild types by using the two stage tumourigenesis protocol, described above.

8.3.2 Extend my study to the potential of other natural products to inhibit squamous cells carcinomas; use of tocotrienols

Vitamins are prominent among the natural compounds considered to be beneficial in cancer Vitamin E exists in nature as two classes of compounds: tocopherols (Toc) and tocotrienols (T3s). Humans are unable to synthesise vitamin E and therefore must obtain the compound from plant sources. Toc is present in nuts and vegetable oils, whereas T3 are primarily derived from palm oil, oat, rye, wheat germ, barley

and rice bran (Constantinou *et al*, 2008; Hiura *et al*, 2008). Vitamin E is relatively nontoxic and well tolerated by humans, and the various isoforms have been reported to lower blood cholesterol and have cardioprotective effects, immunoregulatory activities, antioxidant activity, potent neuroprotective effects and antitumour activity (Jemal *et al*, 2006; Sen *et al*, 2006).

T3 have also been reported to have anti-tumour activity. *In vitro*, T3 showed apoptotic or antiproliferative activities against various cancer cells such as hepatoma Hep3B (Sakai *et al*, 2006), breast carcinoma MCF7 (Nesaretnam *et al*, 1995) colorectal adenocarcinoma DLD-1 (Eitsuka *et al*, 2006), prostate cancer (Srivastava & Gupta, 2006) and chronic myelogenous leukemia KBM-5 cells (Ahn *et al*, 2007). The anticancer properties of T3s were also evaluated *in vivo*. In these studies they inhibited liver and lung carcinogenesis (Iqbal *et al*, 2004; Wada *et al*, 2005) breast tumours (Nesaretnam *et al*, 2004) and suppressed angiogenesis in mice bearing human colorectal cancer cells (Nakagawa *et al*, 2007). γ-T3 enriched diet decreased tumour weight and prolonged the survival rate of C57BL female mice transplanted with melanoma (He *et al*, 1997).

In humans, the antitumour properties of vitamin E were first suspected when studies showed that the risk of colon cancer is lower for people in the Mediterranean area, who consume diets rich in vitamin E isoforms, than for people in Northern Europe and USA (Berrino & Muti, 1989; Khlat, 1995). Another study showed that low dietary intake of vitamin E increases the incidence of prostate cancer (Eichholzer *et al*, 1996). In Malaysia palm oil is the main dietary fat consumed, which is a particularly rich source of T3s. The levels of the δ-T3 in adipose tissue of Malaysian women showed a significant reduction in malignant compared to benign tissue (Nesaretnam *et al*, 2007).

I investigated the potential of T3s in SCC and obtained some interesting and very promising preliminary data. Pure tocotrienols α, γ, δ and Tocomin®, a natural full spectrum Tocotrienol/Tocopherol complex (Carotech BHD, Perak, Malaysia) (see Chapter 2) were used. I investigated whether T3s specifically inhibit SCC growth using the epidermal SCC-13 and the oral SCC-25, and the pre-malignant cell line SVFHK. Treatment with increasing concentrations of T3s for 4 days caused a dose-

dependent inhibition of growth as determined by the MTT assay (APPENDIX 1). γ- and δ-tocotrienols were more potent, causing 80-95% growth inhibition at a concentration of 10 μM, with γ-tocotrienol having a marked effect even at lower concentrations. Tocomin® was also very effective in the SCC cell lines. To our knowledge, I showed that T3s strongly affect oral and epidermal SCC cell growth for the first time. Moreover, under the same culture conditions, the T3s seemed to have little or no effect on the two types of normal epidermal keratinocytes, NHEK-131 and HEK-127, at doses that killed the malignant cells, as showed by the MTT assay.

I also used the flow cytometric annexin V/DAPI apoptosis assay to determine whether the epidermal SCC-13, the oral SCC-25, and the pre-malignant SVFHK cell lines cells were undergoing an apoptotic death after treatment with T3s. There was a marked difference in the percentage of viable cells between treated and untreated cells after only 48 h treatment, for SCC-25 and SCC-13 (APPENDIX 2 and 3). SVFHK seemed to be more resistant to T3s' effect after 48h. However, the percentage of early apoptotic cells (annexin V +ve/DAPI –ve) did not increase much so it is unclear if the cells were undergoing apoptosis, necrosis, terminal differentiation or a combination of them. The experiment needs to be extended to more time points and western blotting needs to be performed in order to investigate whether apoptotic molecules are activated. However, from these early results it appears that T3s do not inhibit SCC growth by the same mechanism as PUFA.

These preliminary data are very promising showing that T3 have a potential as a chemopreventive and therapeutic tool for SCC. However, further investigation is required regarding the type of cell death and the possible growth arrest, and also in order to determine the molecular mechanism of action.

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APPENDIX 1

Appendix 1. The effect of tocotrienols (T3s) on keratinocyte cell growth

*Means± S.E.M of 3 independent MTT experiments for SCC-13, SCC-25, SVFHK, NHEK-131 and HEK-127. Each experiment was conducted in duplicate. Cells were incubated in different concentrations of α, γ, δ tocotrienols and tocomin mix for 4 days in KGM serum-free medium. S.E.M=Standard error of means. * is significantly different from the mean value of untreated control of each cell line (* = p<0.05 and ** = p <0.01 *** = p<0.001 as measured by one-way ANOVA followed by post-hoc Bonferroni test).*

APPENDIX 2

Appendix 2. Apoptosis assay

Representative flow cytometry plots for annexin V-FITC/DAPI for cellular viability. The scatter plot is shown for the untreated control where SSC=side scatter and FSC= forward scatter and the fluorescence plots are shown for each sample SCC-25 cells were used in this example. Untreated control cells and cells treated with 10 μM α, γ, δ tocotrienols and tocomin for 48 h are shown. Q3 shows the viable cell population (annexin V -ve, DAPI -ve), Q4 shows early apoptotic cells (annexin V +ve DAPI –ve), Q2 shows late apoptotic and necrotic cells (annexin V +ve, DAPI +ve) and Q1 shows only necrotic cells (annexin V –ve, DAPI +ve).

APPENDIX 3

A.

B.

Appendix 3. Means of viable and non-viable cells percentages in the apoptosis assay

Means± S.E.M of 3 independent annexin V/DAPI experiments. SCC-13, SCC-25, SVFHK were incubated in 10 μM α, γ, δ tocotrienols and tocomin for 48 h in KGM serum-free medium prior to trypsinisation. Then the cells were stained with annexin V and DAPI and analysed by flow cytometry. The percentages of viable cells (A) and the normalised with the control percentages (B) are shown.