UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

School of Medicine

Effects of Fatty Acids on Inflammatory Markers Studied *in vivo* and *in vitro*

by

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Thesis for the degree of Doctor of Philosophy

December 2008

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES
DEVELOPMENTAL ORIGINS OF HEALTH AND DISEASE

Doctor of Philosophy

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Inflammation involves interactions amongst many different cell types as a defense mechanism of the body. Inflammation is also involved in cardiovascular disease (CVD). The role of long chain n-3 polyunsaturated fatty acids (LC n-3 PUFAs) in modulating the inflammatory response has been proposed. The aim of these studies is to investigate the effects of modest intakes of n-3 PUFAs on CVD risk factors especially inflammatory markers, including soluble adhesion molecules, in adult humans with and without CVD and to identify the effects of selected fatty acids, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), on inflammatory responses, especially adhesion molecule expression in cultured human endothelial cells of different origin (fetal vs. adults; vein vs. artery).

In the first *in vivo* study, healthy middle-aged men aged 35-60 years were randomized to 1.8 g/d EPA plus 0.23 g/d DHA (n = 9) or placebo oil (2.6 g/day medium-chain saturated fatty acids; n = 11) for 8 weeks. In a second *in vivo* study, patients awaiting carotid endarterectomy were randomised to 0.8 g/d EPA plus 0.67 g/d DHA (Omacor; n = 47) or olive oil (n = 53) as placebo for between 7 and 102 days until surgery. Supplementation with fish oil in healthy men resulted in a 363% increase in EPA and only a 13% increase in DHA in plasma phosphatidylcholine (PC). On the other hand, Omacor supplementation resulted in significantly increased EPA and DHA in plasma PC by 161% and 70%, respectively. In healthy subjects, there was very little effect of n-3 fatty acids on the risk factors measured (lipid profiles and inflammatory markers), apart from a reduction in plasma soluble intercellular molecule-1 (sICAM-1) concentration compared with placebo ($P = 0.05$). The change in plasma sICAM-1 concentration was significantly inversely associated with the change in DHA in plasma PC ($r = -0.675$; $P = 0.001$). Supplementation with Omacor, however, significantly decreased total plasma cholesterol, triacylglycerol (TAG) and LDL-cholesterol concentrations ($P < 0.001$) by 13%, 14%, and 5% respectively. In terms of inflammatory markers, supplementation with Omacor significantly decreased sE-selectin by 23% ($P = 0.006$) and sVCAM-1 by 25% ($P < 0.0001$), and had no significant effects on other plasma inflammatory markers including sICAM-1 even though trends toward decreases in these markers were observed. This study suggests some anti-inflammatory actions of moderate dose of Omacor in carotid endarterectomy patients. Based on correlation analysis between mRNA expression of inflammatory markers in plaque and plasma concentrations, it seems that soluble inflammatory markers cannot be used to reflect the expression of these molecules at the cell surface, i.e. in the vasculature or in the plaque.

In the *in vitro* experiments the inflammatory stimulus lipopolysaccharide (LPS) up-regulated all three adhesion molecules studied at the protein (as assessed by ELISA) and the mRNA (as assessed by reverse transcription and real-time PCR) levels. VCAM-1 was affected by fatty acids to a greater extent than ICAM-1 or E-selectin. Amongst the fatty acids, DHA has the greatest and the most consistent effects on adhesion molecule protein expression. EPA was also a potent fatty acid inhibitor of adhesion molecule expression at the mRNA level. Some effects of stearic, oleic and arachidonic acids on adhesion molecules were also seen. The effects of fatty acids on the adhesion molecule expression were fatty acid, adhesion molecule and endothelial cell specific. The inhibitory effects of fatty acids were more pronounced in vein endothelial cells than arterial endothelial cells. The precise underlying mechanism on how fatty acids affect adhesion molecule expression remains to be clarified.

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DECLARATION OF AUTHORSHIP

I, Hayati Mohd Yusof, declare that the thesis entitled EFFECTS OF FATTY ACIDS ON INFLAMMATORY MARKERS STUDIED IN VITRO AND IN VIVO and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly while in candidature for a research degree at this University;
- where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- parts of this work have been published as: Influence of very long-chain n-3 fatty acids on plasma markers of inflammation in middle-aged men, in Prostaglandins, Leukotrienes and Essential Fatty Acids 78 (2008) 219-228

Signed:

Date: 27 December 2008

ACKNOWLEDGEMENTS

First and foremost I offer my sincerest gratitude to my supervisor, Professor Phillip Calder, who has supported me throughout my time in Southampton University, with his patience, guidance, depth of knowledge and special ability to encourage me, which I will never forget. I will remain forever grateful. I have learnt so much from him in so many different ways.

I am also indebted to Dr. Frederick Anthony, Senior Research Fellow for his untiring efforts and patience in teaching me molecular works of mRNA analysis using reverse-transcription and real-time PCR. I would also like to thank Dr. Liz Miles for her invaluable help in the ELISA work, Dr. Paul Noakes with his kind help ordering all my lab materials, Caroline, Annette, and Aleix, for helping me with the fatty acid analyses. I would also like to thank all the participants involved in the study

To Anim, Along and Aniza, thank you for all your support and kindness to take care of all my little cats. To all my beloved students (Food Science Department, UMT), my friends; Maslinda, Nurlida, Laila, Yulita Hanum, Faridah, Farah and all my colleagues of the Nutrition group; Maria, Stella, Amy and Jackie thank you so much for becoming my friends.

I would like to acknowledge the funding I received from the Ministry of Higher Education, Malaysia for my studentship and University Malaysia Terengganu for granting my study leave at Southampton University.

To Fazly Mas Fauizzuddin bin Ramli and family, your constant support and encouragement always will be precious to me. Finally, I cannot end without conveying a deepest thank my whole family, especially to my late parents, Absah binti Abdullah and Mohd Yusof bin Bache' who always inspire me. This thesis would not have been possible without the contribution of so many people; I dedicate this work to them.

LIST OF ABBREVIATIONS

15-HETE 15-monohydroxyeicosatetraenoic acid

15-HPETE	15-hydroperoxyeicosatetraenoic acid
15-LOX	15-lipoxygenase
ARA	arachidonic acid
Ab	antibody
AHA	American Heart Association
ALA	alpha-linolenic acid
AMI	acute myocardial infarction
AP-1	activator protein-1
ASP	acylation stimulating protein
ATP-III	adult treatment panel III
BMI	body mass index
CAM	cellular adhesion molecule
CBG	coronary bypass graft
CD	cluster of differentiation
CDC	Centre Disease Control
CH_3	methyl
CHD	coronary heart disease
CLA	conjugated linoleic acid
CLP	ceacal ligation and puncture
CO	coconut oil
COOH	carboxylic acid
COMA	Committee on Medical Aspects of Food Policy
COX	cyclooxygenase
CRP	C-reactive protein
CVD	cardiovascular disease
DART	Diet and Reinfarction Trial
DASH	Dietary Approaches to Stop Hypertension
DGLA	dihomo-gamma-linolenic acid
DNA	deoxyribonucleic acid
DHA	4,7,10,13,16,19-docosahexaenoic acid
DPA	4,7,10,13,16-docosapentanoic acid
EGF	epidermal growth factor
ELAM-1	endothelial leukocyte adhesion molecule-1
ELISA	enzyme linked immunosorbent assay
EPA	5,8,11,14,17-eicosapentaenoic acid
EPO	evening primrose oil
FOAD	Fetal Origins of Adult Disease
FOS	Framingham Offspring/Spouse Study
GISSI	Gruppo Italiano per lo Studio della Streptochinasi nell'Infarto Miocardico
GLA	gamma-linolenic acid
HAOEC	human aortic endothelial cell
HbA1c	glycated hemoglobin
HCAEC	human coronary artery endothelial cell
HCO	hydrogenated coconut oil
HETE	hydroxyeicosatetraenoic acid
HF	high fat
HLA	human leukocyte antigen
HOMA	homeostatic model assessment
HPETE	hydroxyperoxyeicosatetraenoic acid
hRVE	human retinal vascular endothelial
HSaVEC	human sephanous vein endothelial cell
HUFA	highly unsaturated fatty acid
HUAEC	human umbilical artery endothelial cell
HUVEC	human umbilical vein endothelial cell
ICAM-1	intercellular adhesion molecule-1

ICD	implanted cardioverter defibrillators
IDL	intermediate-density lipoprotein
IFN-γ	interferon gamma
IgSF	immunoglobulin-like superfamily
IL-	interleukine-
iNOS	inducible nitric oxide
IP	interferon-gamma induced protein
JELIS	Japan EPA Lipid Intervention Study
JPHC	Japan Public Health Centre-Based Study Cohort 1
LAD-1	leukocyte adhesion deficiency-1
LAM-1	leukocyte adhesion molecule-1
LCAT	lecithin-cholesteryl ester acyl-transferase
LDL	low density lipoprotein
LF	low fat
LFA-1	leukocyte function associated antigen
LPS	lipopolysaccharide
LT	leukotrienes
mAB	monoclonal antibodies
Mad	mucosal addressin
MARGARIN	Mediterranean ALA Enriched Groningen Dietary Intervention
MCP-1	monocyte chemoattractant protein 1
MHC	major histocompatibility complex
MI	myocardial infarction
Mig	monokine induced by IFN
mLDL	modified LDL
MLP	Met-Leu-Phe
MMP	matrix metalloproteinase
MO	menhaden oil
MRFIT	Multiple Risk Factor Intervention Trial
MUFA	monounsaturated fatty acid
NEFA	non-esterified fatty acid
NF-κB	nuclear factor-kappaB
NIH	National Institute of Health
NO	nitric oxide
OA	oleic acid
OO	olive oil
ox LDL	oxidized LDL
PAF	platelet activating factor
PAI-	plasminogen activator inhibitor
PBMC	peripheral blood mononuclear cell
PECAM-1	platelet-endothelial cell adhesion molecule
PG	prostaglandins
PKC	protein kinase C
PMA	phorbol myristate acetate
PPAR	peroxisome proliferator-activated receptor
PUFA	polyunsaturated fatty acid
PVD	peripheral vascular disease
RNA	ribonucleic acid
ROS	reactive oxygen species
SA	stearic acid
SACN	Scientific Advisory Committee on Nutrition
SAEC	swine aortic endothelial cell
sCD40L	soluble CD40-ligand
SCR	short consensus repeat
SFA	saturated fatty acid

SMC	smooth muscle cell
SNP	single nucleotide polymorphism
SO	sunflower oil
SOFA	Study on Omega-3 Fatty Acids and Ventricular Tachyarrhythmia
SR-A	scavenger receptors A
TAG	triacylglycerol
TCR	transfer-cluster restricted
TG	triglyceride
TIMP	tissue inhibitor of matrix metalloproteinase
TNF-α	tumor necrosis factor-alpha
tPA	tissue plasminogen activator
TXs	thromboxanes
USDA	United States Department of Agriculture
VCAM-1	vascular cellular adhesion molecule-1
VEGF	vasoactive endothelial growth factor
VF	ventricular fibrillation
VLA-4	very late antigen
VLDL	very low density lipoprotein
VT	ventricular tachycardia
vWF	von Willebrand factor
WHO	World Health Organization
WTCRF	Welcome Trust Clinical Research Facility

Chapter 1

Introduction

1.1 Cardiovascular Disease

Cardiovascular disease (CVD) refers to disease of the arterial wall that restricts blood flow to the heart (coronary heart disease; CHD), or to the brain (cerebrovascular disease), or to other regions of the body, such as the legs (peripheral vascular disease; PVD) or the kidneys. From the clinical perspective, CVD has become a major contributor to the causes of death all over the world, accounting for over half and one-third of all deaths in most developed countries in middle and old age groups, respectively.

1.1.1 Pathophysiology

There are two principal components of CVD, namely atherosclerosis and thrombosis. Atherosclerosis refers to the building up and subsequent hardening of fatty streaks in the arterial wall. This process further develops into the occlusion of blood vessels (thrombosis) and therefore prevents the blood flow to the heart or corresponding organs.

As a chronic inflammatory disease that affects large-and medium-sized arteries, atherosclerosis is characterized by the formation of fibrotic, lipid-containing plaques (Ross 1999). In the early stage of atherosclerosis, the process involves the interaction between blood lipids, endothelial cells and inflammatory cells (Figure 1.1). Low density lipoprotein (LDL) has an essential physiological role as a vehicle for the delivery of cholesterol to peripheral tissue. Since it is a relatively small molecule, LDL easily enters into the tissue

space of the vascular endothelium. When LDL particles become trapped in the arterial wall, they can undergo a progressive oxidation to produce oxidized LDL in the presence of proatherogenic enzymes of 15-lipoxygenase (15-LO) and inducible nitric oxide (iNOS). It has been suggested that the LDL particles must be chemically modified in some way before they are taken up the macrophages receptors. Uptake of this oxidized LDL converts macrophages, which are initially derived from monocytes by differentiation, into foam cells. Several proteins may contribute to the overall process of oxidized LDL uptake; however, it has been demonstrated that scavenger receptors A (SR-A) and CD36 play quantitatively significant roles (Febbraio et al. 2000). The removal of modified LDL and its sequestration in macrophages are an important part of the initial, protective role of the macrophage in the inflammatory response to minimize the effects of modified LDL on endothelial smooth muscle cells (SMCs). However, the process of recruitment of macrophages and their subsequent uptake of oxidized LDL in response to inflammatory stimuli are the major cellular events which lead to fatty streak (plaque) formation. Foam cells secrete various types of growth factors and cytokines, resulting in promotion of SMC migration from the medial portion of the arterial wall towards the intima.

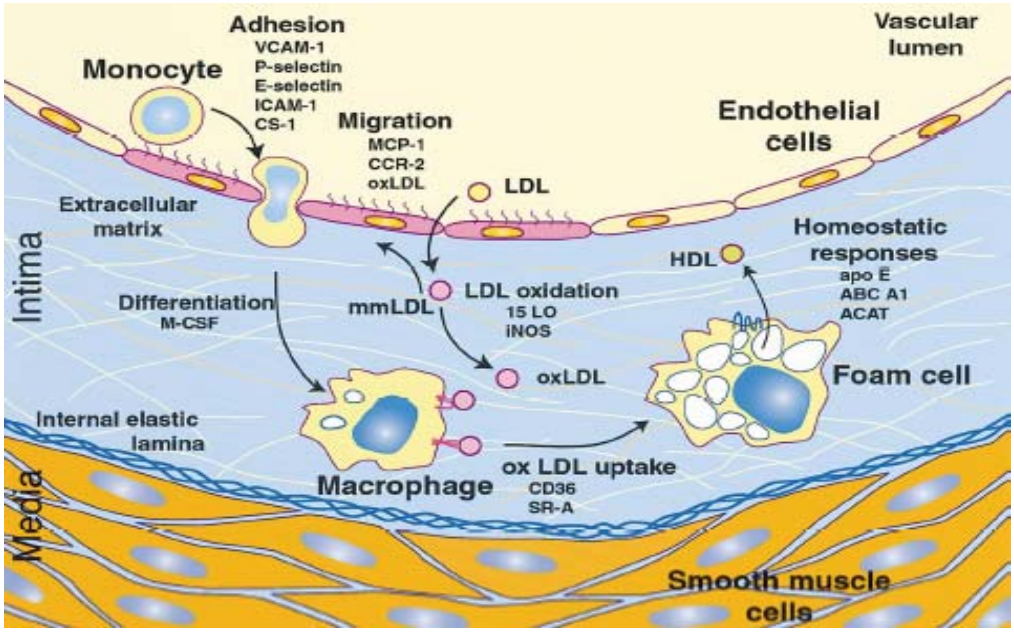


FIGURE 1.1: Initiating Events in the Development of Fatty Streak Lesion
LDL is a relatively small, dense molecule which easily enters into the intima and subsequently becomes oxidized (ox LDL). Adherent monocytes migrate into the intima and differentiate into macrophages. Macrophages take up oxidized LDL and are transformed into foam cells. (Source: Glass & Witztum 1995)

The process of migration of SMCs is then followed by their proliferation and release of extracellular matrix proteins that create a fibrous cap over the growing of fatty lesion (Figure 1.2).

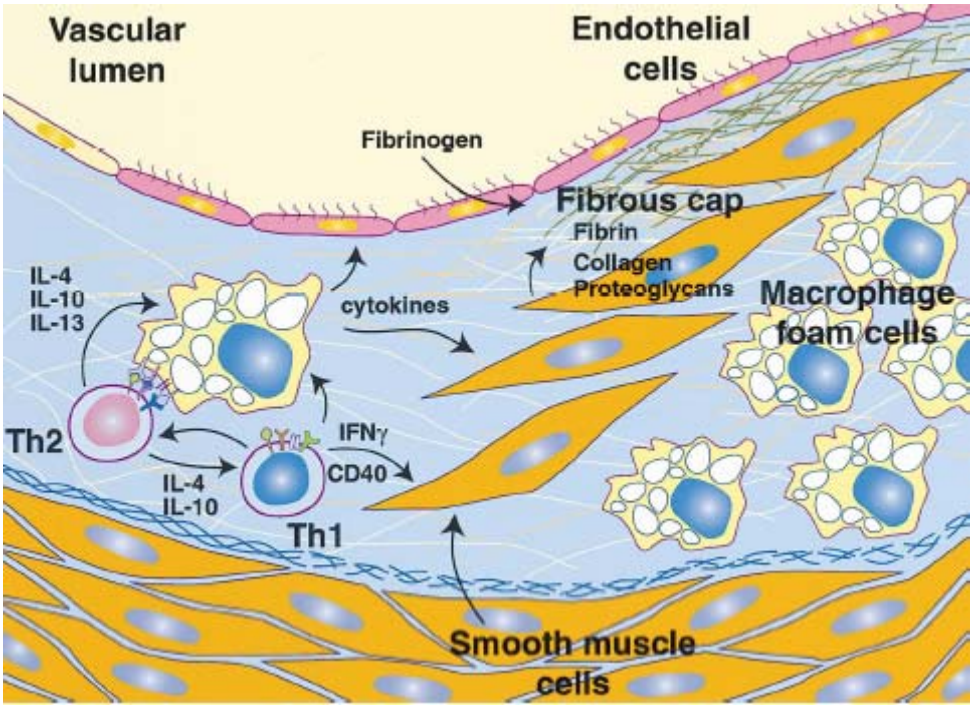


FIGURE 1.2: The Progression of the Plaque Lesion
Interactions between macrophage-derived foam cells and T cells establish a chronic inflammatory process whereby lesional T cells appear to be activated, expressing both Th1 and Th2 cytokines. Smooth muscle cells migrate from the medial portion of the arterial wall, proliferate and secrete extracellular matrix proteins that form a fibrous plaque. (Source: Glass & Witztum 1995)

Over time, the plaque continues to attract cholesterol, white blood cells and other fatty substances, resulting in plaque growth and formation of a relatively thick cap, due to either fibrosis (plaque scarring) or calcification. This particular type of plaque is considered to be stable, with a thick calcified cap and a smaller fatty core. Stable plaques are known as the primary cause of hardened (loss of elasticity) and narrowed arteries (atherosclerosis). On the other hand, another type of plaque is referred to as unstable; unstable plaques have a thin calcified cap covering a relatively larger fatty core. Unstable (thin) plaques are more likely to rupture, releasing fatty particles (from plasma LDL that enters the injured vascular wall) and other non-soluble substances into the bloodstream. The site of plaque rupture may be rapidly covered as blood platelets congregate at the site of the injury (non-occlusive thrombosis) or subsequently can further initiate the process of blood clotting (occlusive

thrombosis), forming a larger blockage (thrombosis) at that site. Thrombosis is associated with angina or myocardial infarction (MI) and sudden death. These pathophysiological observations lead to the 'response to injury' hypothesis of atherosclerosis which emphasizes endothelial dysfunction

The American Heart Association (AHA) has suggested a classification of plaques based upon their morphologic appearance (Stary et al. 1995). Type I, II and III are known as initial lesion, fatty streak and intermediate lesions or preatheroma, respectively. Indeed, type I and II lesions are sometimes combined under early lesions, which generally occur only in infants and children, although they also may occur in adults. On the other hand, type III lesions may further progress after puberty which forms the bridge between early and advanced lesions. In type IV lesions, characterised by lipid accumulation into a lipid core (atheroma), are the first lesion type referred to as advanced in this classification due to the severe intimal disorganization caused by lipid core. Type IV lesions commonly occur from the third decade of life. In type V lesions whereby fibrous connective tissue has formed, are known as fibroatheroma or fibrotic lesions. Type VI lesions are generally characterized by surface disruption, haemorrhage and thrombosis or combination of these, and are also known as complicated lesions. Type V and VI lesions most often occur in middle-aged and older people.

1.1.2 Endothelial Dysfunction

The endothelium is the thin layer of cells lining the interior surface of blood vessels including arteries, veins and also the inner lining of the heart and lymphatics. The endothelium plays a crucial role in the regulation of vascular tone, and inhibition of leukocyte adhesion and platelet aggregation via the action of chemical mediators such as nitric oxide (NO) and prostacyclin (Vane et al. 1990; Moncada & Higgs 1993). Endothelial dysfunction refers to the disturbance of the normal biochemical processes which are carried out by the endothelium. Endothelial dysfunction is believed to be a key event in signaling the occurrence of atherosclerosis, and is characterized by abnormalities in vasomotor control (constriction versus dilation). It associates with impaired fibrinolysis and enhanced thrombosis, and subsequently induces the inflammatory response of leukocyte and platelet

adhesion, along with growth of SMCs (Paterick & Fletcher 2001). Due to its role, the endothelium also forms a selective barrier between the blood and the underlying tissue, allowing oxygen and nutrients, but not dangerous substances, to cross. Therefore, any damage can lead to serious consequences to the vascular system, including the formation of fatty streaks and potentially myocardial infarction, stroke and heart disease.

There are several possible causes of endothelial dysfunction, such as an elevation in circulating oxidized LDL, free radicals caused by cigarette smoking, hypertension and diabetes, genetic alterations, high concentration of plasma homocysteine, infectious organisms such as herpes viruses and *Chlamydia pneumonia,* and a combination of these or other factors. The endothelial dysfunction that results from different forms of injury increases the adhesiveness and also the permeability of the endothelium with respect to leukocytes or platelets, and therefore leads to compensatory responses that alter the normal homeostatic properties of the endothelium. Thus, the injury promotes the endothelium to exhibit procoagulant activity instead of anticoagulant properties and subsequently to form vasoactive molecules, cytokines, and growth factors. However, in the case of an inflammatory response which ineffectively neutralizes or removes the offending/foreign agents, this process continues indefinitely and induces migration and proliferation of SMCs to form an intermediate lesion.

Continuous responses can thicken the arterial wall, which compensates by gradual dilation, and results in the lumen remaining unaltered (Glagov et al. 1987), a phenomenon called 'remodelling'. The response is, however, mediated by monocyte-derived macrophages and specific subtypes of T lymphocytes at every stage of the disease (Jonasson et al. 1986; van der Wal et al. 1989). Increased number of macrophages and lymphocytes emigrate from the blood, and subsequently proliferate within the lesion. Activation of these cells stimulates the release of cytokines, chemokines, hydrolytic enzymes, and growth factors (Libby & Ross 1996; Raines et al. 1996), which eventually can induce further damage, and therefore, lead to focal necrosis (Falk et al. 1996). Cycles of accumulation of mononuclear cells, migration and proliferation of SMCs, and formation of fibrous tissues lead to further enlargement and restructuring of the lesion, so that it becomes covered by a fibrous cap that overlies a core lipid and necrotic tissues, known as an advanced, complicated lesion. At

some point, the artery loses its elasticity which subsequently results in a disturbance in the blood flow.

Not all observations of SMCs of atherosclerotic plaque can be described by the 'response to injury' hypothesis because the proliferation of SMCs is also known as the monoclonal process. Following this finding, Benditt & Benditt (1973) refer to this theory as the 'monoclonal hypothesis'. The theory is supported by several studies (Parkes et al. 1991; Penn et al. 1986) which show the transforming mutational capacity of the *c-myc* gene and other proto-oncogens in the development of atherosclerotic lesions (Marin et al. 1993; Parkes et al. 1991). Indeed, there is strong experimental evidence for mutation theory of atherosclerosis, one of which underlines the similarity of atherosclerotic and carcinogenic processes (Ross et al. 2001), and subsequently supports the role of genetic alterations in the disease (Andreassi & Botto 2003). Moreover, it has been demonstrated that microsatalellite instability (Spandidos et al. 1996; Hatzistamou et al. 1996) and loss of heterozygosity in SMCs of human plaque suggesting that genomic destabilization also plays an important role in atherosclerotic mechanisms (Grati et al. 2001; Flouris et al. 2000; McCaffrey *et al.* 1997; Hatzistamou *et al.* 1996). Thus, in conjunction with this theory, DNA damage may provide new insight as an emerging risk factor in the development of atherosclerosis. Figure 1.3 illustrates the schematic combination of atherosclerosis hypotheses, comprising of 'response to injury' and the 'monoclonal hypothesis'.

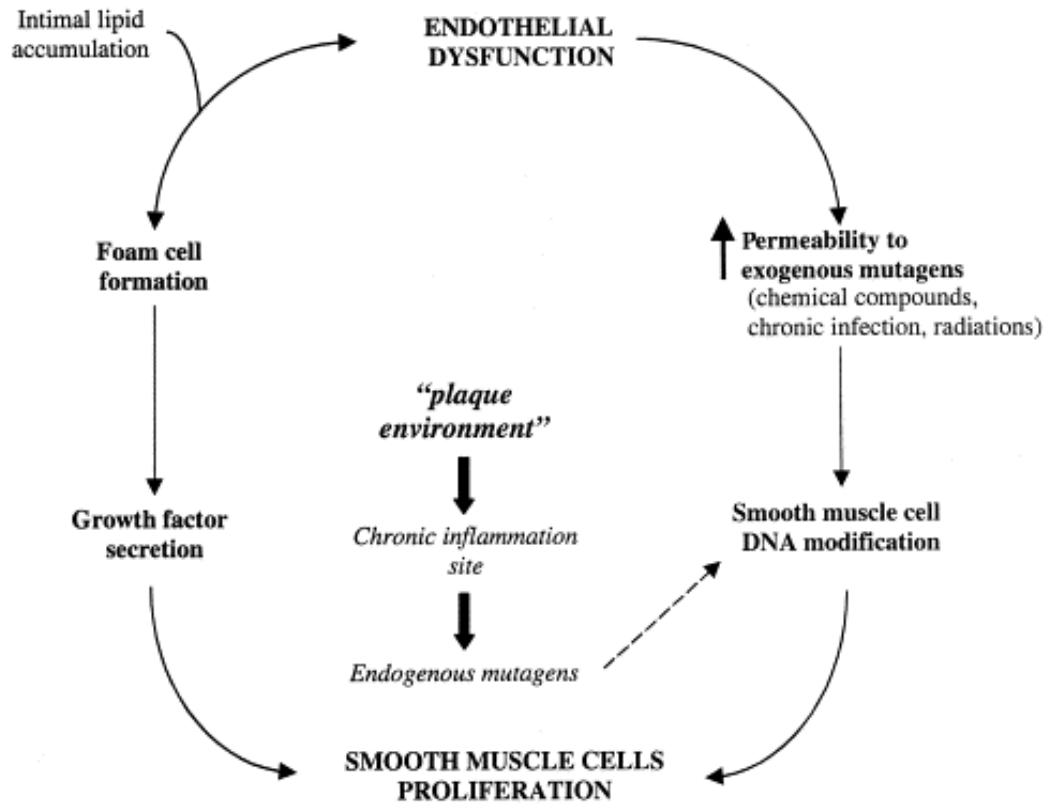


FIGURE 1.3: Scheme of a Unifying Hypothesis of Atherosclerosis

The figure shows that one of the earliest events in atherosclerosis is altered endothelial function (dysfunction) causing increased permeability to lipids. In addition, the endothelial dysfunction and the plaque environment also may induce DNA modification of smooth muscle cells by action of exogenous and endogenous mutagens. (Source: Andreassi & Botto 2003)

1.1.3 Cardiovascular Risk Factors

A risk factor is a measurable characteristic that is predictively related to the subsequent occurrence of a disease. This definition includes modifiable lifestyle, biochemical and physiological characteristics, as well as unmodifiable personal characteristics such as age, sex and family history. The American College of Cardiology has categorized risk factors into four categories (Table 1.1) that match the intensity of risk factor management based on evidence for association with CVD, clinical usefulness, and response to therapy (Pasternak et al. 1996). Category I risk factors are those in which interventions have been proven to lower CVD risk. Category II risk factors are those that interventions are likely to lower CVD risk, whereas Category III risk factors are those in which additional evidence is

required to determine whether interventions can lower risk. However, Category IV risk factors are those that cannot be modified. Alternatively, the British Nutrition Foundation (2005) suggested two categories of risk factors for CVD, namely classical (conventional) and emerging risk factors.

TABLE 1.1: Cardiovascular Risk Factors: Evidence to Support Interventions

Risk factor	Evidence for association with CVD		Clinical measurement	Response to:	
	Epidemiologic	Clinical trials	Usefulness	Nonpharmacologic therapy	Pharmacologic therapy
Category I (Risk factors for which interventions have been proven to lower CVD risk)					
Cigarette smoking	+ + +	+ +	+ + +	+ + +	+ +
LDL-c	+ + +	+ + +	+ + +	+ +	+ + +
High-fat/cholesterol diet	+ + +	+ +	+ +	+ +	-
Hypertension	+ + +	+ + + (stroke)	+ + +	+	+ + +
Left ventricular hypertrophy	+ + +	+	+ +	-	+ +
Thrombogenesis factor	+ + + (fibrinogen)	+ + + (aspirin, warfarin)	+ (fibrinogen)	+	+ + + (aspirin, warfarin)
Category II (Risk factors for which interventions are likely to lower CVD risk)					
Diabetes mellitus	+ + +	+	+ + +	+ +	+ + +
Physical inactivity	+ + +	+ +	+ +	+ +	-
HDL-c	+ + +	+	+ + +	+ +	+
Triglycerides; small, dense LDL	+ +	+ +	+ + +	+ +	+ + +
Obesity	+ + +	-	+ + +	+ +	+
Postmenopausal status (women)	+ + +	-	+ + +	-	+ + +
Category III (Risk factors associated with increased CVD risk that, if modified, might lower risk)					
Psychosocial factors	+ +	+	+ + +	+	-
Lipoprotein (a)	+	-	+	-	+
Homocysteine	+ +	-	+	+ +	+ +
Oxidative stress	+	-	-	+	+ +
No alcohol consumption	+ + +	-	+ +	+ +	-
Category IV (Risk factors associated with increased CVD risk that cannot be modified)					
Age	+ + +	-	+ + +	-	-
Male gender	+ + +	-	+ + +	-	-
Family history of early-onset CVD	+ + +	-	+ + +	-	-

1.1.3.1 Classical Risk Factors

Classical or conventional risk factors (Table 1.2) include unmodifiable characteristics, such as age, gender, genetic predisposition, and regional, social and ethnic differences. These unmodifiable risk factors are paralleled with modifiable risk factors, such as cigarette smoking, raised blood cholesterol, high blood pressure, obesity and physical inactivity. Modifiable risk factors not only predict the occurrence of the disease in healthy adults, but also contribute to the disease process in those who already have atherosclerosis.

TABLE 1.2: Conventional Risk Factors for Cardiovascular Disease

Risk factor	Direction of association	Modifiable by dietary factors?	Relevance to cardiovascular disease
Age	↑ with age	No	↑ prevalence as population ages
Gender	Males at ↑ risk	No	Risk in men and women is equalized after the menopause, possibly due to protection by estrogens in the younger women or to increases in male pattern (central obesity) amongst post-menopausal women
Socioeconomic status	Lower socioeconomic status ↑ the risk	Some dietary changes may help to reduce the higher risk in lower socioeconomic groups	Highly related to other factors (*e.g.* diet, smoking, physical inactivity)
Ethnic group	People from the Indian subcontinent are at particularly ↑ risk of CHD. People of African-Caribbean descent in the UK are at greater risk of stroke	No	May reflect abdominal obesity and insulin resistance Possibly reflects genetic predisposition to hypertension. Rates are falling as the result of better detection and treatment of hypertension
Smoking	Smoking ↑ risk	Yes (fruits & vegetables rich in antioxidant are helpful)	Increases oxidative stress and impairs endothelial function
Serum total cholesterol concentration	↑ blood cholesterol level ↑ risk	Yes (limit fat, cholesterol & alcohol intake)	Uptake of cholesterol by macrophages is the origin of the core of the atherosclerotic plaque
Serum LDL-cholesterol concentration	↑ LDL-c level ↑ risk	Yes (limit fat, cholesterol & alcohol intake)	LDL particles carry cholesterol that may be deposited in arterial walls
Serum HDL-cholesterol concentration	Lower HDL-c ↑ risk, particularly amongst women	Yes	HDL may transport excess cholesterol to the liver for excretion
Serum triglyceride concentration	↑ TG level ↑ risk	Yes (limit fat, carbohydrate & alcohol intake)	Strongly inversely related to HDL-c although serum TG is also an independent risk factor, perhaps reflecting more subtle alterations in lipid metabolism
Blood pressure	↑ BP increases risk	Yes (potassium rich food is helpful, limit fatty food and sodium intake)	Hypertension ↑ the risk of haemorrhagic/ ischaemic stroke, induces endothelial dysfunction, exacerbates the atherosclerosis process and contributes to the instability of the atherosclerotic plaque
Diabetes	Diabetes ↑ risk	Partly	Causes a unique pattern of microvascular complications and the ↑ frequency of macrovascular disease.
Physical inactivity	Being inactive ↑ risk	No	Physical fitness has been demonstrated to be a powerful predictor of low rates of subsequent vascular disease.
Obesity	Overweight and obesity ↑ risk partly via other CVD risk factors (*e.g.* blood pressure, diabetes, blood cholesterol)	Yes	Some of the co-morbidities of obesity are strong risk factors for CVD.

1.1.3.1.1 Age and Gender

Evidence shows that with increasing age, higher mortality rates from CVD are observed in both genders. Indeed, the risk of CVD increases markedly with ageing, which may be facilitated by adiposity and increased inflammatory reactions. Fat accumulation or deposition peaks in middle-age and substantially declines in advanced old age. Whilst the total body fat is reduced, the percentage of body fat remains unchanged or even increases in old age because of fat redistribution (British Nutrition Foundation 2005). Importantly, more fat is lost from subcutaneous than visceral fat storage after middle-age due to a decrease in physical activity level, which therefore, increases the risk of atherosclerosis and type 2 diabetes.

On the other hand, gender is one of the risk factors that is prominent for the assessment of risk. It has been reported that the incidence of death from premature CHD in men aged 35 to 44 years is three times higher compared with women of the same age. For women, however, the increased risk usually occurs after the menopause. Endogenous estrogen has been speculated to confer an important protective role against CVD in pre-menopausal women, probably by preventing vascular injury (Krummel 2004). In contrast, Davison and Davis (2003) reported that oral estrogen had a negative role in primary and secondary prevention trials of cardiovascular events. Van Beresteijn et al. (1993) revealed that average serum cholesterol levels increased by 19% in peri-menopausal women. For instance, during the menopausal period, total cholesterol, LDL cholesterol and triglyceride levels increase, whereas level of HDL cholesterol decreases, especially in women who gain weight (Krummel 1996). However, rates of CHD amongst women increase between the ages of fifty and sixty, indicating that the hormonal protective effect is lost after this period. Undoubtedly, this may be correlated with changes in body fat distribution as longitudinal data supports an increase in central adiposity after menopause (Barrett-Connor 1997).

1.1.3.1.2 Genetic Predisposition

Genetic factors refer to the fact that one individual will be more prone or susceptible to a disease than another, whatever environment they share. A family history of premature CVD

is a strong risk factor, even when other risk factors are considered. This genetic predisposition is suggested to be correlated when myocardial infarction (MI) or sudden death occurs before the age of 55 years in a male first-degree (close) relative or the age of 65 years in a female first-degree relative (parents, siblings, offspring). Studies in mice show that susceptibility to atherosclerosis depends on strain background (Dansky et al. 1999) and most of this variation is unlikely to be due to changes in known risk factors, such as lipoprotein concentrations, suggesting the involvement of genetic differences.

Current study of newly emerging risk factors may associate with understanding changes in the environment and the genes that determine which individuals are likely to be most affected, which is known as a gene-environment interaction. Studies have shown that regulation of metabolic consequences and risk of CVD in relation to food intake is genetically regulated. Interaction between genes and diet can occur when some dietary component switches on genes; for example certain fatty acids or their derivatives stimulate genes that are involved in adipocyte differentiation and behaviour (British Nutrition Foundation 2005). New technology that is capable of profiling the expression of thousands of genes in one experiment leads to in-depth understanding of developmental, homeostatic and pathological changes of gene expression (Lockhart & Winzeler 2000; Young 2000). Thus, this approach leads to identification of new candidate genes that can be tested for their roles in atherosclerotic development and further generates discovery of new antiatherogenic drugs, for instance, a specific therapy using gene expression profiles.

1.1.3.1.3 Socioeconomic and Ethnic Group

In terms of socioeconomic status, CVD within the UK and other developed countries is consistently more common among less affluent groups. Studies show that amongst male manual workers, the premature CHD mortality rate is 58% higher than that of trained professionals (Frayn 2005). Moreover, evidence demonstrates that the socioeconomic gradient in CVD mortality appears to be widening. For example, the difference in CHD risk between men in social classes V and I for sudden death due to heart attack was two-fold in the 1970s; however, this had risen to three-fold by the 1990s (Department of Health 2000). One component of this may be the smoking pattern which is higher in lower socioeconomic

groups for both genders compared with the more privileged ones. However, there are other factors that may influence the impact of socioeconomic status, such as diet, obesity, blood pressure and geographical differences. In addition, other suggested explanations for the social class differences include early life factors, physiological elements and access to health care services (Marmot et al. 1991). Therefore, no single factor accounts for the socioeconomic status since there are inter-relationships between one risk factor and another.

Ethnic group additionally plays an important role in determining disease incidence and prevalence. For example, studies reveal that South Asian men, such as Indians, Bangladeshis, Pakistanis and Sri Lankans living in the UK, have higher premature death rates from CHD and stroke compared with the national average (Department of Health 2001). Moreover, the difference in the death rates between South Asian men and the rest of population is rising due to the fact that this death rate in South Asians is not falling as fast as in the rest of the population. These studies have suggested that the high rates of CHD, simultaneously with a high rate of type 2 diabetes, are primarily explained by the existence of an insulin resistance syndrome. Insulin resistance syndrome is prevalent in South Asian populations and is also related to a pronounced susceptibility to central obesity in this group (Wild & McKeigue 1997). Conversely, premature death rates from CHD in Caribbeans and West Africans living is the UK are much lower than those of the average population, which are approximately half and two-thirds of the rate in the general population, for men and women, respectively. Notwithstanding, those of African-Caribbean ancestry have an increased risk of stroke, hypertension and diabetes. The underlying reasons for this phenomenon are not fully understood; however, genetic predisposition or other novel stroke risk factors are believed to contribute. On the other hand, it was found that early detection and control of hypertension can be attributed to the decline in stroke mortality in this group (Wild & McKeigue 1997).

1.1.3.1.4 Cigarette Smoking

Smoking is the number one cause of preventable death; one in five CVD deaths is related to smoking (AHA 2001). The increased risk of CVD from smoking has been identified for more than 30 years, with conclusive evidence presented in numerous reports. Furthermore,

smoking is synergistic with other risk factors and directly influences acute coronary events such as formation of the thrombus, plaque instability and arrhythmias. This risk also increases with the number of cigarettes smoked each day, and low-tar brands do not reduce the risk. Any exposure, including passive smoking also increases the risk (Howard & Thun 1999). Based on the Framingham Offspring/Spouse Study (FOS), it was found that women who consumed a heart-healthy diet and never smoked, demonstrated the overall lowest odds of sub-clinical heart disease (Millen et al. 2004). Cigarette smoke consists of complex mixture, and only a few components have been studied extensively. Nicotine and carbon monoxide appear less damaging than whole smoke, although nicotine up-regulates the expression of several pro-inflammatory and pro-oxidative molecules (Pitillo 2000).

Studies in animals suggest that the acetylcholine relaxation, which is dependent on endothelial function, is impaired by smoking (Celermajer et al. 1996; Pittilo 2000; Wang et al. 2001) an effect which may be attenuated by the antioxidant vitamin C (Mays et al. 1998). Furthermore, Wang et al. (2001) postulate that excessive endothelial apoptosis may contribute to endothelial injury that is induced by cigarette smoke. On the other hand, cigarette smoke also contains large amounts of nitric oxide, which subsequently is mainly converted to a powerful oxidant of nitrogen dioxide radical during the respiratory process in the lungs (transfer through the alveoli). Once the oxidation takes place, the level of antioxidant will drop simultaneously. Tappia et al. (1995) reported a 21% lower plasma vitamin C concentration was observed in smokers despite a similar intake of this vitamin C as compared to non-smokers.

1.1.3.1.5 Total Cholesterol (TC)

A total cholesterol (TC) determination measures cholesterol comprised in all lipoprotein fractions; of this TC 60-70% is carried on low density lipoprotein (LDL), 20-30% on high density lipoprotein (HDL) and 10-15% on very low density lipoprotein (VLDL). It has been reported that a 10% reduction in total cholesterol could decrease CHD incidence by up to 30% (CDC 2001). However, there are several factors that affect the level of serum cholesterol, for example age, dietary fat intake, genetics, endogenous and exogenous hormones or steroids, body weight, glucose tolerance and physical activity. Therefore,

although total cholesterol was previously recommended as a screening tool, recently Adult Treatment Panel III (ATP-III) has endorsed a complete lipoprotein profile for screening purposes.

Dietary cholesterol increases the ratio of total cholesterol to HDL, and higher ratios are associated with CHD (Dawber et al. 1982). The effects of dietary fats on total:HDL cholesterol ratio may differ markedly from their effects on LDL. Still, these effects on CVD risk markers should not be considered to reflect changes in risk and further conclusion needs to be confirmed by prospective observational studies or clinical trials (Katan et al. 2003). By that standard, risk is reduced most effectively when *trans* fatty acids and saturated fatty acids are replaced with *cis* unsaturated fatty acids (Katan et al. 2003). Since *trans* fatty acids are only a minor proportion of the diet, even marked relative reductions in intake will probably have less effect on LDL compared with a reduction in saturated fatty acids and cholesterol (Mensink & Katan 1993). However, in the long term or after several years, it was found that dietary intervention with different fatty acid types is ineffective in substantially lowering TC (Okuyama et al. 2007). This is because serum lipoproteins change shortly after dietary changes, but enzymes adapt to new dietary conditions. Thus, based on data from several studies (Suzuki et al. 1970; MRFIT 1982; Strandberg et al. 1991; Keys et al. 1950), the consequences of short- and long-term nutritional manipulations differ markedly. Even though it can be argued that the effectiveness of long-term dietary interventions due to progressively decreased compliance, other studies showed that a lack of compliance is not likely to be a major cause for the failure to lower TC values. In terms of cholesterol ratio, LDL/HDL balance is better than total cholesterol as a predictor of atherosclerosis. Although TC and LDL-c are highly directly correlated, the causal relationship between them and CHD is unclear. TC is probably a measure of CHD risk in groups with relatively large proportions of familial hypercholesterolemia (Okuyama et al. 2007). Moreover, even positive associations are noted between the TC and CHD events, but not all populations behave the same since CHD events differ approximately 4 to 8 fold at the same TC value.

1.1.3.1.6 LDL-cholesterol (LDL-c)

LDL-c is essentially a cholesterol carrier in the blood; therefore, total cholesterol and LDL-c levels are highly correlated. In humans, the majority of serum cholesterol is carried by LDL particles. LDL has an important physiological role as a vehicle for the delivery of cholesterol to the peripheral tissues. Therefore, increased LDL-c levels are associated with increased risk of CVD. There are a large number of case-control studies which demonstrate strong relationships between LDL-c and atherosclerosis (Crouse et al. 1985; Austin et al. 1988; Tornwall et al. 1991; Campos et al. 1992; Coresh et al. 1993; Stampfer et al. 1993; Griffin et al. 1994; Gardner et al. 1996; Stampfer et al. 1996; Lamarche et al. 1997; Skoglund-Andersson et al. 1999). Studies found that 95% of the apolipoproteins in LDL are apo B-100, known as apo B. It has been argued that apo B might exhibit as an even better risk marker than LDL-c (Sniderman et al. 1992; Lamarche et al. 1998; Walldius et al. 2001; Sniderman et al. 2003). However, issues of standardization and the fact that apolipoproteins have not been employed in large-scale studies restrain the use of apolipoproteins for these purposes.

Two LDL subclasses with different risks for CVD have been identified. Phenotype A is manifested by very large LDL particles, which are not associated with CVD risk. Meanwhile, phenotype B is characterized by small and dense LDL particles which are triglyceride rich and cholesterol depleted, that are predictive of CHD risk in both genders. The formation of phenotype B is almost exclusively demonstrated when the plasma level of TAG is mildly to moderately elevated. Therefore, this process is apparently slow when only few TAG-rich lipoproteins are available. The subsequent changes in chemical characteristics of the small, dense LDL particle trigger binding to the subendothelial matrix, resulting in easy oxidative modification thereby leading to foam cell formation (Tribble et al. 1992; Anber et al. 1996) (also see Section 1.1.1). Postmenopausal women have a greater prevalence of phenotype B than pre-menopausal women at the same age (Campos et al. 1988). In addition, phenotype B that is seen in 30% of general population tends to occur with low HDL-c and high levels of triglyceride, VLDL and intermediate-density lipoprotein (IDL).

1.1.3.1.7 HDL-cholesterol (HDL-c)

HDL particles contain more protein than any other lipoprotein. Apo A-I, the main apolipoprotein in HDL, is involved in tissue cholesterol removal by activating lecithin-cholesteryl ester acyl-transferase (LCAT). Therefore, HDL-c is also used as a predictor of lower CVD risk. Both apo C and apo E are minor apolipoprotein components, synthesized by the liver and are transferred to chylomicrons. Apo E allows receptors to recognize chylomicron remnants facilitating their removal from the circulation and metabolism. Elevated HDL levels are therefore associated with low levels of chylomicrons, VLDL remnants, and small, dense LDL.

Like LDL, HDL can also be divided into sub-fractions. There are two classifications of HDL by which is often described based on size and/ or density. HDL_2-c, which is relatively large and lipid rich, and HDL_3-c (subdivided in to HDL_{2a}, HDL_{2b}, HDL_{3a}, HDL_{3b}, HDL_{3c}), which is small and comparatively dense, that predominates in human plasma. The presence of HDL_2-c is critically dependent on low concentrations of triglyceride. Therefore, either HDL-c or HDL_2-c is strongly inversely related to plasma triglyceride concentrations. Only a limited number of studies designed to explore the relationship between HDL sub-fractions and CVD are available due to methodological difficulties involved in assessing the HDL sub-fraction concentrations accurately. Studies found that the effects of low fat diets on the plasma concentration of HDL_2 are inconsistent (Ehnholm et al. 1984; William et al. 1994), whereas physical activities are important strategies raising the HDL_2 concentration (William et al. 1994) or to improve the HDL inflammatory index from pro- to anti-inflammatory (Roberts et al. 2006). The Quebec Cardiovascular Study, however, concludes that HDL sub-fractions do not provide additional information on the risk of CHD compared with HDL-c *per se* (Lamarche et al. 1997); hence, total HDL-c remains the best predictor.

1.1.3.1.8 Triglyceride (TG)

The triglyceride rich lipoproteins include chylomicrons, VLDL and any remnants or intermediary products formed in catabolism of chylomicrons or VLDL. Chylomicrons provide the primary means of transport of dietary lipid, while VLDL functions to transport

endogenous lipids. In addition, the function of TG-rich VLDL particles that also contain apo B-100 and apo E, is to transport fatty acids to adipose tissue and muscle. There are some studies which suggest that postprandial triglyceride measurements may be more likely to predict the CHD risk compared with fasting levels (Ginsberg 1994). It was also found that although patients with CHD have higher fasting and postprandial levels of triglycerides compared with controls, the response based on percent increase to a fat-rich meal is similar in both groups (Schaefer et al. 2001). Endothelial dysfunction observed after consumption of a high-fat meal indicates one possible explanation for this proposition of higher levels of postprandial triglycerides. There is, however, no doubt that circulating triglyceride levels in fasting and postprandial states are related to the severity and advancement of atherosclerosis (Hodis 1999) and are recognized as independent risk factors for CHD (Hokanson & Austin 1996; Karpe 1997).

1.1.3.1.9 Hypertension

Hypertension is defined as an average blood pressure of over 140/90 mm Hg, or use of antihypertensive drugs (AHA 2001). It has been documented that higher systolic and diastolic blood pressure coincides with an increased incidence of ishaemic and hemorrhagic stroke (MacMahon 1996). In conjunction with this finding, salt (sodium), for instance, is viewed as the most important dietary intervention for controlling blood pressure. Sodium is the principal cation in the extracellular fluid and plays a pivotal role in maintaining the body homeostasis. Nevertheless, there is a maximum level of intake above which excretion of excess sodium cannot occur. This phenomenon, therefore, causes an increase in body sodium content and water retention and ultimately promotes the development of raised blood pressure.

The Committee on Medical Aspects of Food Policy (COMA) has recommended a reduction in salt intake to the average of 6 g/day for the adult population (Department of Health 1994). Based on the review of the available studies, more recently, the UK's Government of Scientific Advisory Committee on Nutrition (SACN 2003) concluded that the strong association between salt intake and elevated blood pressure strongly supports the recommendation. The convincing evidence comes from the Dietary Approaches to Stop

Hypertension (DASH) Sodium Trial, in which stepwise reductions in blood pressure were demonstrated in response to lowering of dietary salt levels (Sacks et al. 2001).

The relationship between hypertension and atherosclerosis can be explained by means of endothelial dysfunction. This mechanism may lead to changes in the production of local vasodilators, such as nitric oxide (NO) and of vasoconstrictors, such as endothelin-1 and vasoactive endothelial growth factor, VEGF. Hypertension also has pro-inflammatory action, a potential to increase the formation of hydrogen peroxide and free radicals, such as anions and hydroxyl radicals in plasma (Griendling 1997; Lacy et al. 1998; Swei et al. 1997). These substances, consequently, reduce the formation of NO (Vanhoutte et al. 1995), and thus the function of the endothelial layer may be impaired (British Nutrition Foundation 2005). Furthermore, leukocyte adhesion and peripheral resistance are also increased in response to these pro-inflammatory mediators (Swei et al. 1997).

Chobanian & Dzau (1996) found that concentrations of angiotensin II, a potent vasoconstrictor and the prominent product of renin-angiotensin system, are often elevated in hypertensive patients. In addition to causing hypertension, this vasoconstrictor can activate atherogenesis by inducing the growth of smooth muscle. Furthermore, angiotensin II binds to specific receptors on the smooth muscle, resulting in activation of phospholipase C. This process, subsequently, can lead to increases in intracellular calcium concentrations and in smooth-muscle contraction (Chobanian & Dzau 1996), and increased protein synthesis and smooth-muscle hypertrophy (Gibbons et al. 1992). Moreover, the activity of smooth-muscle lipoxygenase also increases and this can promote inflammation and oxidation of LDL.

1.1.3.1.10 Diabetes

Diabetes, defined as a dysregulation of glucose metabolism, is both a disease in its own right and a risk factor for CVD. Studies demonstrated that rates of CVD are four times higher in diabetic women aged 18 to 44 years compared with women without the disease (CDC 2001; Hu et al. 2001). After age-adjustment, prevalence of CVD in women with diabetes is twice that in women without diabetes. Diabetes is also responsible for the increased frequency of macrovascular disease, with the relative risk being around two-to-

three fold and three-to-five fold for men and women, respectively (Kannel & McGee1979; Morish et al. 2001). Some of the increased risk for CHD observed in diabetic patients is attributable to the concurrent presence of other risk factors, such as dyslipidemia, hypertension and obesity.

1.1.3.1.11 Physical Inactivity

Physical inactivity, or low level of fitness, is the most prevalent modifiable and independent risk factor for CVD. It is generally believed that physical activity levels have declined in the UK over the past 20 years (Department of Health 1999; Ruston et al. 2004). Incidence of obesity can only occur when energy intake is not harmonized by energy utilisation, thus reflecting a decline in average physical activity. Despite public health recommendations of thirty minutes of moderate-intensity daily activity (Pate et al. 1995), 29% to 38% of adults in national surveys reported to be physically inactive (Schoenborn & Barnes 2002). Physical activity minimizes CHD risk factor by hindering atherogenesis (Austin et al. 1990), increasing the vascularity of the myocardium, promoting fibrinolysis (Clarkson et al. 1999; Higashi et al. 1999) and modifying other risk factors, such as raising HDL cholesterol (Durstine et al. 2001), improving glucose tolerance and insulin sensitivity (Hsueh et al. 2004), supporting in weight management (Department of Health and Human Services 1996; Esposito et al. 2003; Nicklas et al. 2004) and reducing blood pressure (Whelton et al. 2002).

1.1.3.1.12 Obesity

Body mass index (BMI) and CVD are well documented to be positively related; as the BMI goes up, the risk of CVD also increases. The WHO has adopted a definition of adult obesity, this being a BMI over 30 kg/m^2 (WHO 1998). This method of defining obesity, however, cannot differentiate between muscle mass and adiposity. Thus, heavily muscled individuals may be categorized as obese even though their total body fat percentage is low. It is quite certain that obesity is a risk factor for CVD and weight loss has been associated with lower fibrinogen (Ditschuneit et al. 1995) and C-reactive protein levels (Tchernof et al.

2002), both of which are indicators for atherosclerosis. Interestingly, adipose tissue mass which is below what is categorized as obese, is still found to associate with raised inflammatory mediators (Kazumi et al. 2003), reflecting a role of adipose tissue in the inflammatory processes involved in CVD.

In some circumstances, visceral obesity, which is usually determined by the waist/hip ratio or waist circumference, is closely related to excess morbidity (Rexrode et al. 1998). A waist circumference of less than 35 and 40 inches for women and men, respectively, is therefore, recommended (NIH 1998). The relationship between morbidity and high waist/hip ratio is believed to be because visceral fat has especially unfavorable metabolic actions, such as mobilization of non-esterified fatty acid (NEFA) directly to the liver. Apparently, a complex mechanism is involved in this association since high waist/hip ratio is also accounted for by low physical inactivity, weight gain and high alcohol intake (Seidell & Bouchard 1997). Hence, studies correcting for these factors will be required before acknowledging that visceral fat *per se* has some adverse metabolic effect.

1.1.3.2 Emerging Risk Factors

Emerging risk factors have been initiated because it was found that existing risk factors for CVD do not completely explain the risk. For example, the distribution of serum cholesterol levels, believed to be one of the important classical risk factors, does not clearly distinguish those with from those without the disease. Indeed even some individuals with low cholesterol levels CVD is still manifested. Therefore, other factors must underlie the risk of CHD and it is also likely that other novel risk factors may play a substantial role and also account for a significant proportion of CVD cases. Moreover, new investigations on the underlying mechanism of atherosclerosis have identified the presence of other independent risk factors, such as inflammatory markers and oxidative stress, in the aetiology of CHD. Table 1.3 summarizes the parameters which are considered as emerging risk factors.

TABLE 1.3: Emerging Risk Factors for CVD

Risk factor	Relevance to CHD
Individual factors	
Lipid-related factors (apart from cholesterol concentrations)	Associates with plaque development and possibly thrombosis. Factors other than LDL-c may play a role in atherosclerosis
Endothelial dysfunction	Impaired endothelial dysfunction may allow entry of monocytes and LDL particles to subendothelial space. Mechanical stress on blood vessels due to high blood pressure is likely to be an important factor.
Oxidative stress	LDL oxidation may be involved in atherosclerosis.
Inflammation	May reflect atherosclerosis and inflammatory process.
Coagulation-related factors	Formation of thrombus leads to MI or stroke.
Homocysteine	Not clear
Adipose tissue-derived factors	Not clear
Common mechanism	
Insulin resistance	Related to dyslipidaemia, pro-coagulant state and endothelial dysfunction
Maternal and/or fetal undernutrition	May relate to insulin resistance and hypertension
Abdominal obesity	Related to insulin resistance

1.1.3.2.1 Lipid-related Factors

Lipid-related risk factors, essentially, are related to the classical risk factors of high plasma TC and LDL-c, low plasma HDL-c and elevated plasma TG (Woods et al. 1988). Interestingly, studies found that many of the emerging risk factors are directly related to plasma TG, a conventional risk factor for which it has been essentially difficult to exhibit independent associations with CVD. There are also interrelationships between post-prandial lipaemia, remnant-like particles, small dense LDL-c and HDL-c and the levels of triglycerides in plasma. Post-pradial (non-fasting) lipaemia is a condition in which atherogenic lipoproteins are generated, such as chlymicron and VLDL remnants. After meals, in the presence of TG-rich lipoproteins as substrates, LDL is likely to form due to the action of hepatic lipase activity that becomes prevalent whereas HDL depletes. If the

removal of post-prandial lipoproteins is slow, therefore, less *de novo* synthesis of HDL components are formed. It has been documented that a large number of case-control studies have shown excessive post-prandial lipaemia in people with CHD compared with controls (Karpe 1999). Moreover, positive relationships between markers of post-prandial lipoproteins and degree of CVD have been consistently shown in cohort studies (British Nutrition Foundation 2005).

1.1.3.2. Endothelial Function

It is postulated that the size of the lipid particles determines the degree by which the endothelium can be penetrated; small particles are more easily accumulated in the arterial wall compared with the larger ones. This point is already discussed under the sub-topic of endothelial dysfunction (see section 1.1.2).

1.1.3.2.3 Oxidative Stress

Oxidative stress is usually implicated in the aetiology of chronic diseases that are related to inflammatory markers. Certainly, this is true in the progression of atherosclerosis, where there is a participation of monocytes and macrophages in plaque formation. This invasion, subsequently, results in the release of reactive oxygen and nitrogen species which are believed to be a key process linked with many pathological events. Involvement of oxidative stress in the development of atherosclerosis, for example, can be ascribed in LDL oxidation. LDL also contains esterified polyunsaturates from dietary intake, and these polyunsaturates are prone to oxidative attack by free radicals. On the other hand, many studies support the protective nature of antioxidants, especially in the form of foods rich in these nutrients upon various types of CVD. However, since LDL also carries a high proportion of fat-soluble antioxidants, such as tocopherols and carotenoids, oxidation and propagation of fatty acid oxidation can be prevented in normal circumstances (British Nutrition Foundation 2005).

1.1.3.2.4 Inflammation

Cardiovascular disease is increasingly being recognised as a disease with a major immune-inflammatory component in its pathogenesis (Witztum & Steinberg 2001). There are various stimuli which can up-regulate the expression of number of genes in the endothelium and circulating blood mononuclear cells that are responsible for the reaction of the tissues to stress such as oxidative stress, oxidised lipids, haemodynamic flow and inflammatory cytokines. These genes include those encoding for adhesion molecules, inflammatory cytokines, heat-shock proteins, eicosanoids and redox enzymes (Yaqoob 1998; Wahle & Rotondo 1999; Witztum & Steinberg 2001).

Since atherogenesis is an inflammatory process, markers of inflammation such as C-reactive protein, have been measured. From epidemiological studies, CRP was found to be associated with angina, risk of cardiac events, stroke and peripheral vascular disease, independent of other risk factors (Wood 2001). Tchernof et al. (2002) demonstrated that weight loss lowers CRP, which suggests another physiological benefit of weight management as a preventative strategy for CHD control.

In relation to changes in endothelial cell function and inflammation, much attention has been devoted to the role of cellular adhesion molecules (CAMs) in CVD risk. Indeed, this focus has generated considerable evidence for altered endothelial CAM expression in animal models of CVD (Krieglstein & Granger 2001; Libby et al. 2002); identified levels of circulating soluble intercellular ICAM-1 (sICAM-1) as a marker of inflammation severity in clinical setting (Mulvihill et al. 2002); CAM expression as a critical determinant of the stage of organ damage and vascular dysfunction associated with experimental CVD (Krieglstein & Granger 2001; Tailor et al. 2000; Libby et al. 2002) and the relation between CAM expression and other factors involved in the development of CVD (Cai & Harrison 2000; Cooper et al. 2002).

1.1.3.2.5 Homocysteine

Homocysteine, a sulphur-containing amino acid exists in all cells. The mechanism by which high circulating homocysteine concentration is initially thought to be associated with the CVD risk factor is incompletely understood. Homocysteine is proposed as a risk factor when it was observed that children who were deficient in the essential catabolic enzymes for homocysteine, cystathionine β-synthase or methylenetetrahydrofolate reductase, were found to have premature atherosclerosis, albeit in veins and not arteries, and mortality.

In vitro and *in vivo* studies suggest that homocysteine may accelerate plaque formation by enhancing the process of lipid oxidation, exerting procoagulant activity and promoting collagen synthesis and smooth muscle cell proliferation (Mangoni & Jackson 2002; Faraci 2003). Homocysteine is also responsible for determination of acute and chronic endothelial dysfunction by promoting the production of hydrogen peroxide and other highly reactive oxygen compounds, upregulating cell adhesion molecules and inhibiting the release of nitric oxide or reducing nitric oxide availability (Faraci 2003; Upchurch et al. 1997). Researchers have also focused on the relationship between homocysteine and inflammation. Evidence shows that concentrations of acute phase reactants, such as fibrinogen, CRP and ∝-1 chymotrypsin, are closely related with circulating concentrations of homocysteine (Bates et al. 1997; Evans et al. 1997; de Jong et al. 1997; Rohde et al. 1999). Preclinical studies indicate that interleukin 6 (IL-6) may interact with vitamin B_6 metabolism and compromise cystathionine β-synthase activity (McCarty 2000). Interestingly, high concentrations of proinflammatory cytokines are associated with a high risk of medical conditions that have also been associated with the hyperhomocysteinemia, such as ischemic stroke, myocardial infarction and more recently, osteoporosis. Thus, it may be hypothesized that hyperhomocysteinemia and cardiovascular risk may be both mediated by inflammatory state.

Conversely, previous studies that addressed the association between inflammation and homocysteine also reported conflicting findings. Therefore, homocysteine as a risk factor for cardiovascular disease is now controversial for several reasons (Braattstrom 2000). First, elevated plasma homocysteine is strongly and positively related to the other major

risk factors for CVD (i.e., age, smoking, blood pressure, high blood cholesterol, and lack of exercise) (Bots 1997). Second, serum creatinine as an indicator of renal function is a strong predictor for plasma homocysteine levels. Thus, poor renal function due to atherosclerosis raises homocysteine levels, not *vice versa*. Third, whereas low serum or red cell folate or vitamin B_{12}, cause elevations in plasma homocysteine levels, deficiency of either of these vitamins does not produce vascular disease. In conclusion, homocysteine is categorized as a modest independent risk factor for cardiovascular disease and more research in this area is warranted.

1.1.3.2.6 Adipose Tissue-derived Factors

As discussed earlier (See Section 1.1.3.1.12), obesity associates with increased risk of CVD morbidity and mortality, and is becoming an expanding dilemma in developing countries (Kushner 2002). This link involves a complex of diverse elements that can be partially explained by novel signaling molecules (adipokines), formed in adipose tissue. It has been suggested that the secretion of these molecules, such as, leptin, IL-6 and adiponectin, together with the expression of TNF-α in obese individuals, possibly underlies the association of insulin-resistance with endothelial dysfunction and coagulopathy, which ultimately lead to the progression of CHD (Mohamed-Ali et al. 1998). Furthermore, the relationship between obesity and CVD can be illustrated by popular intermediaries such as hypertension, type 2 diabetes and dyslipidaemia, as well as the less well-known mediators, such as chronic inflammation and hypercoagulation. Apparently, these proteins, synthesized by adipose tissue are synchronized with energy balance and metabolism. In obesity, peripheral tissue resistance to leptin, together with the deficiency of adiponectin, may result in decreased fat oxidation in skeletal muscle, macrophages and liver, thus leading to increased fat accumulation within these tissues.

Acylation stimulating protein (ASP) or adipsin, is a small serum protein that may function mainly as an adipocyte autocrine factor and plays a significant role in adipose tissue metabolism. Studies show that adipsin is positively related to various CVD risk factors including, insulin glycated hemoglobin (HbA1c), leptin, CRP, plasminogen activator

inhibitor 1 (PAI-1) and tissue plasminogen activator (tPA) (Mavri et al. 1999; Ebeling et al. 2001; Ylitalo et al. 2001).

1.1.3.2.7 Maternal/Fetal Undernutrition

The roots of cardiovascular disease can be tracked back to fetal life and infancy, and also to the effects of poverty on mothers, as first proposed by Barker (1998). Previous, studies have shown that lower birth weight and weight at one year are associated with an increased risk of death in adult life from CHD and stroke (Barker et al. 1989; Osmond et al. 1993), and also are independent of socioeconomic status (Frankel et al. 1996; Rich-Edwards et al. 1997). However, there is some evidence in Finland that the proportions of body at birth exhibit a stronger relationship, for example, low ponderal index (weight/ length^3) predicts CHD better than birth weight *per se* (Forsen et al. 1997; Eriksson et al. 1999), whereas, in UK, low birth weight/head circumference ratio predicts stroke mortality (Martyn et al. 1996).

Figure 1.4 shows the association between small size at birth and CVD risk factors that reflects a permanent effect of fetal undernutrition, called the 'Fetal Origins of Adult Disease (FOAD)' Hypothesis (Barker 1998), also known as 'thrifty phenotype' hypothesis (Hales & Barker 1992). Low birth weight babies tend to catch up, and the rapidity of post-natal growth may simply indicate the severity of growth retardation at birth (Leon et al. 1996). Indeed, it is also postulated that the catch-up growth hormones have adverse cardiovascular and metabolic effects (Lever & Harrap 1992). In contrast, the 'thrifty phenotype' hypothesis proposes 'fetal programming', that the undernourished fetus develops insulin resistance and other metabolic changes as a strategy for immediate survival, to down-regulate and prioritize growth, for which it pays a price later in life, generally after the reproductive period. However, evidence is insufficient to make recommendations about catch-up growth during infancy in low birth weight babies. Therefore, monitoring childhood BMI should be a public health concern, since accelerated weight and BMI gain in childhood is reported as a clear risk factor.

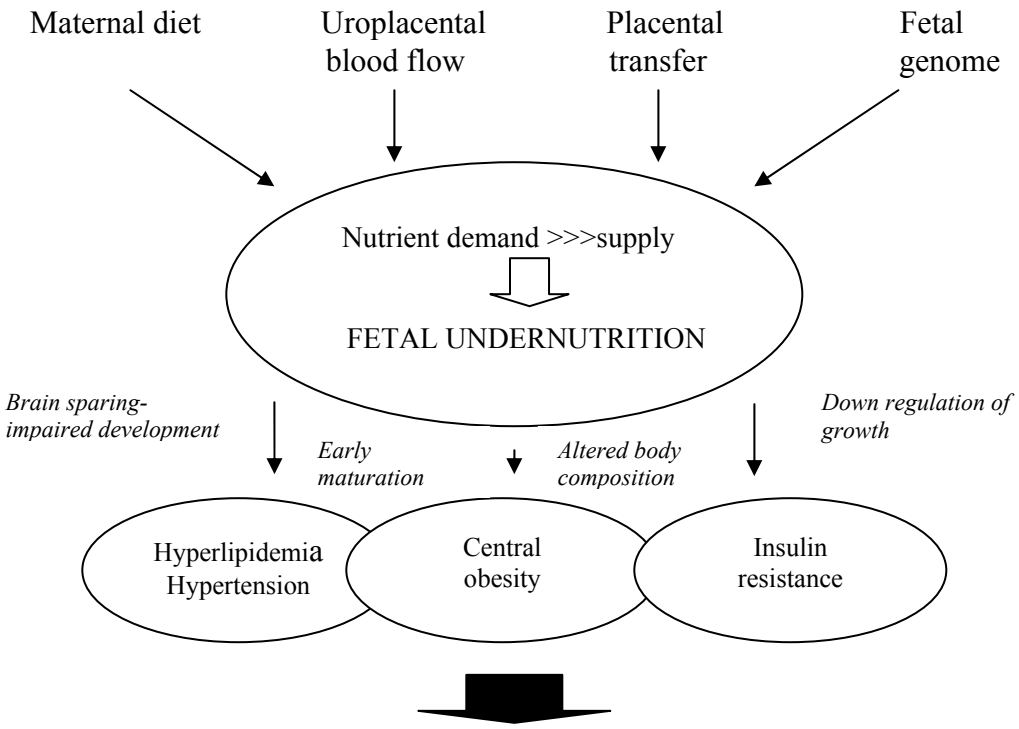


FIGURE 1.4: The Fetal Origins Hypothesis

(Adapted with modification from British Nutrition Foundation 2005)

1.2 Fatty Acids and Cardiovascular Disease

Fatty acids are hydrocarbon chains varying in total chain length from 2 to 30 or more carbons, with a methyl group at one end of the chain and carboxyl group at the other (Calder 2005). A vast number of fatty acids occur in nature and they differ in chain length, and in number, type and position of double bonds. Figure 1.5 shows the molecular structure of some fatty acids. Stearic acid containing 18 carbon atoms and no double bonds is referred as a saturated fatty acid (SFA); the term 'saturated' refers to hydrogen, in that all carbons, apart from the carboxylic acid (-COOH) group, are linked to as many hydrogen atoms as possible. On the other hand, fatty acids with at least with one double bond between adjacent carbon atoms are called unsaturated. There are two types of unsaturated fatty acids; monounsaturated fatty acids (MUFA) contain a single double bond and polyunsaturated fatty acids (PUFAs) with at least two double bonds. Double bonds can be either in *cis*- or

trans- conformation and also occur in different positions within the chain. The corresponding unsaturated fatty acids for stearic acid are oleic acid, linoleic acid and linolenic acid which contain one, two and three double bonds, respectively, as shown in Figure 1.5. In the commonly used fatty acid nomenclature, the double bonds are counted from the methyl (CH_3) end of the molecule, noted by n-x (x is the position of double bond) or ω-x (ω for the terminal carbon). For example, oleic acid in the nomenclature is written as 18:1n-9 or 18:1ω-9, since has 18 carbon atoms and one double bond with the double bond on carbon nine counting from terminal methyl group. For n-3 fatty acids, the first double bond is located between the third and fourth carbons from the methyl end of the chain.

CH_3-$(CH_2)_{16}$-COOH
Stearic acid (18:0)

CH_3-$(CH_2)_7$-CH=CH-$(CH_2)_7$-COOH
Oleic acid (18:1n-9)

CH_3-$(CH_2)_4$-CH=CH-CH_2-CH=CH-$(CH_2)_7$-COOH
Linoleic acid (18:2n-6)

CH_3-CH_2-CH=CH-CH_2-CH=CH-CH_2-CH=CH-$(CH_2)_7$-COOH
Linolenic acid (18:3n-3)

FIGURE 1.5: Molecular Structures and Names of Some 18 Carbon Fatty Acids

There are probably less than twenty fatty acids that are quantitatively important in the human diet. Table 1.4 presents the most common occurring fatty acids, grouped as SFA, MUFA and PUFA**.**

TABLE 1.4: Common Dietary Fatty Acids

Fatty acid	**Systemic name**	**Formula**	**Common food source**
Saturated			
Caprylic	Octanoic	8:0	Human breast milk, coconut oil
Capric	Decanoic	10:0	Animal fats. Coconut oil
Lauric	Dodecanoic	12:0	Coconut oil, palm kernel oil
Myristic	Tetradecanoic	14:0	Coconut oil, palm kernel oil, dairy products
Palmitic	Hexadecanoic	16:0	Dairy products, meat, palm oil
Stearic	Octadecanoic	18:0	Cocoa butter, palm oil, meat
Monounsaturated			
Oleic	9-Octadecanoic	18:1n-9	Olive oil, rapeseed oil, palm oil, meat
Elaidic	*Trans* 9-Octadecanoic	t-18:1	Partially hydrogenated fats
Gadoleic	11-Eicosaenoic	20:1n-9	
Erucic	13-Docasaenoic	22:1n-9	Mustard oil
Nervonic	15-Tetracosaenoic	24:1n-9	
N-6 polyunsaturated			
Linoleic (LA)	9,12-Octadecanoic	18:2n-6	Sunflower, maize, safflower and soybean oils
γ-linolenic (GLA)	6,9,12-Octadecatrienoic	18:3n-6	Borage and evening primrose oils
Dihomo-γ-linolenic (DGLA)	8,11,14,17-Eicosatetraenoic	20:3n-6	
Arachidonic	5,8,11,14-Eicosatetraenoic	20:4n-6	Ruminant meats (low levels)
N-3 polyunsaturated			
α-linolenic (ALA)	9,12,15-Octadecatraenoic	18:3n-3	Linseed, walnut and soybean oils, walnuts, vegetables
Eicosapentaenoic (EPA)	5,8,11,14,17-Eicosapentaenoic	20:5n-3	Fish oil, oil-rich fish, marine mammals
Docosapentaenoic (DPA)	4,7,10,13,16-Docosapentaenoic	22:5n-3	Fish oil, oil-rich fish, marine mammals,
Docosahexaenoic (DHA)	4,7,10,13,16,19-Docosahexaenoic	22:6n-3	Fish oil, oil-rich fish, marine mammals

Most fatty acids can be synthesised in the body. However humans and mammalian cells, but not plants, lack the desaturase enzymes required to produce two fatty acids: α-linolenic acid (n-3) and linoleic acid (n-6). These are known as essential fatty acids which must be acquired from the diet. The body can convert one n-3 fatty acid to another n-3 fatty acid; for example α-linolenic acid can be elongated and subsequently desaturated to the long chain n-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Figure 1.6), but the conversion rate may not be sufficient to meet requirements and only occurs when the n-6/n-3 ratio is low. For example, less than 8% of ALA is metabolised to EPA and the capacity for the body to synthesise DHA appears to be limited. It is estimated that only between 0.02% and 4% of dietary ALA is metabolised to DHA (Vermunt et al. 2000; Pawlosky et al. 2001; Burdge et al. 2002), with women having greater capacity for DHA synthesis than men (Budge & Wootton 2002). Thus, it is recommended that good sources of these fatty acids, for example oil-rich fish, need to be included in the diet.

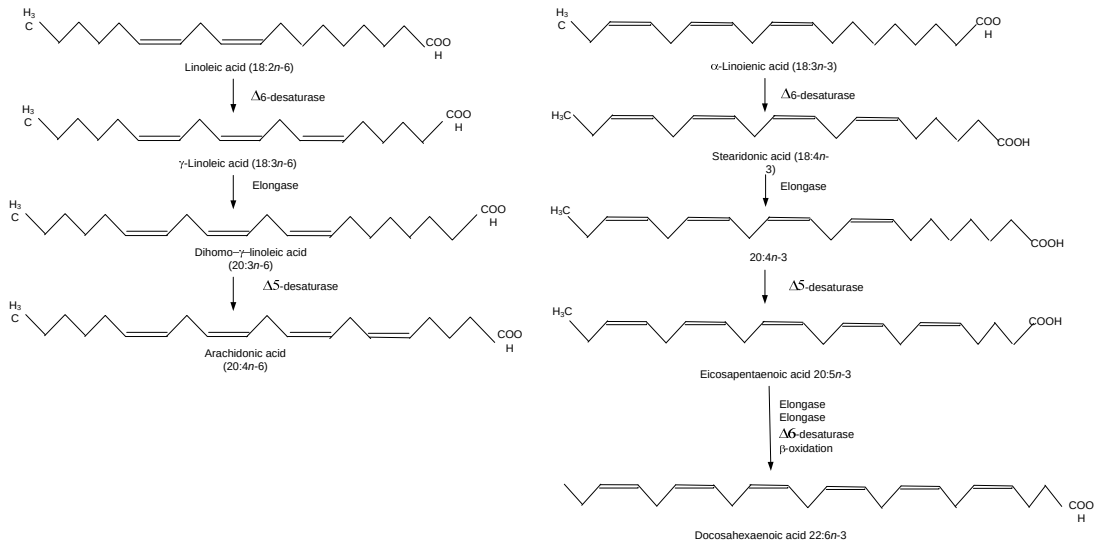


FIGURE 1.6: Metabolism of n-6 and n-3 PUFAs in Mammals

The pathway in Figure 1.6 shows that there is a competition between n-6 and n-3 fatty acid families due to sharing of the same series of enzymes in their conversion. Although the preferred substrate for Δ6-desaturase is α-linolenic acid (ALA), due to linoleic acid being much more prevalent in most human diets as compared to ALA, the metabolism of n-6 fatty acids is quantitatively more important (Calder 2005). Morover, excessive n-6 fatty acids in

the diet saturates the enzymes and prevents conversion of ALA into EPA and DHA (Kris-Etherton 2000). In mammals, this pathway occurs mainly in the liver.

1.2.1 Long Chain n-3 Polyunsaturated Fatty Acids

LC n-3 PUFAs refer to fatty acids with chain length of 18 carbons or more, with the first double bond located between the third and the fourth carbons counted from the methyl end. For example, EPA (20:5n-3) is an omega(ω)-3 or (n-3) fatty acid with 20 carbon atoms in the entire chain and with five double bonds, with the first double bond located at the third carbon atom from the methyl end of the chain (n-3).

Dietary EPA and DHA primarily come from marine sources by which algae as prime source: mackerel, salmon and sardines, as well as crab, shrimp and oysters and also fish oils and some fish liver oils like cod liver oil (Table 1.5). The content of n-3 PUFAs varies between species, and also depends on the location, diet, season of capture and cooking methods.

TABLE 1.5: n-3 PUFAs Contents in 3-oz Serving Seafood

	EPA+DHA content, g/3-oz serving seafood (edible portion) or g/g oil	Amount required to provide ≈ 1g of EPA+DHA/d, oz (seafood) or g (oil)
Seafood		
Sardines	0.98-1.70	2-3
Mackerel	0.34-1.57	2-8.5
Halibut	0.4-1.0	3-7.5
Haddock	0.2	15
Flounder/sole	0.42	7
Lobster	0.07-0.41	7.5-42.5
Crab, Alaskan King	0.35	8.5
Shrimp, mixed species	0.27	11
Clam	0.24	12.5
Scallop	0.17	17.5
Tuna		
Light, canned in water, drained	0.26	12
White, canned in water, drained	0.73	4
Fresh	0.24-1.28	2.5-12
Salmon		
Chum	0.68	4.4
Sockeye	0.68	4.5
Pink	1.09	2.5

Chinook	1.48	2
Atlantic, farmed	1.09-1.83	1.5-2.5
Atlantic, wild	0.9-1.56	2-3.5
Herring		
Pacific	1.81	1.5
Atlantic	1.71	2
Trout, rainbow		
Farmed	0.98	3
Wild	0.84	3.5
Cod		
Pacific	0.13	23
Atlantic	0.24	12.5
Catfish		
Farmed	0.15	20
Wild	0.2	15
Oyster		
Pacific	1.17	2.5
Eastern	0.47	6.5
Farmed	0.37	8
Capsules		
Cod liver oil	0.19	5
Standard fish body oil	0.30	3
Omega-3 fatty acid concentrate	0.50	2
Omacor (Pronova Biocare/Solvay)	0.85	1

The intakes of fish given above are very rough estimates because oil content can vary markedly (>300%) with species, season, diet, and packaging and cooking methods.
Adapted from USDA Nutrient Data Laboratory.

1.2.2 Fish Consumption and Risk of CVD

It is increasingly recognized that regular fish consumption is associated with a reduced risk of sudden cardiac death and of cardiovascular disease (von Schacky 2003; Harris 2005). Table 1.6 illustrates that epidemiological studies consistently show that consumption of at least one serving of fish per week may reduce the risk of CHD death by approximately 40% compared with no consumption of fish (Kromhout et al. 1985; Rodriguez et al. 1996; Daviglus et al. 1997; Albert et al. 1998). In the US Physicians' Health Study of 20,551 US male physicians (aged 40-85 years) free from CVD, who were followed-up for 11 years, it was found that weekly fish consumption (up to one meal) was associated with a lower risk of sudden cardiac death and a 30% reduction in total mortality as compared to consumption of less than one fish meal per month (Morris et al. 1995). Similarly, Hu et al. (2002) concluded in the Nurses' Health Study of 84,688 healthy women (aged 34-59 years) with 16-year follow-up that a higher intake of fish, was associated with a lower risk of CHD and

CHD mortality. In a 30-year follow-up of the Chicago Western Electric Study (N=1822, aged 40-55 years), men who consumed more than 35 g fish daily as compared to those who consumed none had a relative risk of death from CHD of 0.62 (Daviglus et al. 1997). In a case-controlled study in the elderly (> 65 y), the beneficial association between marine-derived n-3 PUFAs and CHD was shown for fatal CHD, but not for non-fatal MI (Lemaitre et al. 2003). In another study, Iso et al. (2006) in a 10-year follow-up on a large cohort study comprising of 41,575 Japanase men and women (aged 40-59 years) reported that a higher consumption as compared to modest intake of fish once a week of 20 g/day was associated with a substantially reduced risk of CVD, primarily of nonfatal cardiac events among middle-aged people. Moreover, a meta-analysis of prospective studies counted a total of 116,764 individuals has confirmed the association, and again only in high-risk for CVD groups (Marckmann & Gronbaek 1999). In this analysis, it was estimated that consumption of 40-60 g of fish daily could reduce the risk of CHD death by 40-60% in these groups.

TABLE 1.6: Evidence of Cardio-protective Effect of Fish and N-3 PUFAs from Epidemiological Studies

Studies	**Population (n)**	**Follow up (y)**	**Association between fish or n-3 PUFA intake and risk of CHD death**	**Main conclusion-protectio against CHD death (Yes/No)**
Kromhout et al. 1985 (Zutphen Elderly Study)	n=852, men, age 57-76 y	20	Consumption of ≥ 30 g fish/day was associated with 50% lower mortality for CHD compared with those who did not eat fish.	Yes
Rodriguez et al. 1996 (The Honolulu Heart Program)	n=7513, men, 45-65 y, heavy smokers	23	Mortality among high smoking group with high fish intake was half that those with low fish consumption. No inverse relationship between fish intake and CHD in the overall population	No
Daviglus et al. 1997 (Chicago Western Electric)	n=1822, men, 40-55 y	30	Consumption of > 35 g fish/d compared was associated with 38% lower risk of death from CHD compared with those who did not eat fish.	Yes
Albert et al. 1998/ Morris et al. 1995 (US Physicians' Health Study)	n=43757, men, 40-75 y n=20551, men, 40-84 y	11	Compared with < 1x fish/mo, relative risk for sudden death was 0.64 for those consuming 1-3x/mo and 0.48 for those consuming ≥ 1x/wk Consumption of ≥ 1 fish meal/wk was associated with lower risk of sudden cardiac death and a 30% reduction in total mortality as compared with < 1 fish meal/mo.	Yes
Lemaitre et al. 2003 (Cardiovascular Heart Study)	n=4778 men and women > 65 y	Up to 10-y	Inverse association between marine n-3 PUFAs and CHD for fatal CHD but not for non-fatal MI	Yes
Iso et al. 2006 (JPHC-Japan Public Health Centre-Based Study Cohort 1)	n=41575, 40-59 y	10	Higher consumption as compared to modest intake of fish 1x/wk of 20 g/d was associated with reduced risk of CVD primarily non fatal cardiac events.	Yes

TABLE 1.7: Evidence of Cardio-protective Effect of N-3 PUFAs from Clinical Trials

Studies	**Population (n)**	**Follow up (y)**	**Outcome**	**Main conclusion-protection against CHD death (Yes/No)**
Burr et al. 1989 (DART; Diet and Reinfarction Trial)	n=2033 men with recent MI, advised to increase their oily fish intake to twice a week or received 3 fish oil capsules/day if they could not tolerate the fish	2	The fish advice resulted in a 29% reduction in total mortality, 32% decrease in fatal myocardial reinfarction. Based on assumption that EPA contributes ≈ 40% of the total EPA and DHA in oily fish, daily intake of EPA plus DHA was estimated ≈ 900 mg/d. No significant reduction in the incidence of recurrent non-fatal MI.	Yes
Sacks et al. 1995	n=59, 4.8 g/day EPA + DHA (n=31) *vs* olive oil (n=28)	2.4	Fish oil supplementation does not promote major favourable changes in the diameter of atherosclerotic coronary arteries.	No
Singh et al. 1997 (Indian Study on Infarct Survival)	n=360, 1.8 g/day EPA + DHA (n=122) *vs* 2.9 g/day ALA (n=120) or non-oil placebo (n=118)	1	Total cardiac events (sudden cardiac death, total cardiac death, non-fatal reinfarction) were reduced by 25% in the fish oil group.	Yes
Leng et al. 1998	n=120, 0.27 g/day EPA (n=60) *vs* sunflower seed oil 3 g/day (n=60)	2	N-3 PUFAs significantly reduced a systolic blood pressure, but no other significant benefits on risk factors.	No
von Shacky et al. 1999	n=223 patients with CAD, 3.3 x mo, then 1.65 g/day EPA + DHA (n=112) *vs* vegetable oil blend (n=111)	2	Fish oil supplementation modestly reduces the course of coronary atherosclerosis.	Yes
De Lorgeril et al. 1999 (The Lyon Heart Study)	n=233 survivors of first MI, Mediterranean diet (rich in ALA) *vs* prudent Western-type diet.	4	50-70% lower risk of recurrent risk cardiac death and non-fatal MI in group who had Mediterranean diet supplemented with ALA	Yes
Nilsen et al. 2001	n=300 hospitalized patients, 3.36 g/day n-3 PUFAs *vs* corn oil.	1.5	No clinical benefit from fish oil was demonstrated, but participants lived in a coastal area of Norway where they consumed a baseline diet rich in n-3 PUFAs.	No

Studies	**Population (n)**	**Follow up (y)**	**Outcome**	**Main conclusion-protection against CHD death (Yes/No)**
GISSI, 2001; Marchioli et al. 2002 (GISSI-Prevenzione Trial)	n=11323 persons having survived a MI for a median of 16 days; a factorial design of 850-882 mg/d EPA and DHA (n=5665) *vs* 300 mg vitamin E/usual care (n=5658)	3.5	A significant reduction for all-cause mortality, cardiac death and sudden death by 21%, 35% and 45% respectively. No significant reduction in the incidence of recurrent non-fatal MI.	Yes
Bemelmans et al. 2002 (MARGARIN; The Mediterranean ALA Enriched Groningen Dietary Intervention)	n=266, 6.3 g/day ALA (n=109) *vs* control-ALA 1.0 g/day (n=157)	2	The effect of ALA supplementation on estimated IHD risk was at least similar to, or even greater than, that of a standard LA-rich diet	No
Burr et al. 2003 (DART-2)	DART-2 n=3114 men with angina, advice to eat fish or providing fish oil capsules, estimated of 1.07 g/day EPA + DHA (n=1571) *vs* control (n=1543)	12-16	Less sudden death in control (under-funded, thus not properly conducted or reported study, compliance was checked in only 2% of the cohort and only at 6 months)	No
Raitt et al. 2005	n=200 patient with implantable cardioverter defibrilator (ICD) and a recent episode of sustained ventricular tachycardia (VT) or ventricular fibrillation (VF), 1.8 g/day EPA + DHA (n=100) *vs* olive oil (n=100)	2	Fish oil supplementation does not reduce the risk of VT/VF and may be pro-arrhythmic in some patients.	No, fo reducing th risk o VT/VF.
Leaf et al. 2005	n=402 of ICD patients, 2.6 g/day EPA + DHA (n=200) *vs* 4 g/ day olive oil (n=202)	1	Fish oil supplementation may significantly reduce fatal ventricular arrhythmias in individuals at high risk fatal ventricular arrhythmias although no significance was achieved for the primary end point (ICD events for VT/VF and/ or death from any cause)	Yes, in high risk individuals.

Studies	**Population (n)**	**Follow up (y)**	**Outcome**	**Main conclusion- protection against CHD death (Yes/No)**
Brouwer et al. 2006 (SOFA; The Study on Omega-3 FA and Ventricular Tachyarrhythmia)	n=546 ICD patients, 2g/ day EPA + DHA (n=273) *vs* 3.4 g/day sunflower oil (n=273)	1	Fish oil supplementation does not indicate a strong protective effect against ventricular arrhythmia in patients with ICD	No, on li threatening cardiac arrhythmia
Yokoyama et al. 2007 (JELIS; Japan EPA Lipid Intervention Study)	n=18645 patients with hyperlipidemia of which 3664 had already established CAD, 1.8 g/day EPA *vs* usual care	4.6	The primary endpoint was 'major coronary events'- sudden cardiac death, fatal and non- fatal MI, unstable angina, events of angioplasty/stenting or CBG. This primary endpoint was reduced by 19% compared with control. ** *All hyperlipidemia patients was treated with statins*	Yes

1.2.3 n-3 PUFAs and CVD

Observational findings of a lower incidence of CVD in Inuits of Greenland despite a high saturated fat intake (about 40% from total energy intake) (Dyerberg & Bang 1979) were believed to be related to the high LC n-3 PUFAs consumption. Further experiments showed that the cardio-protective effects were derived from EPA and DHA (Billman et al. 1999). However, in a cross-sectional study involving 454 Alaskan Eskimos, Ebbesson et al. (2005) found that there was no association between current n-3 fatty acid consumption/blood concentrations and the presence of CHD. The authors suggested that the so-called 'Eskimos paradox' should be replaced with more modest hypotheses based on the information currently available: high consumption of n-3 PUFAs does not protect against the development of CHD if other risk factors are present.

Table 1.7 shows a compilation of clinical studies of the effect of n-3 PUFAs on CVD risk. Burr et al. (1989) reported a 29% reduction in all-cause mortality over a 2-year period in male MI survivors who were advised to increase their oily fish intake (200 to 400 g of oily fish per week), equivalent to 500-800 mg/d of n-3 PUFAs. Subsequently, Singh et al. (1997) reported a 25% reduction in total cardiac events with 1.8 g/d of EPA plus DHA. The pioneer large randomized controlled trial before JELIS to test the efficacy of n-3 PUFAs was the GISSI-Prevention Study. It was found that after 3.5 years of follow-up, there was a 20% reduction in all-cause mortality and a 45% reduction in sudden death in post-MI patients receiving 850-882 mg/d EPA plus DHA (GISSI 2001). An effect of EPA *per se* (1.8 g/day) is suggested in a large study involving more than 18,000 patients with hyperlipidemia, of which 3664 had established CAD; there was a significant reduction in unstable angina, non-fatal coronary events and major coronary events (Yokoyama et al. 2007) (Table 1.7).

Some meta-analyses conclude that n-3 PUFAs reduce overall mortality, mortality caused by myocardial infarction (MI) and sudden cardiac death in patients with CHD by 30% (Bucher et al. 2002). In contrast, Hooper et al. (2006) concluded in a recent meta-analysis, which reviewed 48 randomised controlled trials and 41 cohort studies, that no strong evidence of a reduced risk of: total mortality, CVD events or cancer. This finding however has been debated and caused considerable controversy since the finding of no demonstrable

protective effect of n-3 PUFAs on CVD contrasts with other meta-analyses and the outcomes appear to be influenced by the inclusion of the DART 2 study involving more than 3000 men with angina. In this DART 2 study, an unexpected 26% increase in cardiac deaths in men taking the long chain n-3 PUFAs supplements was reported (Burr et al. 2003) but this finding was not explained by the authors. Among several recent reviews of the relationship between fish or fish consumption and CHD, that by Wang (2006) appears to be the most thorough and comprehensive. Indeed, the authors stated that most studies reported that n-3 PUFAs significantly reduce all-cause mortality, MI, cardiac and sudden death or stroke. Primary prevention of CVD was reported in one randomised controlled trial, in 25 prospective cohort studies and in seven case-controlled studies. In this review, it was concluded that the evidence fish oil beneficial is stronger in secondary-prevention than in primary-prevention setting.

1.2.3.1 Possible Mechanism: Arrhythmias

Cell culture and animal models, observational studies and human intervention trials all suggest that the major effect these fatty acids may protect against is fatal arrhythmias (Burr et al. 1989; Charnock 1994; Nair et al. 1997) rather than being anti-atherothrombotic. The underlying mechanism involved may due to a stabilizing effect on the myocardium itself (Nair et al. 1997; Kris-Etherton et al. 2002; Lee & Lip 2003) and n-3 PUFAs are suggested to alter the electrical properties of the surface membrane of the cell, causing a raising threshold for the generation of an action potential. Consequently, any stimulus that could lead to arrhythmia would have to be larger in order to exceed the increased threshold (McLennan et al. 1992) and therefore these fatty acids exert a protective effect; DHA is recognised as the most potent agent (McLennan et al. 1992; Demaison et al. 2002). Leaf (2007) concluded that even though the evidence that EPA and DHA are able to prevent fatal ventricular arrhythmias in humans is probable, this is not yet totally convincing.

Heart-rate variability and absolute heart rates have been associated with altered risk for sudden cardiac death. This was investigated by Mozaffarian et al. (2006) in a meta-analysis of randomised trials; it was found that n-3 PUFAs decreased heart rate significantly by 2.5 beats/min compared with placebo in trials with baseline heart rate of more than 69

beats/min. In trials with heart rate less than 69 beats/min only a small effect was seen. Furthermore, fish oil reduced heart rate by 2.5 beats/min in trials with duration greater than 12 weeks compared with 0.7 beats/min reduction in trials less than 12 weeks. Therefore it is concluded that based on randomised, controlled trials in humans, fish oil reduces heart rate, particularly in those with higher baseline heart rates and longer treatment duration.

The intake of 1 g/day of EPA and DHA for CVD prevention, treatment after a MI, prevention of sudden death, and as a secondary prevention of CVD is recommended by cardiac societies (von Schacky & Harris 2007). In accordance to this scientific evidence, a new risk factor has been proposed to be considered for sudden cardiac death, the omega-3 index which is measured in red blood cells and presented as a percentage of EPA + DHA of total fatty acids. An index of > 8% is associated with 90% less risk for sudden cardiac death, as compared to an omega-3 index of < 4% and this index has striking similarity to LDL as a risk factor for CAD (von Schacky & Harris 2007).

1.2.4 n-3 PUFAs and Blood Pressure

n-3 PUFAs appear to have a small dose-dependent hypotensive effect (Howe 1997), and the ability to reduce the blood pressure appears to be dependent on the initial extent of hypertension (Howe 1997). It has been reported that consuming a high dose of 5.6 g n-3 PUFAs daily, a reduction in blood pressure of 3.4/2.0 mm Hg in hypertensive subjects was observed (Morris et al. 1993). Moreover, Geleijne et al. (2002), in a meta-analysis of 36 randomised trials showed that blood pressure was reduced by -2.1/1.6 mm Hg following n-3 PUFAs supplementation and the effects were greater in older (> 45 years; -3.5/-2.4) and hypertensive (≥ 140/90; -4.0/-2.5) subjects. On the other hand, in 11 trials involving normotensive subjects, Appel et al (1993) estimated a reduction by -1.0/-0.5 mm Hg and by -5.5/-3.5 mm Hg in untreated hypertensives (6 trials) following an average intake > 3 g/day n-3 PUFAs.

It was still unclear until recently whether EPA and DHA are similarly important in blood pressure lowering. Mori et al. (1999) demonstrated that high doses (4 g/day) of DHA, but not EPA, significantly reduced blood pressure (-5.8/-3.3 mm Hg) in mildly

hypercholesterolemic patients. Conversely, in a similar study design, neither EPA nor DHA was able to demonstrate this effect (Woodman et al. 2002). Further research is needed to determine the effect of low doses of n-3 PUFAs which are achievable by normal dietary intake, on blood pressure since those studies reported blood pressure lowering tended to use high doses in region > 4 g/day.

1.2.4 n-3 PUFAs and Lipid Profile

The hypotriglyceridemic effects of n-3 PUFAs from fish oils are well established. This effect is reported to be dose-dependent. Harris (1997) in a comprehensive review of human studies reported that approximately 4 g/day of n-3 PUFAs from fish oil decreased serum triglyceride levels by 25-30% with an accompanying increase in LDL-c of 5%-10% and in HDL-c of 1%-3% In terms of different type of n-3 PUFAs, EPA has been reported to have little impact on total cholesterol, LDL-c and HDL-c levels. One study, however, revealed a small but significant 2.5% decrease in total cholesterol with EPA (Grimsgaard et al. 1997). Despite HDL-c not being changed by EPA supplementation, HDL_3-c decreased and HDL_2-c increased in dyslipidaemic (Mori et al. 2000) and HDL_3-c decreased in type 2 diabetic (Woodman et al. 2002) patients. Supplementation with DHA, on the other hand, reduced triglycerides in most controlled studies by approximately 17-33% (Woodman et al. 2002; Nestel et al. 2002; Mori et al. 2000; Grimsgaard et al. 1997; Agren et al. 1996; Conquer et al. 1996) with the largest decrease occurring in subjects with the highest baseline triglycerides (1.6 mmol/L) (Mori et al. 2000). Moreover, unchanged triglycerides were observed in studies using lower purity DHA (Conquer et al. 1998; Hamazaki et al. 1996), a relatively low dose (< 2 g/day) (Conquer et al. 1998) and in normotriglyceridemic subjects.

1.2.5 n-3 PUFAs and Inflammation

Inflammation is part of the complex immunological response of host tissues to injury or infection and is characterized by pain, swelling, redness and heat. This reaction is a normal and protective mechanism in order to remove the harmful stimuli and also to initiate the healing process for the host. However, when inflammation occurs excessively or in

inappropriate manner, it can lead to the development of acute and chronic diseases characterised by the production of inflammatory cytokines, arachidonic acid (ARA)-derived eicosanoids, other inflammatory agents such as reactive oxygen species (ROS) and expression of adhesion molecules.

The relationship between PUFAs and inflammation is based on the fact that a high intake of LC n-3 PUFAs results in decreased production of cytokines, inflammatory eicosanoids, ROS and also adhesion molecule expression (Calder 2006). The eicosanoids, which are among the most potent mediators and regulators of inflammation, are derived from 20-carbon PUFAs. Since inflammatory cells typically contain a high proportion of n-6 PUFA ARA and low proportions of n-3 PUFAs, particularly EPA and DHA, ARA is the main substrate for eicosanoid synthesis (Calder 2006). For example, PGs (prostaglandins), TXs (thromboxanes), LTs (leukotrienes), HETEs (hydroxyeicosatetraenoic acid) and other derivatives as summarized in Fig 1.7, are among ARA-derived eicosanoids which are generated from free ARA derived from cell membrane phosphatidylcholine.

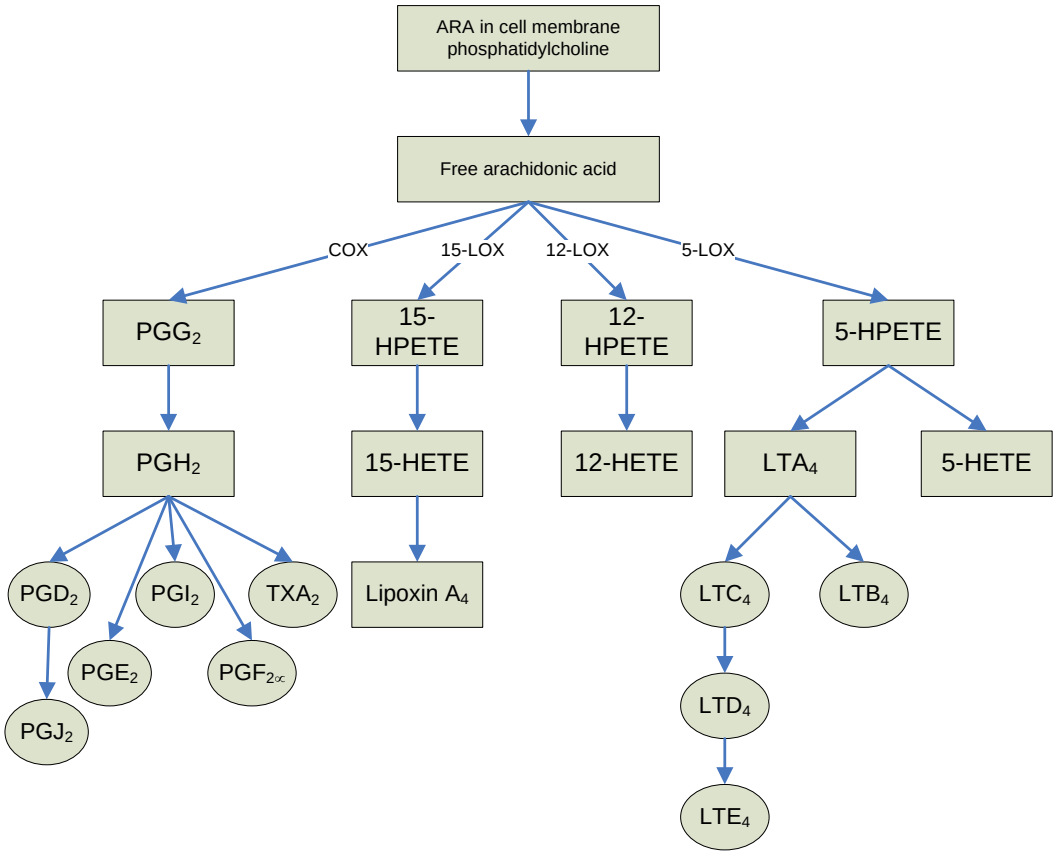


FIGURE1.7: Synthesis of Eicosanoids from ARA

It has been reported that eicosanoids are responsible for modulating the intensity and duration of inflammatory responses (Lewis et al. 1990; Tilley et al. 2001), with those from ARA most commonly have pro-inflammatory actions. Bagga et al. (2003) have demonstrated the effects of PGE_2 as pro-inflammatory mediators whereby PGE_2 stimulates cyclooxygenase 2 (COX-2) which is induced in inflammatory conditions, in fibroblasts cells and thus up-regulates its own production and also induces the production of IL-6 by macrophages. Indeed, increased rates of ARA-derived eicosanoids production are found under inflammatory states in blood and tissues from patients with acute and chronic inflammatory conditions. On the other hand, PGE_2 suppresses 5-lipoxygenase (5-LOX) and thus decreases the production of the 4-series LTs (Levy et al. 2001) and stimulates 15-LOX and thus leads to the formation of lipoxins (Levy et al. 2001; Vachier et al. 2002), which have been shown to have anti-inflammatory effects (Gewirtz et al. 2002; Serhan et al. 2003). Therefore, PGEs has both pro- and anti-inflammatory actions.

In order to suppress the generation of pro-inflammatory mediators from ARA-derived eicosanoids, increased n-3 PUFAs intake should be considered. Supplementation with fish oil has been shown to result in decreased production of PGE_2 (Endres et al. 1989; Caughey et al. 1996; Meydani et al. 1991; Trebble et al. 2003), TXB_2 (Caughey et al. 1996), LTB_4 (Lee et al. 1985; Sperling et al. 1993), 5-HETE (Lee et al. 1985; Sperling et al. 1993) and LTE_4 (von Shacky et al. 1993) by inflammatory cells. Studies showed that 6 g DHA/day resulted in decreased production of PGE_2 and LTB_4 by 60% and 75%, respectively in endotoxin-stimulated mononuclear cells (Kelley et al. 1998). Furthermore, DHA-derived mediators, known as D-series resolvins, docosatrienes and neuroprotectins, produced by COX-2 have been identified and these have anti-inflammatory actions (Hing et al. 2003; Marcheselli et al. 2003; Mukherjee et al. 2004).

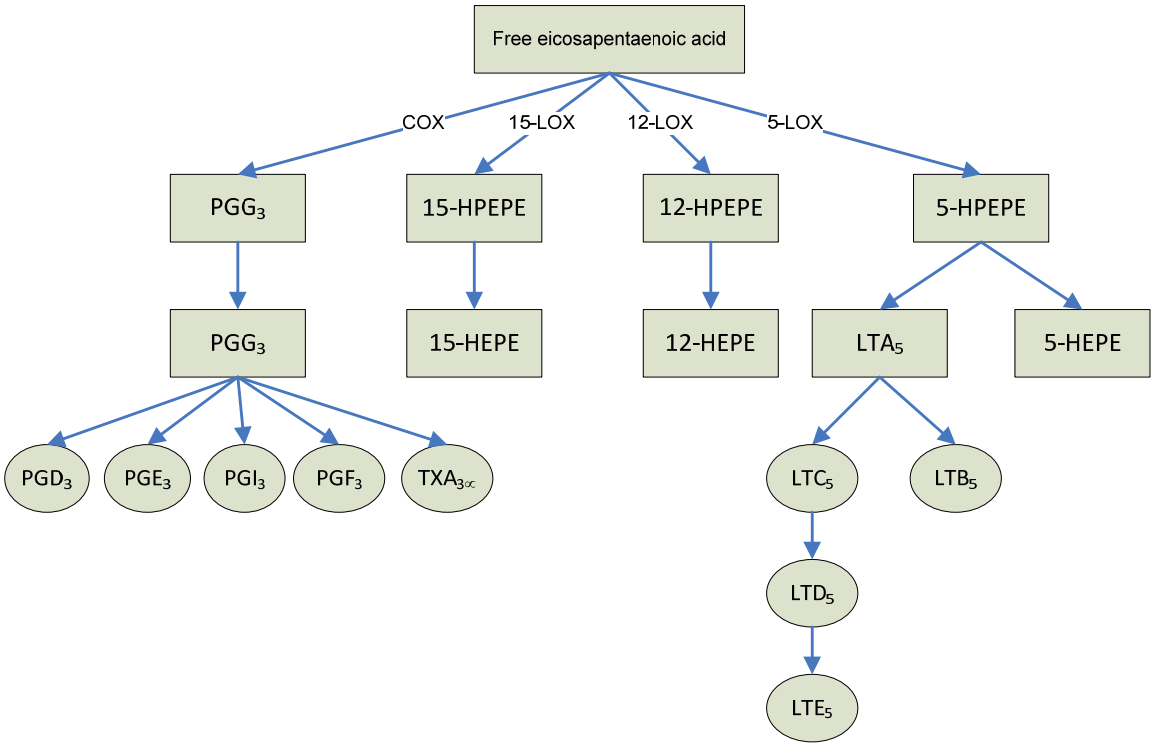


FIGURE 1.8: EPA-derived Eicosanoid Synthesis

On the other hand, Figure 1.8 shows EPA can also acts as substrate for both COX and 5-LOX which leads to increases in EPA-derived eicosanoids, which have a slightly different structure from those generated from ARA (Figure 1.7). Accordingly, increased production of the less inflammatory LTB_5, LTE_5 and 5-hydroxyeicosapentaenoic acids (5-HEPE) by inflammatory cells with fish oil supplementation have been shown (Lee et al. 1985; Sperling et al. 1993; von Shacky et al. 1993), although production of PGE_3 has been more difficult to demonstrate (Hawkes et al.1991). The EPA-derived eicosanoids are believed to be less potent than those generated from ARA (Goldman et al. 1983; Lee et al. 1984). Resolvins (resolution phase interaction products) are introduced for the bioactive compounds that demonstrate potent anti-inflammatory and immunoregulatory actions. The compounds derived from EPA carrying potent biological actions are designated as E series, given their EPA precursor, and denoted as Resolvins of the E series (Resolvin E1 or RvE1), and those biosynthesized from the precursor DHA are Resolvins of the D series (Resolvin D1 or RvD1) (Serhan et al. 2000; Serhan et al. 2002; Hong et al. 2003). Thus, not only decreased production of ARA-derived mediators following fish oil supplementation is important in understanding the modulation of immune system by EPA and DHA, but also

the production of D- and E-series resolvins have led to the idea that fish oil is anti-inflammatory (Fig 1.9).

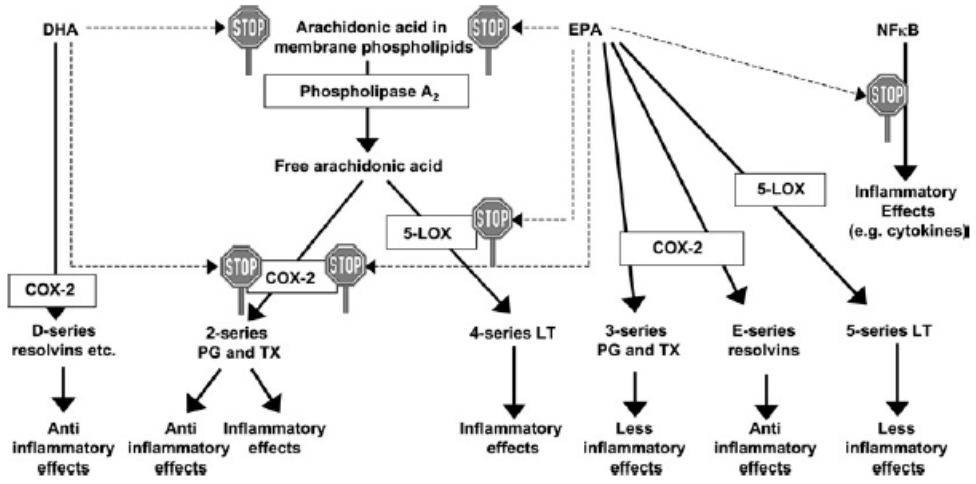


FIGURE 1.9: Mechanism of the Anti-inflammatory Action of DHA and EPA. (Source: Calder 2005)

Of note, the anti-inflammatory effects of n-3 PUFAs might also be independent of eicosanoids production. For example, studies have been shown that n-3 PUFAs from fish oil result in decreased leukocyte chemotaxis, decreased production of reactive oxygen species and proinflammatory cytokines and decreased adhesion molecule expression (See Section 1.3.7). However, although some data suggest a beneficial effect of n-3 PUFAs on endothelial function (Schmidt et al. 2005; Kris-Etherton et al. 2002; Seierstad et al. 2005), there are few indications that n-3 PUFAs reduce CRP levels (Madsen et al. 2003; Geelen et al. 2004). The association between n-3 PUFAs and inflammatory markers remains unclear. For instance, some studies reported no effect of high doses of EPA and DHA (4 g/d) on CRP, IL-6 and TNF-α (Mori et al. 2003), whereas other studies have found that intake of ALA about five times higher than average reduced levels of inflammatory marker such as sICAM-1 and sE-selectin (Zhou et al. 2004). Moreover, Hjerkinn et al. (2005) found that relatively high intake of EPA plus DHA reduced serum thrombomodulin and adhesion molecule levels in elderly, hyperlipidemic Norwegian men treated for 3 years.

In vitro studies showed that DHA reduces endothelial expression of VCAM-1, E-selectin, ICAM-1, IL-6 and IL-8 in response to exposure to IL-1, IL-4, tumor necrosis factor or

bacterial endotoxin (De Caterina 2000). Conversely, no inhibitory effects were observed with saturated fatty acids. They also concluded that n-3 PUFAs seem to have the greatest inhibitory effect, with n-6 fatty acids being intermediate, followed by MUFAs. The effect of n-3 PUFAs particularly EPA and DHA on adhesion molecule expression is further discussed in Section 1.3.7.

1.3 Cellular Adhesion Molecules and Atherosclerosis

Cellular adhesion molecules (CAMs) are trans-membrane proteins that mediate cell-cell or cell-matrix interactions. In the early pathogenesis of atherosclerosis, CAMs participate in leukocyte-endothelial interactions and infiltration of monocytes; thus suppression of their expression protects against atherosclerosis (Nageh et al. 1997). A variety of these, named endothelial-leukocyte adhesion molecules, participate in the process of selective attachment of leukocytes to the blood vessel wall, which is involved in tissue inflammation (Gonzalez-Amaro et al. 1998), atherogenesis (Cotran et al. 1998) and immune function (Wang et al. 1998).

1.3.1 Adhesion Molecules of the Selectin Family

The selectin family includes: L-selectin (also known as leukocyte adhesion molecule-1, (LAM-1) and CD62L), E-selectin (also known as endothelial leukocyte adhesion molecule-1 (ELAM-1) and CD62E) and P-selectin (also known as granule membrane protein 140 (GMP-140) and CD62P). The expression of L-selectin is limited to haematopoietic cells, with most leukocytes expressing L-selectin at some stage of differentiation. E-selectin plays a central role in the binding and infiltration of neutrophils and a subset of leukocytes from the bloodstream into sites of inflammation (Kaszubska et al. 1993). Expression of E-selectin is both cell specific since it is only expressed on endothelial cells and also stimulus specific since it is expressed in response to induction by the cytokines interleukin-1 (IL-1), TNF-α, lipopolysaccaride (LPS) and phorbol myristate acetate (PMA) (Bevilacqua et al. 1989). P-

selectin, on the other hand, is constitutively found in Weibal-Plade bodies of endothelial cells and in alpha granules of platelets (Table 1.8).

The molecular mass of selectins varies between 75-140 kDa and these receptors are composed of single-chain transmembrane glycoproteins with a calcium dependent amino-terminal lectin-binding domain, an epidermal growth factor (EGF)-like domain and varying numbers of domains known as short consensus repeats (SCR). Each selectin has a membrane-spanning region and a short cytoplasmic region of 2, 6 and 9 amino acids for L, E and P- selectin, respectively. The roles of each of these domains, however, remain unclear since monoclonal antibodies (mAB) to the lectin domain block selectin mediated adhesion but a mAb to the SCR also blocks L- and E-selectin mediated adhesion (Hogg & Landis 1993). However, if the EGF domain is deleted, cell adhesion is also abolished (Pigott et al. 1991).

1.3.2 Adhesion Molecules of the Immunoglobulin-like Superfamily (IgSF)

Most of these proteins are located as cell surface molecules with the exception of some intracellular and secreted proteins. The IgSF can be divided into groups based on their functions. For example, the C1-type receptors participate in antigen recognition and include the T cell receptor (CD3), CD4 and CD8 or in complementary binding include major histocompatibility complex (MHC) class 1 and class 2. The C2-type receptors are involved with CD56 and CD58. Five receptors of this subclass have been demonstrated to be involved in leukocyte adhesion: ICAM-1, ICAM-2, VCAM-1, platelet-endothelial cell adhesion molecule (PECAM-1) and the mucosal addressin (MAdCAM-1) (Krieglstein & Granger 2001).

ICAM-1 (also known as CD54) has a core protein of 55 kD with five extracellular Ig-like domains. The main ligands for ICAM-1 include LFA-1, Mac-1 and CD43 (Carlos & Harlan 1994; Diamond et al. 1991). The main binding site for LFA-1 is located in the NH_2-terminal first domain of ICAM-1, meanwhile the second binding site for Mac-1 is located in the third domain. In all, eight possible sites for N-linked glycosylation have been found in the Ig-like

domains. ICAM-1 is expressed by epithelial cells, fibroblasts, leukocytes and endothelial cells.

VCAM-1 has a core protein of 81 kDa with seven extracellular Ig-like domains expressed in the dominant form and six in the other sites. Ligand binding sites have been located in the NH_2-terminal first and fourth domains of seven Ig-like domains (7D) VCAM-1. The major ligands are VLA-4 (integrin α_4/β_1) (Elices et al. 1990) and α_4/β_7 (Ruegg et al. 1992). VCAM-1 is expressed by endothelial cells, tissue macrophages, dendritic cells, bone marrow fibroblasts and myoblasts.

1.3.3 The role of Adhesion Molecules in Normal Physiology

Adhesion molecules mediate the processes of cell activation, migration, proliferation and differentiation which require direct cellular and cell-matrix interactions (Mousa & Cheresh 1997). This discovery has been a key factor in understanding the underlying mechanisms of inflammation and immune system activity. Even though CAMs are expressed mainly on the vascular endothelium, their density is greatest in post capillary venules. Larson and Springer (1990) reported that engagement of these receptors to ligands on leukocytes results in intracellular signals which can affect the phenotype and gene expression, movement or activation of a cell. Consequently, this process may be partly mediated through intracellular transcripts of several genes associated with transcriptional control, such as IκBα and activator protein-1 (AP-1) which are altered after monocyte adhesion to an endothelial cell or by stimulation with cytokines such as TNF-α or IL-1. Activation of the cell leads to phosphorylation and proteolysis of IκBα, the cytoplasmic inhibitor of nuclear factor-κB (NF-κB). Ultimately, this activation allows NF-κB to be translocated to the nucleus where it binds to elements in gene promoters. Moreover, it was found that NF-κB and AP-1 can be modulated by redox-sensitive signal tranducers such as PKC, α-tocopherol and glutathione (Mondal et al. 1995; Lum & Roebuck 2001).

Members of the selectin family have been implicated in mediating adhesion to endothelial cells while E- and P-selectin participate in coagulation (Springer 1990). When selectins mediate the initial weak interactions between the vascular endothelium and leukocytes as

shown in Figure 1.10, this process is known as rolling since the leukocytes decelerate from the blood stream by rolling along the endothelium. Rolling causes other CAMs to be activated by chemokines which are produced by cells in the tissue and attached to the endothelium and subsequently leading to firm adhesion and transmigration. Anti-L-selectin mAb decreases the adherence of monocytes and neutrophils to cytokine-stimulated endothelium and reduces recruitment of leukocytes *in vivo* (Abassi et al. 1991). Anti-E-selectin mAb blocked neutrophil accumulation in the inflamed peritoneum and lung of normal animals (Mulligan et al. 1991), anti-P-selectin mAb decreased leukocyte rolling *in vivo*, whereas P-selectin activates neutrophil adhesion to endothelial cells. In accordance with this finding, spontaneous rolling was almost absent in mesenteric venules of P-selectin deficient mice (Carlos & Harlan 1994) and leukocyte rolling was found to be exclusively mediated by P-selectin in colonic venules (Wan et al. 2002). Therefore, it is concluded that differences in expression and utilization of CAMs in different parts of the body are depending on the ligands and regulators of L-, P- and E-selectin, such as listed in Table 1.8.

TABLE 1.8: Examples of Adhesion Molecule Ligands, Distributions and Regulators of Expression

Adhesion molecule	Ligands	Cellular distribution	Regulation and expression
Selectin family: E-selectin (ELAM-1, LECAM-2)	Sialyl-Lewis X and A antigen, CLA	Endothelium	Upregulated by inflammatory diseases, cytokines (TNF-α/LPS/IL-1β), thrombin, phorbol esters.
L-selectin (LAM-1, LECAM-1)	CD34, MadCAM-1, GlyCAM-1	Haematopoietic cells	Upregulated by phorbol esters, cytokines or chemoattractants. Down regulated by shedding after activation of Mac-1 receptor.
P-selectin (LECAM-3)	Sialyl-Lewis X and A antigen	Platelets, EC, megakaryocytes	Upregulated by thrombin, histamine, PMA, LTC_4, bradykinin or peroxides.
Integrin family: β1 VLA-1 α1β1	Laminin, collagen	T cells, monocytes, neuronal cells, smooth muscle cells B & T cells, platelets, fibroblasts, EC & cell lines.	Upregulated by retinoic acid causing differentiation of SY5Y and IMR32 neuroblastoma cell lines.
VLA-2 α2β1	Laminin, collagen		Upregulated by mitogen or antigen on lymphocytes, serum on fibroblasts, TGFβ.
VLA-3 α3Aβ1,α3Bβ1	Laminin, collagen, fibronectin		Upregulated by attachment of some cells to the extracelular matrix. TGFβ can down regulate in some cell types.
VLA-4 α4β1	Fibronectin, VCAM-1	Broad, not lymphoid cell lines	Expression on T cells slowly increased after activation.
VLA-5 α5β1	Fibronectin	Broad leukocytes, fibroblasts, platelets, muscle cells platelets, monocytes, T cells, thymocytes, EC, eosinophils	Constitutively expressed and regulated during B cell development.
VLA-6 α6β1	Laminin		Constitutively expressed. Activity depends on Mg^{2+} dependent activation, not quantitative changes triggered by TCR/CD3 complex.
β2 LFA-1 αLβ2	ICAM-1, ICAM-2, ICAM-3	Leukocytes	Upregulated by inflammatory stimuli e.g. fMLP, C5a, leukotreine B_4. Increased expression in maturation and differentiation of monocytes.
Mac-1 αMβ2	ICAM-1, C3bi, factor X, LPS, fibrinogen	Monocytes, macrophages, granulocytes, NK cells	Upregulated by inflammatory mediators, phorbol esters on B cells, on activation of B cells, differentiation of monocytes to macrophages.
p150,95 αXβ2	C3bi, fibrinogen	Macrophages, granulocytes, activated B lymphocytes.	Constitutively expressed. Can only bind fibrinogen when platelet unstimulated.
β3 GpIIb/IIIa β3 α11bβ3,	Fibrinogen, factor VIII, thrombospondin, vitronectin	EC, osteoclasts, platelets, some B/ macrophages/ tumour cells	Activated by thrombin, collagen, ADP.
CD51/CD61 αvβ3	Vitronectin, factor VIII, fibrinogen, thrombospondin, collagen	Epithelial.	Upregulated by phorbol esters.
β4 α6Aβ4/α6Bβ4, α4β7	Laminin	Activated B & T cells, intraepithelial lymphocytes.	Constitutively expressed. Laminin binding requires activation.
Ig-like superfamily ICAM-1 (CD54)	LFA-1, Mac-1	Broad, most prominently expressed on vascular endothelium EC, subpopulations of lymphocytes, monocytes,	Upregulated by inflammatory diseases, cytokines (IFNγ, IL-1β, TNF-α), LPS, phorbol esters, activated platelets and decreased shear stress *in vitro*.
ICAM-2,	LFA-1		Constitutively expressed on EC, T

ICAM-3		dendritic cells, fibroblasts, myoblasts, myotubes platelets, EC, monocytes, granulocytes and some T cells	cells
VCAM-1	VLA-4, α4β7		Upregulated by inflammatory diseases, cytokines (IFNγ, IL-1β, TNF-α), LPS.
PECAM-1 (CD31)	Unknown		Developmentally regulated during myogenesis. Constitutively expressed. Down regulated on active granulocytes with fMLP treatment and active T cells.

Abbreviations: ICAM-, intercellular adhesion molecule-; C3bi, inactivated complement component C3; VCAM-, vascular cell adhesion molecule-; VLA, very late antigen; fMLP, fMet-Leu-Phe; PECAM-, platelet endothelial cell adhesion molecule-; IL-, interleukin-1; TNF-, tumour necrosis factor-; IFN-, interferon-EC, endothelial cell; TGF- transforming growth factor; LPS, lipopolysaccaride; NK, natural killer; MadCAM, mucosal addressin cell adhesion molecule; TCR, Transfer-Cluster Restricted; CD, cluster differentiation.

In the adhesion cascade (Fig 1.10), integrins mediate firm adhesion of leukocytes to the endothelium and subsequently assist in transmigration. Chin et al. (1992) reported that integrins are first expressed during differentiation of hematopoietic stem cells and are involved in lymphocyte trafficking to peripheral lymph nodes and Peyer's Patches.

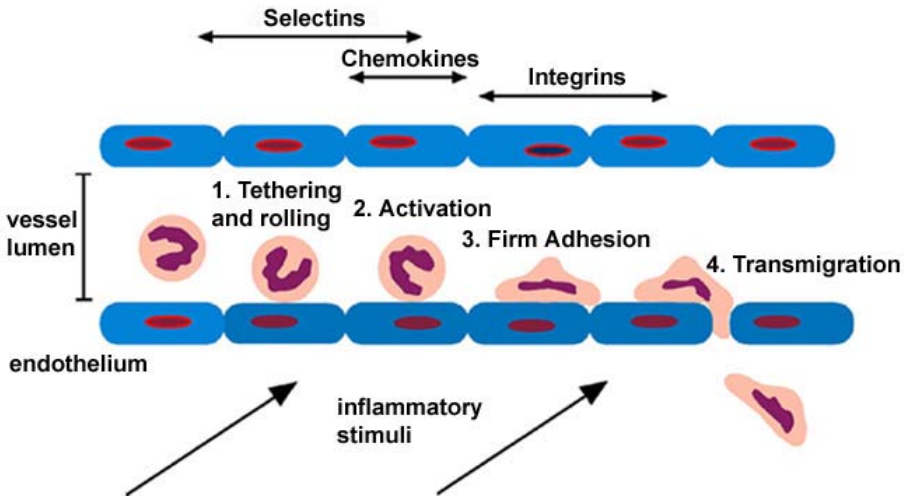


FIGURE 1.10: The Adhesion Molecules Cascade
This figure shows the process of leukocyte adhesion cascade beginning with 1) capturing and subsequently rolling of leukocytes to the blood vessel by selectins, 2) chemotactic factors such as chemokines on the endothelial cell surface leads to the activation of leukocytes, 3) firm adhesion of the leukocytes at endothelial cells before transmigration, 4) finally leukocytes migrate through endothelial cells into the underlying tissue. (Source: Wild 2008)

Beta-2 integrins play a critical role in inflammation, mediating leukocyte adhesion, migration and diapedesis. LFA-1 is important in lymphocyte adhesion to CNS derived endothelial cells (Greenwood et al. 1995) and mediates a wide range of antigen-dependent

and independent reactions. Moreover, LFA-1 may be required for thymic development of lymphocytes whereby LFA-1/ICAM-1 interactions were revealed to be important in differentiation of $CD4^+8^+$ cells from $CD4^-8^-$ cells (Fine & Kruisbeek 1991).

The IgSF is also important in cell activation, differentiation and cell-cell interactions. Anti-ICAM-1 mAb partially decreased the recruitment and adhesion of neutrophils in phorbol ester-induced inflammation (Barton et al. 1989) and it was found that ICAM-1 deficiency led to marked granulocytosis and impaired neutrophil migration and subsequently caused in utero fatality due to the developmental of heart abnormalities in mice (Albelda et al. 1994). Carlos and Harlan (1992) reported that VCAM-1 binds mononuclear cells, but not neutrophils. PECAM-1 is vital in regulating diapedesis and further transmigration via the basal lamina, whereby blocking studies demonstrated that cells remained bound to endothelial cells over cell junctions (Muller & Randolph 1999). In this process, the known regulatory mechanisms are again listed in Table 1.8.

Referring to Table 1.8, of relevance to this discussion, it is clear that ICAM-1, VCAM-1 and E-selectin are all upregulated by inflammatory mediators. NF-κB binding sites have been identified in the promoter region genes for each of these and AP-1 binding sites in the genes for E-selectin and ICAM-1 (Connor & Connor 1997).

1.3.4 The Role of Adhesion Molecules in Pathological Conditions

From a clinical point of view, a deficiency state is needed to confirm the importance or role of these molecules. In this case, leukocyte adhesion deficiency-1 (LAD-1), a lack of β_2 integrins caused by a heterogenous mutation in the CD18 subunits, leads to marked neutrophilia and inability of neutrophils and monocytes to migrate across the endothelial cells. However, it was found that lymphocytes, eosinophils and plasma cells are still able to migrate, and so cell-mediated immunity remains intact. In LAD-2, a congenital defect in endogenous fucose metabolism that leads to an inability to synthesis fucosylated carbohydrate molecules and a crippled selectin system, leukocytes show marked defects in rolling and adherence (Albelda et al. 1994).

Adhesion molecules play an important role in protozoal, fungal, bacterial and viral infection. *Plasmodium falciparum*, for example, causes infected cells to sequester in deep tissue by binding to endothelial cells and subsequently leads to cerebral malaria which is mediated by CD36, ICAM-1 and VCAM-1. *Mycobacterium tuberculosis* binds to CR3 and CR4 receptor, whereby CR3 binding reduces IL-2 production and inhibits the macrophage response and in the end leads to phagocytosis. Viruses often bind to CAMs as well, for example, rhinoviruses (91 serotypes) bind to ICAM-1 and HIV-1 binds to CD4 and IgSF members. Kerr (1999) comprehensively reviewed the role of CAMs in infection.

In chronic inflammatory conditions such as rheumatoid arthritis, E-selectin, VCAM-1 and ICAM-1 are expressed on the microvasculature and synovial lining cells. PECAM-1 is expressed on lining cells and macrophages (Cronstein 1994) whereas integrins, on the other hand, mediate the adhesion of lymphocytes to synovial endothelial cells (Haskard 1995). Graft rejection is another inflammatory state where CAMs play a central role, whereby VCAM-1 and ICAM-1 are found to be upregulated in rejecting renal allografts (Eriksson et al. 2001). In allergic conditions, VLA-1/VCAM-1 and LFA-1/ICAM-1 interactions contribute to eosinophil migration (Albelda et al. 1994). Mononuclear cells from asthmatics show upregulation of LFA-1 and VLA-1 (Tomita et al. 1997). In atopic dermatitis and other types of eczema, ICAM-1 and VCAM-1 are increased in *in vitro* studies (Montefort et al. 1993). Upregulations of ICAM-1, VCAM-1 and E-selectin are found on the small vessels around colon neoplasms. The role of these molecules in metastatis and neovascularisation is supported by the elevation of E-selectin expression around metastatic sites of the endothelium.

1.3.5 The Roles of Adhesion Molecule in Pathogenesis of Atherosclerosis

Leukocyte binding to the endothelium is of fundamental importance in many acute and chronic inflammatory disorders (Libby 2002) and this leukocyte binding has been studied extensively *in vitro* to obtain an understanding of the mechanism(s) involved. Activation of leukocytes, endothelial cells, or both results in increased adhesion of polymorphonuclear leukocytes, monocytes or lymphocytes to the endothelium. It has been found that several protein families with different functions trigger signals for leukocytes. These include 1) the

selectin family of adhesion molecules which act to recognize carbohydrate analogues (sialyl Lewis^x ligands) (Lasky 1993; Rosen 1993); 2) chemoattractants, some of which (classical chemoattractants such as N-formyl peptides, complement components, leukotriene B_4 and platelet-activating factor) act mainly on neutrophils, eosinophils, basophils and monocytes; 3) the immunoglobulin superfamily members on the endothelium (intercellular adhesion molecule 1 (ICAM-1), ICAM-2, ICAM-3 and vascular adhesion molecule 1 (VCAM-1)) which recognize the integrin ligands on the leukocyte surface in a model first established with ICAM-1 binding to leukocyte function associated antigen (LFA-1)) (Springer 1990).

In the pathogenesis of atherosclerosis, increased E- and P-selectin, ICAM-1 and VCAM-1 expression is observed. Moreover, it was found that mice deficient in these adhesion molecules have fewer fatty streaks even after consuming a high fat diet as compared to normal mice (Nageh et al. 1997). E-selectin and ICAM-1 are increased in the fatty streaks, but in fibrous plaques ICAM-1 and E-selectin are less well expressed which might be due to lack of macrophages producing IL-1 and TNF-α. In aortic hypercholesterolemic mice, Nakashima et al. (1998) reported that there is an increased VCAM-1 expression at the atherosclerotic lesion preceeding fatty streak formation. Of interest, however, studies in atherosclerosis show that VCAM-1 can mediate rolling, firm adhesion and migration of monocytes and VLA-4/VCAM-1 interactions without chemoattractants. In a model of cat myocardial ischemia and reperfusion, L- and P-selectin inhibitors also reduced endothelial dysfunction, decreased infarct size and improved myocardial function (Lefer 2000).

1.3.6 Soluble Adhesion Molecules

The soluble forms of ICAM-1, VCAM-1 and the selectins are all present in the blood with similar structure to extracellular transmembrane domain of their membrane-bound counterparts. Soluble adhesion molecule concentration in the blood is believed to reflect the expression of these molecules at the cell surface and theoretically, shedding of the endothelial surface may also increase the concentration of the soluble forms. Thus, these molecules may be useful as markers of inflammation. Since ICAM-1 is expressed widely on different types of cells, the origin of soluble ICAM-1 (sICAM-1) is difficult to identify *in vivo*. Mononuclear cells and endothelial cells release soluble ICAM-1 (as sICAM-1) as

demonstrated in *in vitro* studies and sVCAM-1 and sE-selectin are shed by activated endothelial cells (Fabrega et al. 2000). P-selectin is produced from an alternatively spliced mRNA found in endothelial cells and megakaryocytes. Apparently, the source of sP-selectin is from activated platelets, whereas sL-selectin is shed rapidly after leukocyte activation (Haught et al. 1996). It has been reported that TNF-α, IL-1 and IFN-γ stimulate the shedding of adhesion molecules from endothelial cells. These molecules act to regulate the immune response by providing alternative ligands for leukocyte adhesion. sVCAM-1 can partly inhibit *in vitro* monocyte adhesion to activated human umbilical vein endothelial cells (Haught et al. (1996). sL-selectin retains functional activity and in high concentration, this molecule can suppress leukocyte adhesion to cytokine-stimulated endothelial cells (Haught et al. 1996).

1.3.7 Soluble Adhesion Molecules in Pathological Conditions

In general, soluble adhesion molecule concentrations tend to be higher in men compared with women (Abe et al. 1998; Eschen et al. 2004). In the elderly, Miles et al. (2001) found that sVCAM-1 and sICAM-1 are elevated in older compared with young subjects. sVCAM-1 and sE-selectin are increased in all types of acute infections, although sICAM-1 is not affected in viral infections (Kulander et al. 2001). In chronic inflammatory diseases, for example rheumatoid arthritis, sICAM-1, sVCAM-1 and sE-selectin can be raised (Wallberg-Jonsson et al. 2002). sVCAM-1 and sICAM-1 are elevated in renal transplants and liver graft rejection, but not sE-selectin (Eriksson et al. 2001; Adams et al. 1993). sICAM-1, sVCAM-1 and sE-selectin can also be raised in metabolic conditions, such as in diabetes (Bagg et al. 2001), even though patients are not obese and non-hypertensive (Chen et al. 1999) and in individuals with high BMI (Chen et al. 1999; Abe et al. 1998). All these three soluble forms are elevated in certain types of cancer including lymphoma, gastric, breast and hepatocellular (Gruss et al. 1993; Velikova et al. 1997; Wittig et al. 1996; O'Hanlon et al. 2002) and sICAM-1 and sVCAM-1 are also found to be raised in colorectal cancer (Velikova et al. 1998).

In one study, an association has been noted between atherosclerosis and increased sICAM-1, sVCAM-1 and sE-selectin (Hwang et al. 1997). Even though all three can be raised in

coronary artery disease (CHD) (Lang et al. 1994), there is debate about whether their concentrations can provide predictive information in CHD outcomes in comparison with more established risk factors (Malik et al. 2001). One study found sICAM-1 to be positively correlated with age, smoking, hypertension, diabetes, serum triglycerides, fibrinogen, homocysteine and tissue-type plasminogen activating factor which are all well established risk factors (Rohde et al. 1998). Interestingly, regular exercise was found to be beneficial whereby levels of sICAM-1 are more likely to be lowered and estrogen replacement therapy results in decreased serum levels of sICAM-1, sVCAM-1 and sE-selectin (Lang et al. 1994), demonstrating a possible link to hormonal regulation. The role of sP- and sL-selectin in pathological conditions also has been studied. Generally, sP-selectin is elevated in thrombotic consumptive platelet disorder and unstable CHD, whereas sL-selectin is raised in acute inflammatory processes like sepsis, but is down-regulated in chronic inflammatory states like CHD (Haught et al. 1996).

1.3.8 The Effects of Unsaturated Fatty Acids on Adhesion Molecule Expression

Dietary components such as micronutrients and fatty acids can influence vascular endothelial cell function (Cuevas & Germain 2004). Indeed, in recent years, studies have led to new thinking about the relationship between fatty acids, particularly unsaturated forms in modulating the immune system and inflammatory processes. This thinking has wide implications for pathogenesis of human disease, immunonutrition and therapeutics, with special reference to inflammation and atherosclerosis. Fatty acids have been shown to affect inflammatory diseases (Calder 2006), and n-3 fatty acids in particular, are receiving increasing attention as potential anti-atherogenic and anti-inflammatory agents. There have been several investigations of the effects of fatty acids on adhesion molecule expression on endothelial and inflammatory cells and on sCAM concentrations.

In general, *in vitro* studies with stimulated endothelial cells (Table 1.9) or short term human studies (Table 1.11) (Turpenin et al. 1998; Huang et al. 1997) showed that saturated fatty acids have no effect on the expression of adhesion molecules even though these fatty acids are commonly associated with a higher incidence of cardiovascular disease (Nordoy et al.

1990; Nordoy 1999; Kromhout et al. 2000). However, Bemelmens et al. (2003) reported that decreasing saturated fat intake over two years decreases the concentration of sICAM-1.

1.3.8.1 The Effect of Monounsaturated Fatty Acids on Adhesion Molecule Expression

The monounsaturated fatty acid, oleic acid, has been demonstrated to inhibit ICAM-1 and VCAM-1 expression by human saphenous vein endothelial cells (HSaVECs) *in vitro* (De Caterina & Libby 1996; Carluccio et al. 1999). In contrast, Shaw et al. (2007) reported that oleic acid caused up-regulation of VCAM-1, but had no effect on E-selectin expression. Apart from oleic acid, De Caterina et al. (1998) showed that palmitoleic acid (*cis*16:1n-7) C16:1Δ7cis, elaidic acid (*trans*18:1n-9) C18:1Δ9trans and ricinoleic acid (*cis*18:1n-9-12-OH) C18:1Δ9cis-12-OH, also suppress expression of VCAM-1. De Caterina et al. (1998) concluded that the configuration (*cis*/*trans*) has no effect on inhibitory potency since oleic acid (*cis*18:1n-9) and its *trans* stereoisomer elaidic acid (*trans*18:1n-9) are of similar potency. Increased consumption of oleic acid in the diet decreased ICAM-1 expression on peripheral blood mononuclear cells by 20%, even though this effect was not significant (Yaqoob et al. 1998). On the other hand, some studies report no effect of oleic acid on ICAM-1, VCAM-1 or E-selectin expression in human umbilical vein endothelial cells (HUVECs) (Holthe et al. 2005) or on the soluble forms in plasma (Turpeinen et al. 1998; Eschen et al. 2004). To date, there is no beneficial effect of oleic acid in human studies regarding the inhibition of adhesion molecule expression, even though *in vitro* studies revealed that oleic acid exerts a similar effect to EPA and DHA.

1.3.8.2 The Effect of n-6 PUFAs on Adhesion Molecule Expression

Toborek et al. (2002) found that linoleic acid enhanced messenger RNA levels of VCAM-1 and ICAM-1 in HUVECs whereas Chen et al. (2003) reported an increase of ICAM-1 in human retinal vascular endothelial (hRVE) cells, but not in HUVECs. In another study with HUVECs, no change in the expression of these adhesion molecules was reported with linoleic acid after exposure to LPS (Holthe et al. 2005). For arachidonic acid, one *in vitro* study reported reduced ICAM-1, VCAM-1 and E-selectin in HUVECs (Huang et al. 1997),

whereas no effect was observed in other studies with either HUVECs (Sethi et al. 1996) or HSaVECs (De Caterina & Libby 1996; De Caterina et al. 1998). However, Shaw et al. (2007) reported that ARA can produce a neutral or down-regulatory effect on VCAM-1 and E-selectin in HUVECs. Their findings partly agree with Stuhlmeier et al. (1997) who reported reduced E-selectin expression with ARA in porcine AOEs and HUVECs. Arachidonic acid increased ICAM-1 expression in hRVE cells, but not in HUVECs (Chen et al. 2003) and in cultured human keratinocytes (Lu et al. 1995).

n-6 PUFAs such as linoleic acid, γ-linolenic acid and arachidonic acid, generally, have no effect on adhesion molecule expression *in vivo* (Cazzola et al. 2006; Thies et al. 2001; Yaaqob et al. 2000).

Thus the literature on n-6 PUFAs and adhesion molecules is rather contradictory. It seems important to try to resolve these discrepancies since they may provide information about these fatty acids and cardiovascular risk. Reasons for discrepancies, especially in the *in vitro* studies, may be due to different types of cell being studied and the fatty acid used and its concentration, as well as in methods of study (Goua & Wahle 2007).

1.3.8.3 The Effect of CLA on Adhesion Molecule Expression

Conjugated linoleic acids (CLAs), a group of positional and geometrical isomers of linoleic acid characterised by the presence of conjugated double bonds, can prevent and regress atherogenesis in several animal models of the disease (Kritchevsky et al. 2004; Toomey et al. 2006). The CLAs consist of two main isomers; *cis*-9, *trans*-11 CLA (CLA c9,t11) and *trans*-10, *cis*-12 CLA (CLA t10,c12) which is less common, but of particular interest since it appears to be the more biologically-active isomer in several respects (Choi et al. 2001; Eder et al. 2003). The cytokine-induced expression of ICAM-1, VCAM-1 and E-selectin in HAOECs was not reduced by either CLA isomer (Schleser et al. 2006). In contrast, Sneddon et al. (2006) found that attenuation of cytokine-induced ICAM-1, VCAM-1 and E-selectin gene expression was mirrored in a reduced capacity of co-incubated monocytes to adhere to HUVECs and that the CLA t10,c12 isomer had the greatest efficacy. However, Guoa et al. (2008) reported decreased VCAM-1 and ICAM-1 protein and mRNA

expression by CLA (t10, c12 and c9, t11), and this attenuation was paralled with decreased NF-κB activy.

1.3.8.4 The Effect of LC n-3 PUFA on Adhesion Molecule Expression

As for the LC n-3 PUFAs, many studies have been performed and most research has focused on EPA and DHA since it was hypothesized that n-3 PUFAs may modulate atherogenesis via reducing endothelial activation (De Caterina et al. 1994). Pioneering *in vitro* experiments using HSaVECs activated by cytokines showed that EPA had no effect on endothelial adhesion molecule expression (De Caterina et al. 1994; De Caterina & Libby 1996; De Caterina et al. 1998); however increased ICAM-1 expression on keratinocytes was observed (Lu et al. 1995). It was then demonstrated that EPA, at higher concentrations (> 65 μM, compared with 10-25 μM on HSaVECs), did attenuate the expression of ICAM-1, VCAM-1 and E-selectin by using HUVECs (Collie-Duguid & Wahle 1996; Khalfoun et al. 1996). It is not certain whether EPA at higher concentration may attenuate the expression of adhesion molecules on HSaVECs since there is no study investigating the effect of fatty acid concentrations on this. DHA, on the other hand, was able to decrease ICAM-1, VCAM-1 and E-selectin expression on cytokine-stimulated HSaVECs or HUVECs (Collie-Duguid & Wahle 1996; Khalfoun et al. 1996; De Caterina et al. 1994; De Caterina & Libby 1996; De Caterina et al. 1998; Shaw et al. 2007) and again at higher concentration in HUVECs, and subsequently decrease leukocyte adhesion. Thus, DHA is determined to be the most potent fatty acid inhibitor of adhesion molecules (De Caterina et al. 1995). EPA and DHA separately or in combination decreased HLA-DR/DP and ICAM-1 on stimulated monocytes (Hughes & Pinder 2000; De Caterina et al. 1999; Hughes et al. 1996), and this may be part of the reason for fish oil's beneficial effect seen in rheumatoid arthritis. The combination of EPA and DHA attenuated the expression of the three endothelial adhesion molecules (Nohe et al. 2002; Collie Duguid et al. 1996) and reduced L-selectin and LFA-1 on peripheral blood lymphocytes (Khalfoun et al. 1996). Table 1.9 compiles *in vitro* studies using different type of cells (cell type) and the outcomes observed. Based on *in vitro* studies using HSaVECs, De Caterina et al. (2004) concluded that a minimum of a single double bond is required to inhibit adhesion molecule expression (therefore saturated fatty acids are inactive); the effectiveness does not depend on the chain

length and the degree of effectiveness increases correspondingly with unsaturation (double bond).

In animal studies (Table 1.10), combined EPA and DHA were found to decrease expression of ICAM-1, CD 2/4/8/11a/18, L-selectin and LFA-1 on stimulated lymphocytes (Sanderson & Calder 1998; Sanderson et al. 1995) and resulted in a 50% reduction in Con A-stimulated lymphocyte adhesion to tumour necrosis factor-alpha (TNF-α)-stimulated endothelial cells (Sanderson & Calder 1998). In human studies (Table 1.11), sICAM-1, sVCAM-1, sE-selectin were decreased with fish oil or n-3 PUFA supplementation even though the effects seen were not consistent between studies. Levels of sE-selectin and sVCAM-1 were decreased by ALA, DHA and EPA in one set of studies (Yli-Jama et al. 2002; Thies et al. 2001), but other studies show increased sE-selectin in young men and a decrease in sVCAM-1 and sE-selectin in the elderly (Miles et al. 2001). Fish oil supplementation had no effect on PBMC adhesion molecule expression in human studies (Yaqoob et al. 2000). Interestingly, in patients with CHD, n-3 fatty acids (1.35 of EPA and 1.17g of DHA/ day) increased sE-selectin and sVCAM-1 blood concentrations after 4 weeks, although this was not significant (Johansen et al. 1999). Another study showed no effect of n-3 fatty acids on the expression of monocyte adhesion molecules in people with type 2 diabetes after receiving capsules containing 1.2 g of EPA and 0.8 g of DHA per day for 3 weeks (Sampson et al. 2001). The reason for these discrepancies is unclear, however they may due to several reasons such as dosage, type of fatty acids administered (either EPA, DHA or in combination), gender differences, physiological state (healthy or with different disease conditions), source of supplement (fish oil capsule or consumption of whole fish), age and also the genotypes of the subjects being studied. Interestingly, Grimble et al. (2002) reported that the effect of n-3 PUFAs on cytokine production can vary between individuals, and a small proportion of the population appear to have a genetic polymorphism whereby n-3 PUFAs tend to induce inflammatory cytokine formation, in direct contract to the general responses observed. Therefore, a small proportion of the population may not derive anti-inflammatory benefit from n-3 PUFAs and on the other hand could be at risk of exacerbating any inflammation and its consequences.

1.3.9 The Potential Mechanism for Fatty Acids to Alter Adhesion Molecule Expression

The potential mechanisms for these fatty acids to modulate the expression of adhesion molecules are thought to be related to the type of fatty acids, antioxidant/oxidant modulation, direct actions on gene expression and actions via the eicosanoid pathway. It was concluded that a progressive increase in inhibitory activity was observed for the same chain length but with increased numbers of double bonds (De Caterina et al. 2000). However, Wahle & Rotondo (1999) suggested that double bond theory may be an oversimplification since probucol, a potent antioxidant, decreased the up-regulation of VCAM-1 and E-selectin; ICAM-1 on the other hand was unaffected, indicating the differential redox sensitivity of this adhesion molecule. Regulation of the expression of endothelial pro-atherogenic genes occurs, at least partly through controlling the activity of the nuclear factor-κB (NF-κB) system of transcription factors, secondary to generation of intracellular hydrogen peroxide (De Caterina & Massaro 2005). The NF-κB system of transcriptional factor controls expression of adhesion molecules and of leukocyte-specific chemo-attractants upon cytokine stimulation (De Caterina & Zampoli 2001). Fatty acids can alter the expression of genes coding for adhesion molecules, cytokines and redox enzymes. n-3 PUFAs can reduce the production of TNF-α and IL-1β which up-regulate adhesion molecules, as well as increase glutathione peroxidise activity which is known to inhibit ICAM-1 and VCAM-1 expression and NF-κB activation (Wahle & Rotondo 1999; Hennig & Toborek 2000). For example, DHA causes a reduction in reactive oxygen species, such as hydrogen peroxide, which are considered to be critical mediators of the NF-κB activation pathway. Finally, eicosanoid formation is believed to play a role in adhesion molecule expression, as n-6 PUFAs produce series 2 thromboxanes and prostaglandins and series 4 leukotrienes, which are more potent inflammatory mediators compared with the 3 series thromboxanes and prostaglandins and 5 series leukotrienes produced by n-3 PUFAs. There is, however, some evidence to rule out the effects of eicosanoids since the effects on the endothelium are not attenuated by indomethacin, a blocker of cyclooxygenase (and therefore prostaglandins production). MUFA, such as oleic acid have an effect, but are not precursors of eicosanoids and the DHA has a larger effect than EPA, which would be unexpected since EPA is the direct precursor of the series 3 and 5 eicosanoids (De Caterina et al. 2000; Abeywardena & Head 2001).

As indicated above, different fatty acids have been shown to have a wide range of effects on adhesion molecule expression on endothelial cells, neutrophils, monocytes and lymphocytes. These differences may be due, in part, to variations in study design such as duration of incubation or type of cells used (Table 1.9), type of stimulus used and amount of oil consumed per day. By inhibiting leukocyte adhesion and migration of leukocytes, certain fatty acids, particularly oleic acid, EPA and DHA may have beneficial effects on a wide range of diseases in the western world including inflammatory, autoimmune and neoplastic disorders as well as alleviating symptoms and in the prevention of these illnesses. Soluble adhesion molecules may also be useful in some conditions as a marker of vascular endothelial dysfunction and may help in an early diagnosis of certain disease states, ie graft rejection.

TABLE 1.9: Effects of Unsaturated Fatty Acids on Adhesion Molecule Expression in *InVitro* Studies

HSaVECs *Reference*	*Study design*	*Outcomes studied*	*Findings*
De Caterina et al. 1994	Fatty acids studied: OA, LA, ARA, EPA, DHA (10 μM) Stimuli: IL-1α, TNF-α, IL-1β (1-10 ng/mL), IL-4 (50-100 ng/mL); LPS (1-10 μg/mL)	Adhesion of monocytes and U937 cells. VCAM-1, ICAM-1, E-selectin protein expression	DHA and OA (to a lesser extent) ↓ VCAM-1 induced by IL-1α, TNF-α. DHA ↓ VCAM-1, ICAM-1, sE-selectin in dose-dependent manner. DHA inhibited VCAM-1 for all stimuli tested. VCAM-1>sE-selectin>ICAM-1.
De Caterina & Libby 1996	Fatty acids studied: DHA, EPA, OA, LA, ARA (10 μM) Stimuli: IL-1 (1-10 ng/mL), TNF-α (1-10 ng/mL), IL-4 (50-100 ng/mL); LPS (1-10 ng/mL)	Adhesion of monocytes and U937 cells. VCAM-1, ICAM-1, E-selectin protein expression	DHA ↓ adhesion of monocytes and U937 cells DHA ↓ ICAM-1, VCAM-1, E-selectin EPA, ARA, LA- no effect.
De Caterina et al. 1998	Fatty acids studied: PA, SA, 20:0, ARA, palmitoleic, OA, elaidic, ricinoleic, LA, DGLA, ALA, EPA, DPA, DHA (25 μM) Control: HUVECs alone Stimuli: IL-1α (0.1-10 ng/mL), IL-1β, TNF-α (1-10 ng/mL), LPS (0.01-10 μg/mL)	VCAM-1, ICAM-1, E-selectin protein expression, VCAM-1 mRNA expression	↔ SFA. ↓ expression with ↑ number of double bonds; chain length does not matter. No effect of the double bond position or configuration. Double bond is necessary for inhibition.

HUVECs *Reference*	*Study design*	*Outcomes studied*	*Findings*
Sethi et al. 1996	Fatty acids studied: Oxidised PUFA, EPA, DHA, LA, ALA, PA, SA, ARA (200 μmol/L) HUVEC and SAEC.Stimuli : IL-1α (10 ng/mL), TNF-α (20 ng/mL), LPS (50 ng/mL).	Adhesion of U937 cells VCAM-1, ELAM-1 and ICAM-1 protein expression	Oxidised PUFA but not unoxidised PUFA ↓ the adhesion of U937 cells.
Collie-Duguid & Wahle 1996	Fatty acids studied: EPA, DHA, ARA (65 μM) Stimulus: IL-1β 10 U/mL).	ICAM-1, VCAM-1, E-selectin mRNA expression	DHA ↓ ICAM-1, VCAM-1, sE-selectin. EPA ↓ VCAM-1, sE-selectin (not ICAM-1).

HUVECs continued *Reference*	*Study design*	*Outcomes studied*	*Findings*
Khalfoun et al. 1996	Fatty acids studied: EPA, DHA (30 μM) Stimuli: TNF-α, IL-4 (100 IU/mL); LPS (1 μg/mL)	Peripheral blood lymphocyte adhesion. E-selectin, ICAM-1, VCAM-1 protein expression	EPA and DHA ↓ VCAM-1 and DHA > potent inhibitor.
Toborek et al. 2002	Fatty acids studied; OA, LA, ALA (60-90 μM) Stimulus: LPS (1 μg/mL)	NFκB and activator protein (AP-1) transcriptional activation.	LA ↑ NFκB and AP-1 ta the most (↑ mRNA levels of TNF-α, VCAM-1, ICAM-1) (sig). ALA stimulated a moderate induction of the genes encoding for these inflamm mediators. OA ↔/↓.
Massaro et al. 2002	Fatty acids studied: PA, SA, OA, DHA (50 and 100 μM) Stimuli: IL-1α, TNF-α; LPS (1 μg/mL)	VCAM-1 protein expression	Oleate + LPS/PMA ↓ VCAM-1, ↓ NFκB activation by LPS.
Mayer et al. 2002	Fatty acids studied: EPA, DHA, ARA (10 μM) Stimuli: TNF-α (1 and 10 ng/mL).	Leukocyte adhesion. VCAM-1, ICAM-1 and P-selectin protein expression	↓ adhesion monocyte to endothelial cells by EPA, DHA (sig). Preincubation of HUVEC with EPA or DHA ↓ (sig) PAF synthesis, monocytes rolling and adherence, however ↔ expression of endothelial adhesion molecules.
Chen et al. 2003	Fatty acids studied: LA, ARA, DHA, PA (100 μM) Stimuli: TNF-α (20 ng/mL), IL-1β (5 ng/mL), PMA (10 ng/mL).	ICAM-1 and VCAM-1 protein expression	LA and AA ↑ ICAM-1 in hRVE cells (sig); no ↑ in HUVEC.
Holthe et al. 2005	Fatty acids studied: OA, LA, PA (100 μM) Stimuli: PMA (100 ng/mL), LPS (1 μg/mL)	VCAM-1, ICAM-1, E-selectin protein expression	PA ↓ VCAM-1 after 24 h (sig), but without change of mRNA level
Shaw et al. 2007	Fatty acids studied: ARA, DHA, EPA, LA, OA, PA (10, 25, 100 μM) Stimulus: TNF-α (10 ng/mL)	VCAM-1, E-selectin mRNA	ARA and DHA ↓/↔ expression of VCAM-1 and E-selectin mRNA; LA and OA ↑ VCAM mRNA expression.
Schaefer et al. 2008	Fatty acids studied: ARA and DHA (10 μM) Stimulus: TNF-α (10 ng/mL)	VCAM-1, ICAM-1, E-selectin protein expression	No significant effects.
Guoa et al. 2008	Fatty acids studied: CLA (t10, c12 and c9, t11), EPA, DHA (12.5, 25, 50 μM) Stimulus: TNF-α (5 ng/mL)	VCAM-1, ICAM-1, E-selectin protein expression	↓ VCAM-1 and ICAM-1 protein expression. ↓ ICAM-1 and VCAM-1 protein expression by n-PUFA was less dependent on the NFκB pathway than reduction by CLA

HAOECs *Reference*	*Study design*	*Outcomes studied*	*Findings*
Stuhlmeier et al. 1997	**Note: Porcine AECs and** HUVECs**.** Fatty acids studied: ETYA (synthetic analog of ARA), ARA (35-65 μM). Stimuli: LPS, PMA, TNF-α	ICAM-1, VCAM-1, E-selectin protein and mRNA expression.	ETYA and ARA ↓ E-selectin in AECs; VCAM-1 and ICAM-1. protein expression in HUVECs. ETYA ↓ mRNA for E-selectin, ICAM-1, VCAM-1.
Schleser et al. 2006	Fatty acids studied: CLA (*cis* and *trans*), LA (5 or 50 μM). Stimulus: TNF-α (2 ng/mL)	ICAM-1, VCAM-1, E-selectin protein expression. Adhesion of U937 cells	↔ expression of adhesion molecules or U937 adhesion.
Stentz & Kitabchi 2006	Fatty acids studied: PA, OA, LA, ALA, ARA LA (1, 50, 500 μM) No stimulus used	E-selectin protein expression	PA ↑ E-selectin protein expression (sig)
HCAECs *Reference*	*Study design*	*Outcomes studied*	*Findings*
Reiβig et al. 2003	Fatty acids studied: LA, PA (10 μM) Stimulus: IL-1α (10 ng/mL)	ICAM-1, VCAM-1, E-selectin protein expression Adhesion of monocytes	LA ↓ ICAM-1 and VCAM-1
Monocytes	*Study design*	*Outcomes studied*	*Outcomes*
Hughes et al. 1996	Fatty acids studied: EPA, DHA (0-140 μM) Stimulus: IFN-γ	Cell surface ICAM-1 expression	EPA ↓ ICAM-1 and HLA-DR in unstimulated PMBCs EPA ↓ ICAM-1 and HLA-DR, DLA-DP with EPA + IFN-γ stimulated PBMCs
Hughes et al. 2000	Fatty acids studied: EPA + DHA (3:1, 12 μg/mL EPA + 8 μg/mL DHA) Stimulus: IFN-γ	Cell surface ICAM-1 expression	↓ ICAM-1

Abbreviation: EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; PA, palmitic acid, OA, oleic acid, LA, linoliec acid; ALA, alpha linolenic acid; CLA, conjugated linoleic acid; ARA, arachidonic acid; ETYA, 5,8,11,14-eicosatetraynoic acid; IFN, interferon; VCAM-, vascular cellular adhesion molecule-; ICAM-, intercellular adhesion molecule; LPS, lipopolysaccarides; AOC, aortic endothelial cell; NF-κB, nuclear factor kappa B; IL- interleukin-; TNF-, tumour necrosis factor-;

TABLE 1.10: Effects of Unsaturated Fatty Acids on Adhesion Molecule Expression in Animal Studies

Reference	*Study design*	*Outcomes studied*	*Findings*
Yaqoob et al. 1995	Rats, fed for 10 wks 1) LF 20% by wt 2) HF 20% HCO 3) HF 20% OO 4) HF 20% SO 5) HF 20% EPO 6) HF 20% FO	Blood leukocytes surface expression (LFA-1, ICAM-1, CD2)	No effect of dietary fats
Sanderson et al. 1995	Rats, fed for 3 wks 1) LF 2.5% wt CO 2) HF 20% HCO 3) HF 20% OO 4) HF 20% SO 5) HF 20% EPO 6) HF 20% FO	Spleen lymphocyte surface expression	FO and OO ↓ CD-2, ICAM-1, LFA-1 .
Sanderson et al. 1997	Rats, fed fror 6 wks 1) LF 2.5% wt CO 2) HF 20% SO 3) HF 20% FO	Dendritic cell surface expression	FO ↓ ICAM-1
Sanderson & Calder 1998	Rats fed for 8 wks 1) LF 2.5% wt CO 2) HF 20% HCO 3) HF 20% OO 4) HF 20% SO 5) HF 20% EPO 6) HF 20% FO	Lymphocyte surface expression of ICAM-1 and L-selectin Lymphocyte adhesion to endothelial cells	FO ↓ ICAM-1 and L-selectin. OO and EPO ↓ ICAM-1 and L-selectin,but to a lesser extent than FO
Miles et al. 2000	Mice fed for 12 wks: 1) LF 2.5% wt CO 2) HF 20% HCO 3) HF 20% OO 4) HF 20% SO 5) HF 20% FO	Macrophage ICAM-1 and scavenger reseptor mRNA expression	FO ↓ ICAM-1
Hsu et al. 2006	Mice fed FO vs soybean oil, 3 wks; polymicrobial sepsis induced by ceacal ligation and puncture (CLP)	Plasma sICAM-1 concentrations. Leukocyte integrin expression.	FO ↑ sICAM-1 6h CLP. FO ↑ leukocyte integrin 12-24 h after CLP.

Animal studies continued *Reference*	*Study design*	*Outcomes studied*	*Findings*
Kato et al. 2007	Mice fed laboratory chow with 5% EPA or LA, 4 wks	mRNA expression of ICAM-1, MCP-1, VEGF and IL-6 in the retinal pigmen epithelium (RPE)	EPA diet ↓ ICAM-1 protein and mRNA levels (sig)
Matsumoto et al. 2008	ApoE deficient mice fed with 5% (w/w) EPA, n=5 vs. control (no EPA in diet), 13 wks	VCAM-1, ICAM-1, E-selectin, MCP-1 expression	↓ VCAM-1, ICAM-1, E-selectin, MCP-1 protein expression in EPA group

TABLE 1.11: Effects of Unsaturated Fatty Acids on Adhesion Molecule Expression in Human Studies

Reference	*Study design*	*Outcomes studied*	*Findings*
Hughes et al. 1996	Healthy volunteers 0.93 g EPA + 0.63 g DHA/day (n=6) *vs* control (n=6) 21 days	Adhesion molecule surface expression on ± stimulation *ex-vivo* with IFN-γ.	FO ↓ ICAM-1, LFA-1 and MHC class II (*in vivo* and *ex-vivo*
Abe et al. 1998	4 g EPA + DHA/day (n=21) *vs* placebo (n=20), 6 wks; then 7 mo (n=18)	Plasma sICAM-1, sVCAM-1, sE-selectin concentrations	None at 6 weeks FO ↓ sICAM-1 and sE-selectin after 7 mo , with greatest reduction in diabetic pt.
Seljetfot et al. 1998	Male smokers with hyperlipidemia (n=41) 4.8 g EPA + DHA/day 6 wks	Plasma sP-selectin, sVCAM-1, sICAM-1, tPA ag, vWF, sTM concentrations	FO ↓ vWF and sTM and ↑ sVCAM-1 and sE-selectin
Turpeinen et al. 1998	Healthy subjects (n=38), strictly controlled diet rich in LA (11.5% en) or OO (18% en) *vs* control (n=13); 1st 4 wks - diet rich SFA (normal); 2nd 4 wks – LA or OO	Plasma sICAM-1 concentration	None
Johansen et al. 1999	Patients with CHD 5.1g EPA + DHA/day (n=23) *vs* corn oil (n=31); 6 months	Plasma sP-selectin, sVCAM-1, sICAM-1, tPA ag, vWF, sTM concentrations	FO ↓ vWF and sTM Placebo ↑ sE-selectin and sVCAM-1
Yaqoob et al. 2000	Healthy subjects (n=40) - CO + soybean oil (3:1) as placebo - OO (6.5 g/d OA) - SO (6.5 g/d LA) - EPO (6.3 g/d LA + 1 g/d GLA) - FO (2.1 g/d EPA +1.1 g/d DHA) 12 wk	CD54, (ICAM-1) expression on PBMC	None
Miles et al. 2001	Healthy subjects (n=140) Subset of 16 young males, 12 elderly (6 M, 6 F) 1.2 g EPA + DHA/d *vs* placebo (PO + soybean oil, 80:20) 12 wks	Plasma sICAM-1, sVCAM-1, sE-selectin Concentrations	FO ↓s-Eselectin and sVCAM-1 (elderly) αvδ ↑ sE-selectin (young)

Reference	*Study design*	*Outcomes studied*	*Findings*
Thies et al. 2001	Healthy subjects (n=40) -Placebo (80:20 PO+sunflower oil). -2 g/g ALA -0.7 g/d GLA -0.7 g/d ARA -0.7 g/d DHA -0.72 g/d EPA + 0.28 g/d DHA 12 wks	Plasma sE-selectin, sICAM-1, sVCAM-1 concentrations	FO and ALA↓ VCAM-1 ALA ↓ sE-selectin
Sampson et al. 2001	Male, non-smokers; FO (1.2 g/d EPA + 0.8 g/d DHA; n=29) *vs* control (n=21) 21 days,	Plasma sICAM-1, sE-selectin concentrations	None
Yli-Jama et al. 2002	152 elderly men with increased risk of CHD	Relationship between EPA, DHA and AA in the NEFA fraction serum sCAM concentrations	-ve (r): EPA and DHA vs. sVCAM-1. -ve (r): AA vs. sVCAM-1 and vWF
Berstad et a*l.* 2003	171 elderly men with increased risk of CHD FO (2.4 g/d EPA+DHA) vs. corn oil 18 months	Effect of the FO supplementation on the association between serum NEFA and sCAM concentrations	+ve ™: ΔDHA vs. sVCAM-1 in FO group. -™(r): ΔDHA vs. sVCAM-1 in placebo group.
Lopez-Gracia et al. 2004	Cross-sectional study, 727 women 43-69 y; validated FFQ.	Plasma sE-selectin, sICAM-1, sVCAM-1 concentrations	High n-3 FA intake associated with lower sCAM concentrations
Eschen et al. 2004	Healthy volunteers (n=60) FO (2.0 or 6.6 g EPA+DHA/day) vs. olive oil 12 wks	Serum sP-selectin, sICAM-1, sVCAM-1 concentrations	6.6 g/d n-3 PUFA ↓ sP-selectin in men 2.0 g/d n-3 PUFA ↓ sICAM-1 in women 6.6 g/d n-3 PUFA ↓ sVCAM-1 in women.
Zhao et al. 2004	Moderately hypercholesterolemiac, non-smokers (n=23); cross-over study; 3 periods (6 wks/period): -average American diet (AAD) -↑ PUFA -↑ ALA	Serum sVCAM-1, sICAM-1, sE-selectin concentrations	AAD: Δ VCAM-1 –ve(r) with ΔEPA and ΔEPA + DHA. LA diet: ΔICAM-1 and E-selectin associated with Δany of the serum n-3 FAs.
Paschos et al. 2004	Dyslipidemic males 8.1 g/day ALA 2 group: a) Mediterranean-Cretan diet (n=21) b) Westernised-Greek diet (n=19) 12 weeks	Serum sICAM-1, sVCAM-1, sE-selectin concentrations	Both diets ↓sVCAM-1

Reference	*Study design*	*Outcomes studied*	*Findings*
Seierstad et al. 2005	Patients with CHD (n=60), 6 wks. 0.7 kg/wk of differently fed salmon (fillets): -100% FO, 2.9 g/d n-3 PUFAs -100% RO, 1.5 g/d n-3 PUFAs -50:50 FO/RO, 0.5 g/d n-3 PUFAs	Plasma sVCAM-1, sICAM-1, and sE-selectin concentrations	100% FO salmon ↓ sVCAM-1
Hjerkinn et al. 2005	Elderly male patients with long-standing dyslipidemia (n=562) 2.4 g EPA+DHA/d) 3 y	Plasma sVCAM-1, sICAM-1, sE-selectin, sTM, vWF, tPA ag concentrations ™	FO ↓ sICAM-1, sTM .
Cazzola et al. 2006	Healthy young 18-42 y (n=93), and elderly 53-70y (n=62) males Placebo (5.5 g/d LA) 1.35 g/d EPA + 0.27 g/d DHA 2.7 g/d EPA + 0.54 g/d DHA 4.05 g/d EPA + 0.81 g/d DHA 12 wks	Plasma sE-selectin, sICAM-1, and s VCAM-1 concentrations	High FO ↑ sE-selectin in young
Lindqvist et al. 2008	Healthy men 35-60 y (n=35) Cross-over intervention study, 6 wks 150g baked herring fillet/d, 5d/wk) vs. reference diet (150g baked pork/chicken/d, 5d/wk)	Plasma IL-6, IL-8, ICAM-1	None

1.4 Summary Statement and Study Objectives

The relationship between inflammation and CVD is gaining much attention because it is believed that inflammation plays an important role in initiation and progression of atherosclerosis. Upregulated expression of adhesion molecules following an inflammatory stimulus is one process whereby inflammation could contribute to atherosclerotic plaque initiation and growth. N-3 PUFAs from oily fish and fish oils reduce cardiovascular mortality and may have a primary role in preventing atherosclerosis. Anti-inflammatory effects might be part of the mechanism involved. Although there is some evidence that this is the case there are large discrepancies in the literature concerning the effect of these fatty acids on various components of the inflammatory process (Calder 2006) including adhesion molecule expression on cultured endothelial cells and on soluble adhesion molecule concentrations in human blood. Although *in vitro* studies have shown that EPA attenuates the expression of ICAM-1, VCAM-1 and E-selection in HUVECs (Collie-Duguid & Wahle 1996; Khalfoun et al. 1996), other studies using HSaVECs reported that EPA has no effect (De Caterina et al. 1995). The discrepancy in *in vitro* studies (Table 1.9) could be due to differences in study design: some studies used HUVECs whereas other studies used HSaVECs. They also used different stimulants. There may be differences in effects on adult and fetal ECs. Furthermore, the cells used are venous ECs while atherosclerosis occurs mainly in arteries not veins. To date, there are no studies on the effects of n-3 PUFAs on adhesion molecule expression performed using arterial or aortic endothelial cells. Moreover, conclusive evidence is still lacking as to whether EPA or DHA has the greater role in modulation of inflammatory process. Studies of n-3 PUFA supplementation in adults have typically used high concentrations of the fatty acids, yet epidemiology reports associations with more modest dietary intakes. Therefore there is a need to investigate the effects of modest doses of n-3 PUFAs on CVD risk factors, especially inflammation in adults.

Therefore the overall objectives of the studies that I am undertaking are:

1. To identify the effects of fatty acids, including EPA and DHA, on inflammatory responses, especially adhesion molecule expression in cultured endothelial cells of different origin (fetal vs. adults; vein vs. artery vs. aorta).

2. To investigate the effects of modest intakes of n-3 PUFAs on CVD risk factors especially inflammatory markers, including soluble adhesion molecules in adult humans with and without CVD.

My aims will be to further understanding of the ability of fatty acids, especially n-3 PUFAs, to the modulate adhesion molecule expression in humans and to further investigate the possible mechanisms involved using *in vitro* model. Therefore, the specific objectives of my study will be as follows:

1) To study the effects of fish oil supplementation on CVD risk factors in healthy and CVD patients;
   1a) Effects of fish oil supplementation on fatty acid incorporation in plasma lipid pools,
   1b) Effects of fish oil supplementation on lipid profile,
   1c) Effects of fish oil supplementation on soluble adhesion molecule concentrations,
   1d) The relationship between inflammatory markers and fatty acid status, particularly EPA and DHA.

2) To study the effects of fatty acids on adhesion molecule expression in different ECs;
   2a) Effects of different fatty acids (EPA, DHA, DPA, ARA, OA and SA) on adhesion molecule expression in different cell types using HSaVECs, HUVECs, HUAECs, HCAECs and HAOECs using the same stimulant i.e. LPS,
   2b) Effects at the gene expression level using RT-PCR.

Chapter 2

The Effect of a Moderate Dose of Long Chain n-3 PUFAs on Cardiovascular Risk Factors, Particularly Plasma Lipids and Inflammatory Markers, in Middle Aged Men

2.1 Introduction

2.1.1 Background

Classic risk factors for atherosclerosis and cardiovascular disease include elevated plasma lipids, including triacylglycerols (TAGs) and total and low density lipoprotein (LDL) cholesterol, high blood pressure and insulin resistance (British Nutrition Foundation 2005; see also Section 1.1.3.1). However, it is now recognised that atherosclerosis is an inflammatory process involving movement of leukocytes, especially monocytes and T lymphocytes, from the bloodstream into the intima of the blood vessel wall and subsequent release of inflammatory mediators that contribute to plaque growth and development and ultimately to its rupture (British Nutrition Foundation 2005; Ross 1999; see Section 1.1.1). Prior to entry into the intima, blood leukocytes interact with the endothelial cells lining the vessel wall. These interactions are largely mediated by ligand-ligand interactions between proteins termed adhesion molecules. These interactions serve to slow and then tether the flowing blood leukocytes. Chief amongst the adhesion molecules involved are intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1 and E-selectin. The endothelial expression of these molecules is up-regulated by inflammatory stimuli and they may then be cleaved from the surface of the endothelial cells (Gearing & Newman 1993). In vivo this results in non-surface bound forms of the adhesion molecules circulating

in the bloodstream; these are termed soluble adhesion molecules (e.g. soluble ICAM-1; sICAM-1). It has been demonstrated that elevated plasma

concentrations of soluble adhesion molecules and other inflammatory proteins such as interleukin-6 (IL-6) and C-reactive protein (CRP) are associated with increased cardiovascular risk and are higher in individuals with diagnosed cardiovascular disease compared with those without (Morisaki et al. 1997; Haught et al. 1996; Ridker et al. 1998; Blake & Ridker 2001; see Section 1.1.3.2.4). Thus, a reduction in plasma concentrations of these inflammatory markers, which may reflect a reduction in inflammatory processes at and within the vessel wall, would be interpreted as a lowering of cardiovascular risk.

There is significant epidemiological evidence that consumption of fish, especially oily fish is protective against cardiovascular morbidity and mortality (Kromhout et al. 1985; Shekelle et al. 1985; Norell et al. 1986; Gillum et al. 1996; Daviglus et al. 1997; Albert et al. 1998; Iso et al. 2001; Hu et al. 2002; He et al. 2002). This is believed to be due to the long chain n-3 polyunsaturated fatty acids (n-3 PUFAs) found in oily fish, since both long chain n-3 PUFA consumption in the diet (Albert et al. 1998; Hu et al. 2002; Iso et al. 2001; Dolecek et al. 1982; Siscovick et al. 1995) and blood and tissue concentrations (Pedersen et al. 2000; Albert et al. 2002; Lemaitre et al. 2003) have been shown to be protective against cardiovascular disease morbidity and mortality. The long chain n-3 PUFAs found in oily fish, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are found in fish oils and similar preparations. Studies using fish oils have demonstrated that long chain n-3 PUFAs influence many cardiovascular risk factors, including blood pressure and plasma TAG concentrations, in a manner that could contribute to cardiovascular protection (Kris-Etherton et al. 2002; Calder 2004; von Schacky 2004). There is now much evidence that these fatty acids are anti-inflammatory (Calder 2001; Calder 2003; Calder 2006) While this is believed to be important in the context of cardiovascular disease (Calder 2004) many studies in humans have used very high doses of long chain n-3 PUFAs (e.g. > 3 g/day) which greatly exceed those that could be achieved through fish consumption and so have limited relevance to explaining the epidemiology. Furthermore, in the context of the very important adhesive interactions between leukocytes and the endothelium there is fairly limited information (see Section 1.3.3). In vitro studies have shown that EPA and DHA can inhibit inflammation-induced up-regulation of VCAM-1 on human endothelial cells (De Caterina et al. 1994; Khalfoun et al. 1996; Weber et al. 1995; Collie-Duguid et al. 1996) and of ICAM-1 on human monocytes (Hughes et al. 1996). Feeding studies in laboratory rodents report that fish oil lowers ICAM-1 expression on the surface of

macrophages (Miles et al. 2000) and T lymphocytes (Sanderson & Calder 1998), while fish oil supplementation was shown to decrease ICAM-1 on the surface of human monocytes (Hughes et al. 1996). With regard to soluble forms of adhesion molecules, reports in the literature are mixed (Miles et al. 2001; Seljeflot et al. 1998; Abe et al. 1998; Johansen et al. 1999; see Section 1.3.7.2). Long chain n-3 PUFA dose, duration of exposure, differences between the subjects studied and differences in experimental design might contribute to the different findings of these studies.

Most studies of fish oil and inflammation have used high doses of long chain n-3 PUFAs (Calder 2006). In the UK, the guideline range for oily fish intake among males and adult women not of child bearing age is one to four portions per week (SACN/COT 2004) The long chain n-3 PUFA content of a portion of oily fish ranges from about 1.5 to 3.5 g (British Nutrition Foundation 1999) Thus, one to four portions per week could provide between 1.5 and 14.0 g long chain n-3 PUFAs. This equates to an average daily long chain n-3 PUFA intake of 0.2 to 2 g. It is important to know the effect of consumption of long chain n-3 PUFAs within this guideline intake range on risk factors for cardiovascular disease. Thus, in this study a dose of EPA plus DHA at the upper end of the guideline intake range was used to investigate the effects on selected cardiovascular risk factors, with a focus on plasma lipid concentrations and inflammatory markers. The study was performed in middle-aged men, a group at risk of cardiovascular disease and had a double blind, randomised, placebo-controlled, parallel design.

2.1.2 Aim of This Study

The aim of this study was to identify the effect of an intake of long chain n-3 PUFAs (EPA plus DHA) at the upper end of the UK adult guideline intake range on selected cardiovascular risk factors, with a focus on plasma lipid concentrations and inflammatory markers. To address this aim, a double blind, randomised, placebo-controlled study of 2.1 g/d EPA plus DHA was conducted in middle-aged men, a group at risk of cardiovascular disease. The main outcomes were:

- The fatty acid composition of plasma lipid fractions
- The concentrations of plasma lipids
- The concentrations of plasma markers of inflammation

- The concentrations of plasma glucose and insulin
- Blood pressure

2.2 Methods

2.2.1 Study Design

This was a randomised, double-blind and placebo-controlled study of eight weeks duration. The study ran from March until June 2006, depending on when the subject started. Volunteers were given a detailed written description of the study in the form of an information sheet before they gave their written informed consent. Subjects were required to attend the Welcome Trust Clinical Research Facility (WTCRF) at Southampton General Hospital on two occasions (at study entry ("baseline") and end of the eight week intervention). On both occasions they were in the fasted state (> 12 hours without food and drink apart from water) and gave 20 ml blood samples. Weight, height (only for the first visit) and blood pressure were taken at each visit.

2.2.2 Recruitment of the Volunteers

Ethical approval (05/Q1704/151) was obtained from the Southampton and South West Hampshire Joint Ethics Committee. Volunteers were recruited by a variety of means of advertising, including announcement in the university sussed portal system and in the University Bulletin, and posters around the University of Southampton and Southampton General Hospital. Some of them were contacted from existing databases (SoFIA and FINGEN studies). Volunteer's eligibility for study entry was screened by using a Health and Lifestyle Questionnaire (see Section 2.2.3) and their GP was informed by formal letter if they were suitable to participate in the study. A total of 21 subjects were recruited; however, one subject withdrew after 3 weeks. Figure 2.1 shows the recruitment and participation of the volunteers in the study

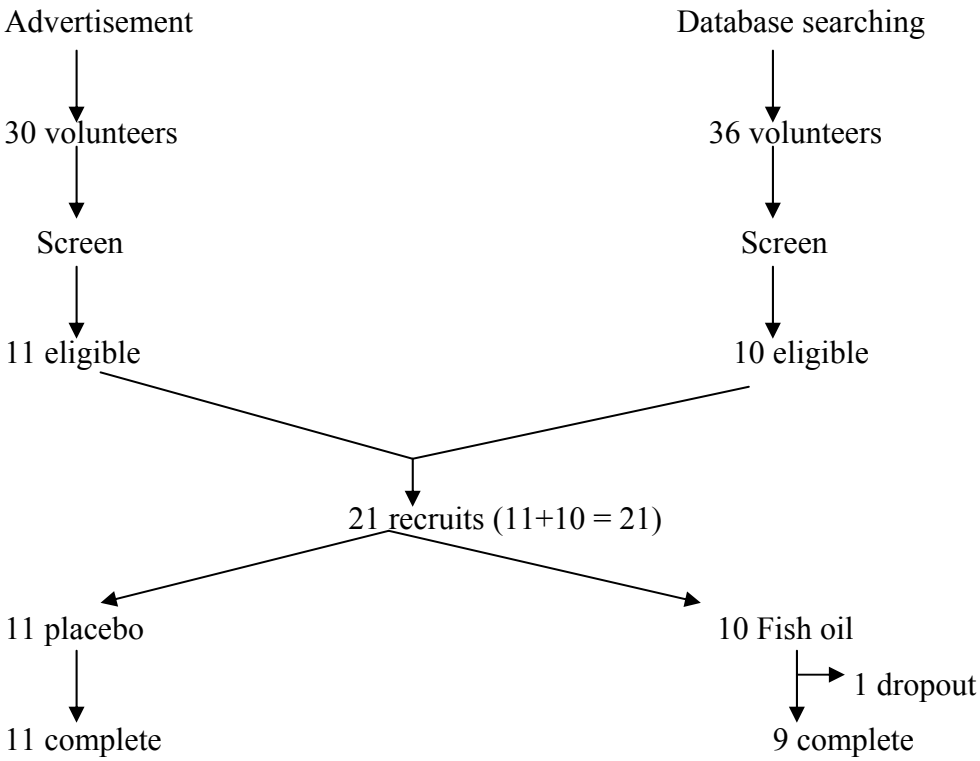


FIGURE 2.1: Summary of Subject Recruitment

2.2.3 Inclusion Criteria

Volunteers were identified as eligible to participate in the study if they were male; within the age range of 35-60 years; had a body mass index (BMI) of 18.5-29.9 kg/m^2; were not on drug treatment for hyperlipidaemia or inflammatory conditions or a regular (daily) aspirin user; were not suffering from any gastrointestinal disorder, diabetes mellitus or other endocrine disorders; were not taking any dietary supplements including fatty acids and vitamins; were not vegetarian or vegan; did not consume more than one serving of oily fish per month; were not a heavy smoker (> 10 cigarettes per day); were not a vigorous exerciser (more than 3 x 30 minutes vigorous sessions per week); were not planning to lose weight; were not a blood donor; and had not participated in a clinical trial in the previous three months.

2.2.4 Capsules

Subjects received in random, minimization assigned group order either fish oil (66% of fatty acids by weight as EPA and 11% as DHA; Cardiozen®) or placebo (97% w/w an oil rich in medium chain fatty acids (MCFAs)). The capsules used were a gift from Equazen UK Ltd. and contained 0.5 g of oil, fish flavoured and gelatine coated. Subjects were provided with more capsules than needed for that period of the study and they consumed six capsules per day. Therefore, subjects in the fish oil group consumed 3 g/d fish oil providing 2.1 g/d of EPA (1.8 g/d) and DHA (0.3 g/d), while subjects in the placebo group consumed 3 g/d placebo oil providing 2.5 g/d MCFAs. Table 2.1 shows the fatty acid composition of the capsules. The placebo oil used was a rich source of MCFAs, comprising 20.8% of fatty acids as caprylic acid (8:0) and 73.9% as capric acid (10:0). MCFAs were selected as placebo because these fatty acids are readily oxidised in the liver and are expected to have little impact on human health related biomarkers.

TABLE 2.1: Fatty Acid Compositions of the Capsules Used (g/100 g Fatty Acid)
Values are means (n = 5) and (SEM)

Fatty acid	Fish oil	Placebo
Caprylic acid, 8:0	-	20.8 (4.4)
Capric acid, 10:0	-	73.9 (3.8)
Lauric acid, 12:0	-	2.2 (0.3)
Palmitic acid, 16:0	0.7 (0.0)	1.5 (0.2)
Palmitoleic acid, 16:1n-7	0.5 (0.0)	-
Oleic acid, 18:1n-9	2.2 (0.0)	-
Elaidic acid, *t*18:1n-9	0.6 (0.0)	-
Linoleic acid, 18:2n-6	1.1 (0.0)	-
γ-linolenic acid, 18:3n-6	0.7 (0.0)	-
α-linolenic acid, 18:3n-3	0.9 (0.0)	-
Eicosanoic acid, 20:1n-9	9.2 (0.0)	-
Arachidonic acid, 20:4n-6	4.0 (0.0)	-
Erucic acid, 22:1n-9	2.5 (0.0)	-
Eicosapentaenoic acid, 20:5n-3	66.8 (0.0)	1.8 (0.1)
Docosahexaenoic acid, 22:6n-3	10.8 (0.0)	-

2.2.5 Compliance

Subject's compliance was measured by counting of the leftover capsules returned. If subjects consumed less than 75% of the capsules given, then they were not used in the

final analysis of the study. No subjects were excluded on this basis. Compliance was also monitored by studying the plasma fatty acid profiles.

2.2.6 Blood Samples and Measurements Made

A 20 mL venous blood sample was taken from the forearm (18 mL into tubes containing lithium-heparin and 2 mL into a tube containing fluoride oxalate). Blood samples were collected after a 12-hour overnight fast and sampling was successful in all subjects. Samples were put on ice and plasma was separated by centrifugation at 3000 rpm for 10 minutes at 4^{o} C. Aliquots of plasma were kept frozen at -80^{o} C until analysis. Glucose concentration was measured in plasma from blood collected into fluoride oxalate (to stabilize glucose). All other measurements were made on plasma from blood collected into heparin.

Weight and height were taken to the nearest 0.1 kg and 0.1 cm, respectively. Body mass index (BMI) was calculated as weight (in kg) divided by standing height (in m^2). Two-blood pressure measurements were obtained from non-dominant side arm and an additional reading was done if values from two consecutive measurements were more than 10 mm Hg apart. Subjects were lying down when blood pressure was measured using a blood pressure meter, Marquette®.

2.2.7 Determination of Fatty Acid Composition of Plasma Lipids

2.2.7.1 Lipid Extraction

Lipids were first extracted from plasma (0.4 mL that had been adjusted to 0.8 mL by addition of 0.9% NaCl), with 5.0 mL chloroform:methanol (2:1, v/v) containing butylated hydroxytoluene (50 mg/L) as antioxidant. Subsequently, 1.0 mL 1 M NaCl was added to promote partitioning of lipids into the organic phase and to facilitate separation of chloroform and aqueous layers. The mixture was then centrifuged at 2000 rpm for 10 minutes at room temperature and the bottom layer (chloroform phase) containing lipid extract was collected and dried under nitrogen gas at 40^{o} C.

2.2.7.2 Separation of Lipid Classes

Lipid classes were separated by solid phase extraction (SPE). The total lipid extract was dissolved in 1.0 mL dry chloroform and then applied onto aminopropylsilica SPE cartridge on a SPE tank. The column was washed with 2 x 1.0 mL dry chloroform under vacuum. The combined washes were collected; these contained the triacylglycerol (TAG) and cholesteryl ester (CE) fractions and subsequently were dried under nitrogen gas at 40^{o} C. Two mL dry chloroform:methanol (60:40, v/v) was applied to the column and the phosphatidylcholine (PC) fraction eluted under vacuum. Then non-esterified fatty acids (NEFAs) were eluted from the column under vacuum after adding 2.0 mL chloroform:methanol:glacial acetic acid (100:2:2, v/v/v). Then, a new aminopropylsilica SPE cartridge was placed on the SPE tank. The column was washed with 4 x 1.0 mL dry hexane. The mixture of TAGs and CEs collected at the beginning was applied onto the column after being re-dissolved in 1.0 mL dry hexane. The column was washed with 2 x 1.0 mL dry hexane under vacuum pump to elute the CE fraction. Subsequently, the TAG fraction was eluted under vacuum following the addition of 2.0 mL dry hexane:chloroform:ethyl acetate (100:5:5, v/v/v). All the collected fractions were dried under nitrogen gas at 40^{o} C.

2.2.7.3 Formation of Fatty Acid Methyl Esters

Lipid fractions isolated according to the description in section 2.2.7.2 were dissolved in 0.5 mL dry toluene and fatty acid methyl esters (FAMEs) formed after reaction with 1.0 mL methylation reagent (methanol containing 2% v/v H_2SO_4) at 50^{o} C for 2 hours. After that, samples were removed from the heating block and allowed to cool at room temperature before adding 1.0 mL neutralizing solution (0.25 M $KHCO_3$, 0.5 M K_2CO_3). Subsequently, 1.0 mL of hexane was added and the mixture was centrifuged at 1000 rpm for 2 minutes at room temperature. The upper phase containing the FAMEs was transferred to a new round bottom glass tube and dried under nitrogen gas at 40^{o} C. Then 2 x 75 μL dry hexane were added; the sample was vortexed and transferred into the insert of a gas chromatography auto-sampler vial.

2.2.7.4 Separation of Fatty Acid Methyl Esters by Gas Chromatography

FAMEs dissolved in hexane were injected (1.0 μL) via a Hamilton syringe into a Hewlett Packard 6890 gas chromatograph (Hewlett Packard, Avondale, PA), fitted with a 30 m x 32 mm BPX 70 capillary column, film thickness 0.25 μm. Helium, at the initial flow of 1.0 mL/min was used as the carrier gas. The split ratios for TAGs and CEs, PC and NEFAs were 100:1, 50:1 and 5:1, respectively. Injector and detector temperatures were 275^{o} C and the column oven temperature was maintained at 170^{o} C for 12 minutes after samples injection. The oven temperature was programmed to increase from 170 to 210^{o} C at 5^{o} C/min. FAMEs were identified by comparison with authentic standards run previously; standards included commercially available menhaden oil FAMEs, and in-house IHN mix, Q3 and a MCFA mixture. Typical profiles for each standard mixture and for each lipid fraction are shown in Appendix 1. Peak areas were quantified using ChemStation software (Hewlett Packard, Avondale, PA). Each fatty acid is expressed as weight % of total fatty acids present.

2.2.8 Measurement of TAG Concentrations in Plasma

Plasma TAG concentrations were measured using a commercial kit from Konelab™. TAGs were first hydrolysed by a lipase to yield glycerol and fatty acids. The glycerol was subsequently phosphorylated to glycerol-3-phosphate by the action of glycerol kinase, and the glycerol-3-phosphate was then oxidized to dihydroxyacetone phosphate and hydrogen peroxide by glycerol-3-phosphate dehydrogenase. The hydrogen peroxide was then reacted with 4-aminoantipyrine and 4-chlorophenol to form a quinoneimine dye, the absorbance of which was measured at 510 nm. The results were calculated automatically by the Konelab™ analyzer using a calibration curve whereby the absorbance was directly proportional to the TAG concentration.

2.2.9 Measurement of Total Cholesterol Concentrations in Plasma

Total plasma cholesterol concentrations were measured using a commercial kit from Konelab™. CEs were enzymatically hydrolysed by cholesterol esterase to cholesterol

and free cholesterol. Then, the free cholesterol was oxidised by cholesterol oxidase to cholesterol-4-en-3-one and hydrogen peroxide. The hydrogen peroxide was then reacted with hydroxybenzoic acid and 4-aminoantipyrine to form a quinoneimine dye the absorbance of which was measured at 500-550 nm. The results were calculated automatically by the Konelab™ analyzer using a calibration curve whereby the absorbance was directly proportional to the cholesterol concentration.

2.2.10 Measurement of LDL-cholesterol Concentrations in Plasma

Plasma LDL-cholesterol (LDL-c) was measured using a commercial kit from Konelab™. LDL-cholesterol was oxidized by cholesterol oxidase to cholesterol-4-en-3-one and hydrogen peroxide. The hydrogen peroxide was reacted with 4-aminoantipyrine and n-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline to form a quinoneimine dye the absorbance of which was measured at 500-550 nm. The results were calculated automatically by the Konelab™ analyzer using a calibration curve whereby the absorbance was directly proportional to the LDL-cholesterol concentration.

2.2.11 Measurement of HDL-cholesterol Concentrations in Plasma

HDL-cholesterol (HDL-c) was measured using a commercial kit from Konelab™. The concentration of HDL-cholesterol was determined enzymatically by cholesterol oxidase to produce cholesterol-4-en-3-one and hydrogen peroxide. This hydrogen peroxide subsequently was combined with 4-aminoantipyrine coupled with HSDA to form a purple blue dye that may be quantitated at 500-550 nm. Results were calculated as above (Section 2.2.9).

2.2.12 Measurement of NEFA Concentrations in Plasma

Plasma NEFA concentrations were measured using a commercial kit from Wako Chemicals GmBH. NEFAs were converted to CoA esters by acyl-CoA-synthetase. Then the acyl-CoAs were oxidised by acyl-CoA-oxidase to 2,3-trans-enoyl-coA and hydrogen

peroxide. The hydrogen peroxide was reacted with 4-aminophenazone and 3-methyl-N-ethyl-N-(β-hydroxyethyl) aniline to form a quinoneimine dye the absorbance of which was measured at 550 nm. The results were calculated automatically by the Konelab™ analyzer using a calibration curve as standard whereby the absorbance was directly proportional to the NEFA concentration.

2.2.13 Measurement of Glucose Concentrations in Plasma

Plasma glucose concentrations were measured using a commercial kit from Konelab™. Glucose was first reacted with hexokinase to form glucose-6-phosphate and adenosine-5'-diphosphate. The glucose-6-phosphate was then oxidised to 6-phosphogluconate by glucose-6-phosphate dehydrogenase. In this reaction, an equimolar amount of NAD^+ was reduced to NADH, with a resulting increase in absorbance at 340 nm. The results were calculated automatically by the Konelab™ analyzer using a calibration curve whereby the absorbance was directly proportional to the glucose concentration.

2.2.14 Measurement of Insulin Concentrations in Plasma

Plasma insulin concentrations were determined using ELISA kits from Biosource Europe, Nivelles, Belgium. Assays were performed according to the manufacturer's instructions and the absorbance was read on a plate reader at 450 nm with the reference filter of 650 nm. The sensitivity of the assay was < 0.15 μIU/mL. To assess insulin sensitivity, homeostatic model assessment (HOMA) was calculated as follows:

[Glucose concentration (mg/dL) x Insulin concentration (μIU/mL)]/405.

Analyses in Section 2.2.8 to 2.2.14 were performed by Mr. Chris, Institute of Human Nutrition, DoHAD.

2.2.15 Measurement of Inflammatory Marker Concentrations in Plasma

Plasma concentrations of IL-6, sE-selectin, sICAM-1, sVCAM-1 and high sensitivity C-reactive protein (hs-CRP) were measured using commercial ELISAs kits. sE-selectin, sICAM-1 and sVCAM-1 kits were purchased from Biosource Europe, Nivelles, Belgium; IL-6 kits were from R&D System Inc., Minneapolis, Minnesota; while hs-CRP kits were from Diagnostic System Laboratories Inc, Texas. For all assays, the manufacturer's instructions were followed and the absorbance was read on a plate reader using 450 nm as the primary wavelength and 610-650 nm as the reference wavelength. The sensitivities of the assays were < 0.039 pg/mL (IL-6), < 0.5 ng/mL (sE-selectin), 0.5 ng/mL (sICAM-1), 0.9 ng/mL (sVCAM-1) and 1.6 ng/mL (hs-CRP), respectively.

2.2.16 Platelet Reactivity Assay

Plasma sP-selectin concentrations were measured using ELISA kits from Biosource Europe, Nivelles, Belgium, following the manufacturer's instructions. The absorbance was read on a plate reader using 450 nm as the primary wavelength and 610-650 nm as the reference wavelength. The sensitivity of the assay was < 1.3 ng/mL.

2.2.17 Statistical Analysis

Sample size was based upon previous studies indicating that a fish oil supplement providing about 2 g EPA/day would be expected to increase the EPA content of plasma phospholipids from approximately 1 to approximately 4% of total fatty acids (Yaqoob et al. 2000). Using standard deviations for EPA contents of plasma phospholipids from previous studies (Miles et al. 2001; Yaqoob et al. 2000; Wallace et al. 2003), it was estimated that a sample size of 7 would give 80% power of detecting this effect as statistically significant with P set at 0.01. To allow for drop-outs it was decided to recruit 10 subjects per group.

The Kolmogorov-Smirnov and Shapiro-Wilk tests were applied to assess normality of data. Data for continuous variables that were normally distributed are presented as mean

values and their standard errors (SEM) whilst non-normally distributed data are presented as medians and 10^{th} and 90^{th} percentiles. Comparison of normally distributed data between groups was performed using the unpaired Student's t-test and within a group using the paired Student's t-test. Not-normally distributed data were compared using the Wilcoxan signed ranks and Mann-Whitney U tests. Relationships between variables were evaluated using Pearson's correlation coefficient. In all cases a value for $P \leq 0.05$ was taken to indicate a significant effect. SPSS version 14.02 (SPSS Inc., Chicago, IL) was used for all statistical analyses.

2.3 Results

2.3.1 Characteristics of the Subjects

Of the twenty-one subjects recruited, twenty were used in the final analysis of the study. One subject withdrew because of time commitments and reported that he was unable to comply. Subject characteristics were not significantly different between the groups at baseline, except for HDL-c concentration, which was higher in the fish oil group (Table 2.2). Based on the counting of the returned capsules, compliance was high; 90.1 ± 4.1% and 93.3 ± 2.2% for fish oil and placebo groups, respectively. Compliance was not significantly different between groups (P = 0.084). Compliance was also confirmed by an increase in the proportions of EPA and DHA in plasma lipids in the fish oil group (Tables 2.3 to 2.6).

TABLE 2.2: Characteristics of the Subjects
Data are mean (SEM) or median (10^{th}-90^{th} percentile)

	Fish oil (n = 9)	Placebo (n = 11)
Age (y)	43.7 (2.3)	44.7 (2.0)
Weight (kg)	80.1 (3.4)	81.6 (3.0)
Height (m)	1.8 (0)	1.8 (0)
BMI (kg/m^2)	25.7 (0.8)	26.5 (1.0)
Systolic BP (mm Hg)	122.5 (102.5 - 140.0)	110.0 (108.7 - 143.4)
Diastolic BP (mm Hg)	71.5 (60.0 - 90.0)	66.5 (64.5 - 92.6)
Total cholesterol (mmol/L)	5.2 (0.4)	4.8 (0.3)
LDL-c (mmol/L)	3.1 (0.3)	3.0 (0.3)
HDL-c (mmol/L)	1.4* (0.1)	1.1 (0.1)
Triglycerides (mmol/L)	1.3 (0.2)	1.1 (0.1)
Total cholesterol:HDL-c ratio	4.1 (0.5)	4.5 (0.3)
LDL-c:HDL-c ratio	2. 5 (0.4)	2.8 (0.3)
Total NEFA (umol/L)	396.0 (209.0 - 458.0)	334.0 (248.6 - 601.6)
Glucose (mmol/L)	5.9 (0.1)	5.7 (0.1)
Insulin (uIU/mL)	5.6 (1.3 - 28.4)	4.7 (4.1 - 6.2)
HOMA	1.3 (0.3 - 7.5)	1.2 (1.0 - 1.7)
sICAM-1 (ng/mL)	256.9 (27.7)	224.3 (16.1)
sVCAM-1(ng/mL)	456.4 (61.1 - 1375.2)	648.7 (436.8 - 957.3)
sE-selectin (ng/mL)	85.1 (16.1)	84.3 (13.2)
sP-selectin (ng/mL)	36.3 (10.3)	33.5 (3.9)
IL-6 (pg/mL)	1.4 (0.9 - 2.8)	1.2 (0.8 - 3.9)
CRP (mg/L)	1.9 (1.9- 4.0)	1.9 (1.9 - 9.3)

*Significantly different from the placebo group (P = 0.045)

Abbreviations: BMI = Body mass index; LDL-c = low density lipoprotein-cholesterol; HDL-c = high density lipoprotein-cholesterol; NEFA = non-esterified fatty acid; HOMA = homeostatic model assessment; sICAM-1 = soluble intercellular adhesion molecule-1; sVCAM-1 = soluble vascular cellular adhesion molecule-1; sE = soluble endothelial; sP = soluble platelet; IL 6 = interleukin-6; CRP = C-reactive protein.

2.3.2 Effect of Supplementation on the Fatty Acid Composition of Plasma TAGs

Table 2.3 shows the fatty acid composition of plasma TAGs. The most abundant fatty acids in this fraction were palmitic acid (16:0), oleic acid (18:1n-9) and linoleic acid (18:2n-6) comprising 27%, 40% and 16% of total fatty acids, respectively. Total of LC n-3 PUFAs were similar in the two groups at baseline (approx. 3.0% of total fatty acids), with EPA plus DHA contributing approx. 1.3% of total fatty acids. There were no significant differences between groups at baseline except for elaidic acid (*t*18:1n-9; P = 0.032). Supplementation with fish oil significantly (P = 0.025) increased EPA, with an average percent change from baseline of 96% (Table 2.7). At the end of intervention, there was a significant difference between groups for EPA (P = 0.042), but not for DHA (P = 0.337), ARA (P = 0.062), or any other fatty acids.

TABLE 2.3: Fatty Acid Composition of Plasma TAGs (g/100 g Fatty Acid)
Values are means and SEM

Fatty acid	Fish oil (n = 9)				Placebo (n = 11)			
	Baseline		After 8 weeks		Baseline		After 8 weeks	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
12:0	0.06	0.06	-	-	0.02	0.02	0.10	0.07
14:0	2.42	0.40	2.32	0.32	1.86	0.18	2.00	0.25
16:0	28.28	1.71	25.15	3.48	26.21	0.62	24.50	2.22
16:1n-7	3.57	0.34	3.50	0.40	3.41	0.31	6.70	2.98
18:0	3.81	0.24	4.07	0.42	3.37	0.10	3.89	0.31
18:1n-9	38.76	1.33	36.22	1.44	41.75	1.01	38.54	0.96
*t*18:1n-9	1.90[a]	0.07	1.80	0.09	2.17[b]	0.09	2.13	0.14
18:2n-6	16.24	2.03	15.88	1.57	16.10	1.07	15.03	1.19
18:3n-6	0.33	0.08	0.31	0.08	0.27	0.09	0.35	0.06
18:3n-3	1.19	0.11	1.72	0.49	1.38	0.22	1.21	0.13
20:4n-6	1.09	0.17	1.41	0.18	1.03	0.14	1.30	0.13
20:1n-9	0.21	0.06	0.34	0.15	0.21	0.06	0.45	0.14
20:2n-6	-	-	-	-	0.07	0.05	0.05	0.03
20:0	0.18	0.08	0.25	0.11	0.31	0.12	0.30	0.08
20:3n-6	0.12	0.05	0.17	0.06	0.17	0.07	0.12	0.05
20:5n-3	0.25	0.14	1.80*[a]	0.47	0.29	0.13	0.50[b]	0.37
22:5n-3	0.52	0.13	0.77	0.17	0.39	0.12	0.69	0.28
22:6n-3	1.07	0.24	1.53	0.44	1.01	0.22	1.55	0.85

*Significantly different from baseline (P = 0.025)

[ab]Values not sharing the same alphabetic superscript at baseline or after 8 weeks are significantly different between groups at this time point ($P \leq 0.05$).

2.3.3 Effect of Supplementation on the Fatty Acid Composition of Plasma NEFAs

Table 2.4 shows the fatty acid composition of plasma NEFAs. The most abundant fatty acids in this fraction were oleic acid, palmitic acid, stearic acid and linoleic acid comprising 31%, 24%, 20% and 12% of total fatty acids, respectively. Total LC n-3 PUFAs were approx. 4% of total fatty acids at baseline, with most as EPA plus DHA. There were no significant differences between groups at baseline for any fatty acid. There were no changes in fatty acid composition of NEFAs in the placebo group. Table 2.7 shows supplementation with fish oil significantly increased EPA, with an average percent change from baseline of 367%. There were significant differences between groups at the end of the intervention for EPA ($P < 0.0001$) and DPA ($P = 0.018$), but not for DHA ($P = 0.288$) (Table 2.4). The ARA:EPA ratio in plasma NEFAs was significantly decreased from 8.71 (at baseline) to 2.49 (after supplementation) in the fish oil group, but no significant change was observed in the placebo group (Figure 2.2).

TABLE 2.4: Fatty Acid Composition of Plasma NEFAs (g/100 g Fatty Acid)
Values are means and SEM

Fatty acid	Fish oil (n = 9)				Placebo (n = 11)			
	Baseline		After 8 weeks		Baseline		After 8 weeks	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
14:0	1.06	0.09	1.10	0.08	1.01	0.16	1.00	0.09
16:0	23.33	0.73	23.06	0.89	25.34	1.72	23.72	0.60
16:1n-7	1.67	0.17	1.58	0.23	2.01	0.22	1.98	0.27
18:0	20.54	1.40	20.67	1.57	19.11	2.43	18.69	1.49
18:1n-9	30.57	1.21	30.95	1.77	31.54	3.56	32.69	2.24
18:2n-6	12.39	0.91	12.11	1.15	12.88	0.72	12.40	0.65
18:3n-3	1.11	0.07	1.54	0.35	1.28	0.15	1.24	0.12
20:1n-9	0.17	0.07	0.17	0.06	0.40	0.19	0.15	0.10
20:3n-6	0.58	0.20	0.31	0.10	0.27	0.06	0.19	0.09
20:4n-6	3.26	0.42	3.18	0.34	2.99	0.33	2.73	0.32
20:5n-3	0.47	0.09	1.63**[a]	0.17	0.74	0.16	0.87[b]	0.24
22:5n-3	0.49	0.07	0.90*[a]	0.10	0.58	0.18	0.48[b]	0.16
22:6n-3	2.19	0.43	2.77	0.19	1.81	0.24	2.01	0.56

*Significantly different from baseline ($P \leq 0.02$)
**Significantly different from baseline ($P < 0.0001$)
Values not sharing the same alphabetic superscript at baseline or after 8 weeks are significantly different between groups at this time point ($^{ab}P \leq 0.05$)

2.3.4 Effect of Supplementation on the Fatty Acid Composition of Plasma CEs

Table 2.5 shows the effect of supplementation on the fatty acid of plasma CEs. The most abundant fatty acids in this fraction were similar to those in the TAG fraction, namely palmitic acid (11% of total fatty acids), oleic acid (19%) and linoleic acid (53%). There were no significant differences between groups at baseline except for DHA ($P = 0.034$). Total LC n-3 PUFAs was approx. 2 to 2.5% of fatty acids at baseline, mainly as EPA plus DHA. Fatty acid composition of CEs was not changed in the placebo group. However supplementation with fish oil significantly increased ($P < 0.001$) the EPA content of plasma CEs by 235% from baseline with a significant difference between groups at the end of intervention ($P < 0.001$) (Table 2.7). There were also small increases in myristic and palmitic acids and a decrease in linoleic acids after 8 weeks supplementation with fish oil ($P < 0.05$). There were no significant changes in ARA or DHA. In terms of the ARA: EPA ratio, this was significantly decreased from 5.0 (at baseline) to 1.35 (after supplementation) in the fish oil group, with no change in the placebo group (Figure 2.2).

TABLE 2.5: Fatty Acid Composition of Plasma CEs (g/100 g Fatty Acid)
Values are means and SEM

Fatty acid	Fish oil (n = 9)				Placebo (n = 11)			
	Baseline		After 8 weeks		Baseline		After 8 weeks	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
14:0	0.73	0.07	0.95*	0.08	0.70	0.04	0.73	0.07
16:0	11.42	0.22	12.21*	0.16	11.63	0.17	11.72	0.21
16:1n-7	2.83	0.37	2.88	0.50	2.42	0.28	2.62	0.36
18:0	1.03	0.08	1.04	0.07	1.06	0.06	0.90	0.06
18:1n-9	19.13	0.65	18.62	1.10	19.56	0.41	19.44	0.51
*t*18:1n-9	0.93[a]	0.05	1.00	0.08	1.07[b]	0.04	1.12	0.06
18:2n-6	53.87	1.78	49.80*	1.83	52.62	1.07	52.32	1.44
18:3n-6	1.13	0.21	0.99	0.17	1.02	0.11	1.03	0.18
18:3n-3	0.53	0.12	0.70	0.07	0.63	0.11	0.69	0.07
20:3n-6	0.71	0.12	0.64	0.12	0.71	0.11	0.75	0.14
20:4n-6	6.10	0.43	6.13	0.34	6.55	0.52	6.91	0.50
20:5n-3	1.22	0.22	4.54**[c]	0.30	1.12	0.16	1.19[d]	0.22
22:6n-3	0.32[a]	0.08	0.51	0.11	0.87[b]	0.21	0.51	0.14

*Significantly different from baseline ($P \leq 0.02$)

** Significantly different from baseline ($P \leq 0.001$)

Values not sharing the same alphabetic superscript at baseline or after 8 weeks are significantly different between groups at that time point ($^{ab}P \leq 0.05$, $^{cd}P \leq 0.001$).

2.3.5 Effect of Supplementation on the Fatty Acid Composition of Plasma PC

Table 2.6 shows the fatty acid composition of the plasma PC fraction. The most abundant fatty acids in this fraction were palmitic acid, stearic acid, oleic acid and linoleic acid comprising 31%, 14%, 11% and 24% of total fatty acids, respectively. There were no significant differences between groups at baseline except for myristic acid ($P = 0.029$). Supplementation with fish oil significantly increased EPA with a percent change from baseline of 363% (Table 2.7), and there were significant differences between groups at the end of the intervention for EPA and DPA (both $P < 0.001$). It was also observed that the n-6 fatty acids (linoleic acid (LA) and dihomo-gamma-linolenic acid (DGLA)) were significantly decreased with fish oil supplementation ($P < 0.001$). In the placebo group, the proportion of DHA was significantly reduced ($P = 0.011$) but there were no other significant effects in this group. Figure 2.3 shows the proportion of EPA in plasma PC for each subject in the fish oil group, indicating the reproducibility of the increase in EPA proportion. The ARA: EPA ratio in plasma PC was significantly decreased from 7.34 (at baseline) to 1.66 (after supplementation) in the fish oil group, but no significant change was observed in the placebo group (Figure 2.2).

TABLE 2.6: Fatty Acid Composition of Plasma PC (g/100 g Fatty Acid)
Values are means and SEM

Fatty acid	Fish oil (n = 9)				Placebo (n = 11)			
	Baseline		After 8 weeks		Baseline		After 8 weeks	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
14:0	0.40[a]	0.03	0.39	0.04	0.32[b]	0.02	0.3	0.04
16:0	31.38	0.44	31.22	0.46	31.09	0.26	32.00	0.73
16:1n-7	0.42	0.09	0.48	0.11	0.33	0.04	0.41	0.07
18:0	13.96	0.34	14.09	0.51	13.73	0.29	14.67	0.90
18:1n-9	10.17	0.47	9.94	0.65	10.64	0.35	10.31	0.52
*t*18:1n-9	1.19	0.07	1.23	0.06	1.32	0.06	1.26	0.06
18:2n-6	23.99	1.06	20.42**	0.80	23.42	0.78	22.15	1.26
18:3n-6	0.04	0.03	0.08	0.05	0.02	0.01	0.08	0.04
18:3n-3	0.20	0.07	0.27	0.05	0.21	0.04	0.25	0.04
20:0	0.08	0.04	0.15	0.04	0.07	0.03	0.16	0.07
20:3n-6	3.43	0.26	2.59**	0.29	3.53	0.22	3.29	0.29
20:4n-6	8.59	0.57	7.99	0.40	8.94	0.49	9.00	0.48
20:1n-9	0.03	0.02	0.08	0.06	0.07	0.03	0.10	0.04
20:5n-3	1.39	0.19	5.01**[c]	0.33	1.31	0.16	1.44[d]	0.38
22:5n-3	0.84	0.07	1.81**[c]	0.11	0.95	0.13	0.82[d]	0.09
22:6n-3	3.64	0.34	4.10	0.25	3.79	0.28	3.30*	0.28
22:1n-9	0.07	0.05	0.08	0.04	0.13	0.04	0.22	0.08
24:0	0.06	0.03	0.03	0.02	0.03	0.02	0.04	0.03

*Significantly different from baseline ($P = 0.011$).
**Significantly different from baseline ($P < 0.001$).
Numbers not sharing the same alphabetic superscript at baseline or after 8 weeks are significantly different between groups at that time point ($^{cd}P \leq 0.001$).

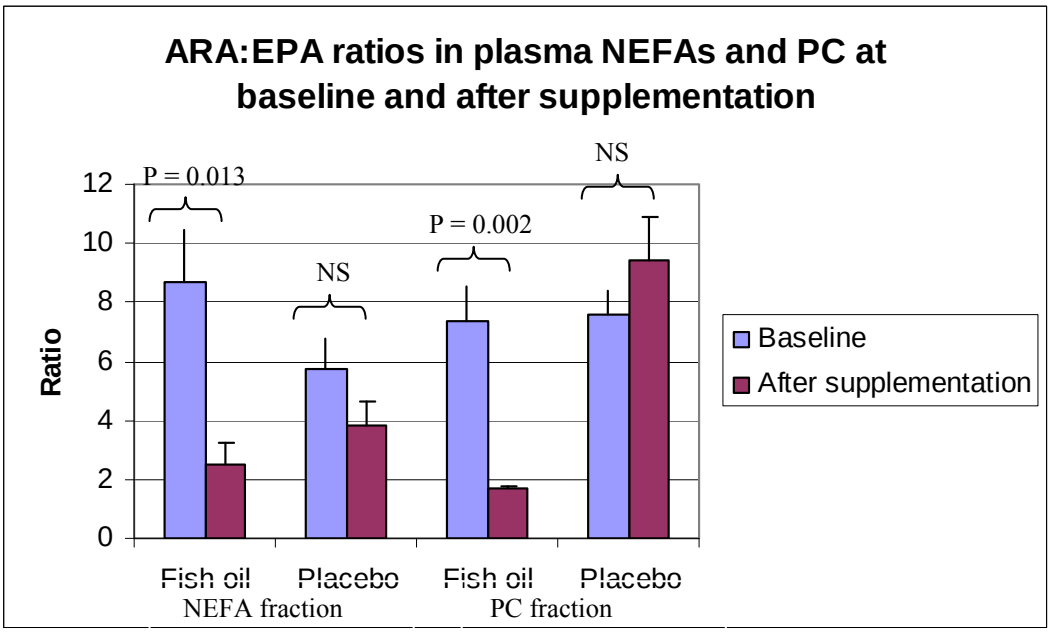


FIGURE 2.2: ARA: EPA Ratio in Plasma NEFAs and PC at Baseline and After Supplementation

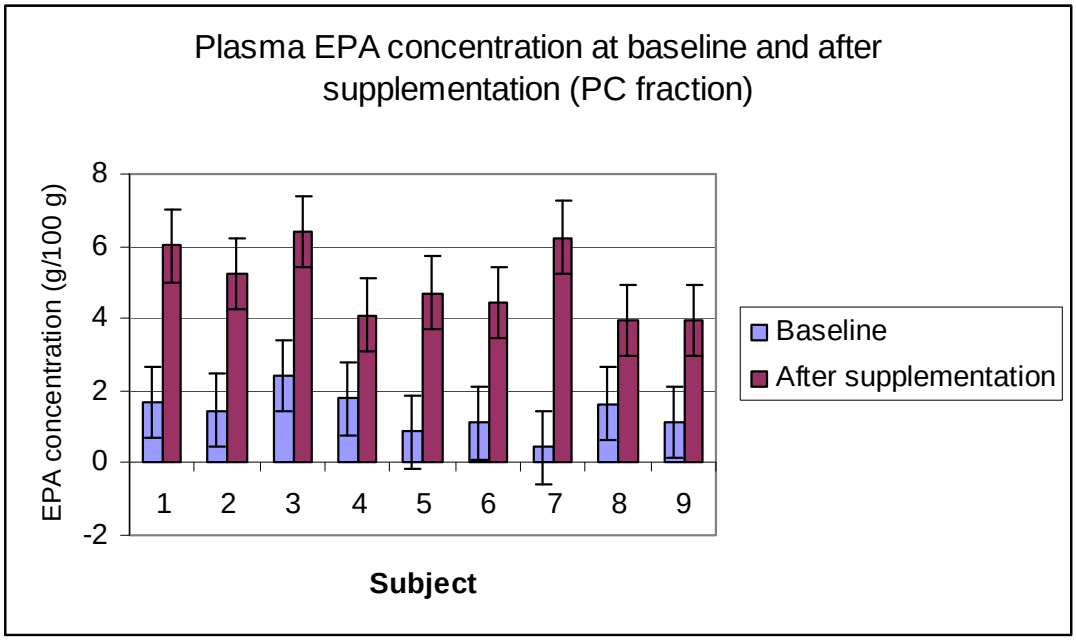


FIGURE 2.3: Effect of Fish Oil Supplementation on EPA in Plasma PC in Each Individual Subject

TABLE 2.7: Percent Change from Baseline (%Δ) of EPA and DHA in the Fish Oil Group

Fraction	%Δ EPA	P	%Δ DHA	P
TAGs	95.62 ± 28.37	0.131	65.17 ± 38.07	0.703
NEFAs	367.98 ± 106.80	0.026*	61.18 ± 37.42	0.885
PC	363.49 ± 123.64	0.011*	19.81 ± 36.84	0.012*
CEs	234.95 ± 45.03	< 0.0001*	28.99 ± 19.67	0.021*

*Significantly different from percent change in placebo group ($P < 0.05$)

Abbreviation: %Δ = percent change from baseline, TAGs = triacylglycerols, NEFAs = non-esterified fatty acids, PC = phosphatidylcholine, CEs = cholesteryl esters.

2.3.6 BMI and Blood Pressure

Table 2.8 shows the BMI and blood pressure values at baseline and after 8 weeks. There were no significant differences in blood pressure between groups at baseline or at the end of intervention or within a group at either time point. For BMI, there were no significant differences between groups at baseline or at the end of the study. However supplementation with fish oil caused a significant increase in BMI ($P = 0.023$).

Body weight was directly associated with systolic blood pressure ($r = 0.556$, $P = 0.011$), diastolic blood pressure ($r = 0.591$, $P = 0.006$) and BMI ($r = 0.739$, $P < 0.001$). In terms of inflammatory markers, only sE-selectin was found to be positively correlated with weight ($r = 0.520$, $P = 0.019$) and diastolic blood pressure ($r = 0.488$, $P = 0.029$).

TABLE 2.8: BMI and Blood Pressure at Baseline and after 8 Weeks Supplementation
Values are means (SEM) or median (10^{th}- 90^{th} percentile)

Outcome	Fish oil (n = 9)		Placebo (n = 11)	
	Baseline (0 weeks)	8 weeks	Baseline (0 weeks)	8 weeks
BMI (kg/m^2)	25.7 (0.8)	26.2* (0.8)	26.5 (1.0)	26.5 (1.0)
Systolic BP (mm Hg)	122.5 (102.5 - 140)	119.5 (101.5 - 152.5)	110 (108.7 - 143.4)	118 (100.9 - 149.6)
Diastolic BP (mm Hg)	71.5 (60 - 90)	72 (59 - 100)	66.5 (64.5 - 92.6)	72 (58.5 - 97.4)

*Significantly different from baseline (P = 0.023)

2.3.7 Plasma Lipid Concentrations

Total, LDL-and HDL-cholesterol concentrations were significantly increased in the placebo group ($P < 0.001$) (Table 2.9). In terms of absolute change, the elevations of total cholesterol and LDL-c ($P < 0.05$) were greater than that of HDL-c (Table 2.10). Fish oil also increased total cholesterol concentration ($P = 0.03$). However, the increases in LDL-c and HDL-c were not significant in the fish oil group. Percent changes in total cholesterol, LDL-c and HDL-c were lower in the fish oil group compared with the placebo group. In the placebo group, the percent change in HDL-c was greater (20.8%), compared with total cholesterol and LDL-c (18.6% and 16.0%, respectively). Nevertheless this change was not significant compared with the fish oil group ($P = 0.172$). Table 2.10 shows the ratio of total cholesterol: HDL-c in the placebo group was decreased (-0.06, -1.35%), due to a significant increase of HDL-c in plasma, but in contrast, this ratio was increased in the fish oil group (0.17, 5.06%). In terms of LDL-c: HDL-c ratio, both groups showed an increase because at the same time, LDL-c was also raised.

2.3.8 Plasma Glucose and Insulin Concentrations and HOMA

There were no significant effects on plasma glucose concentration in either group (Table 2.9). However, in the placebo group, insulin concentration and HOMA were significantly increased ($P < 0.05$) (Table 2.9). Fish oil did not significantly affect insulin concentration or HOMA.

TABLE 2.9: Plasma Lipid, Glucose and Insulin Concentrations and HOMA at Baseline and After 8 Weeks Supplementation
Values are means (SEM) or median (10^{th}-90^{th} percentile)

Outcome	Fish oil (n = 9)		Placebo (n = 11)	
	Baseline	8 weeks	Baseline	8 weeks
Total cholesterol (mmol/L)	5.2 (0.4)	5.6* (0.4)	4.8 (0.3)	5.6*** (0.3)
LDL-c (mmol/L)	3.1 (0.3)	3.3 (0.3)	3.0 (0.3)	3.5*** (0.3)
HDL-c (mmol/L)	1.4 (0.1)	1.5 (0.2)	1.1 (0.1)	1.3*** (0.1)
Triacylglycerol (mmol/L)	1.3 (0.2)	1.7 (0.3)	1.1 (0.1)	1.4 (0.2)
Total cholesterol:HDL-c ratio	4.1 (0.5)	4.2 (0.5)	4.5 (0.3)	4.5 (0.3)
LDL-c:HDL-c ratio	2.5 (0.4)	2.6 (0.4)	2.8 (0.3)	2.8 (0.2)
Total NEFA (umol/L)	396 (209 - 458)	388 (227 - 822)	334 (249 - 602)	485 (209 - 636)
Glucose (mmol/L)	5.9 (0.1)	6.06 (0.1)	5.74 (0.13)	5.8 (0.1)
Insulin (uIU/mL)	5.6 (1.3 - 28.4)	6.3 (1.9 - 26.2)	4.73 (4.1 - 6.2)	6.3** (4.7 - 8.1)
HOMA	1.3 (0.3 - 7.5)	1.7 (0.6 - 6.7)	1.2 (1.0 - 1.7)	1.6** (1.1 - 2.2)

*Significantly different between baseline and after 8 weeks ($P < 0.05$)
**Significantly different between baseline and after 8 weeks ($P < 0.02$)
***Significantly different between baseline and after 8 weeks ($P < 0.001$)
Abbreviations: BMI = Body mass index; LDL-c = low density lipoprotein-cholesterol; HDL-c = high density lipoprotein-cholesterol; NEFA = non-esterified fatty acid; HOMA = homeostatic model assessment.

TABLE 2.10: Changes (Absolute and Percentage) in Plasma Lipid, Glucose and Insulin Concentrations from Baseline
Values are means (SEM) or median (10^{th}- 90^{th} percentile)

Outcome	Change			
	Absolute		%	
	Fish oil	Placebo	Fish oil	Placebo
Total cholesterol (mmol/L)	0.41 (0.17)	0.84* (0.11)	7.90 (4.13)	18.62 (2.75)
LDL-c (mmol/L)	0.16 (0.11)	0.49* (0.07)	6.79 (-16.51 - 32.37)	15.56 (2.73 - 37.92)
HDL-c (mmol/L)	0.08 (0.09)	0.21 (0.03)	5.84 (6.74)	20.76 (4.02)
Triacylglycerol (mmol/L)	0.41 (0.23)	0.26 (0.17)	33.18 (15.76)	27.17 (14.82)
Total cholesterol:HDL-c ratio	0.17 (0.22)	-0.06 (0.08)	5.06 (5.23)	-1.35 (1.72)
LDL-c:HDL-c ratio	0.15 (0.15)	0.11 (0.10)	6.55 (7.05)	2.49 (4.66)
Total NEFA (umol/L)	73.38 (66.02)	93.27(51.06)	25.09(18.60)	33.74 (18.33)
Glucose (mmol/L)	0.13 (-0.37 - 0.63)	0.05 (-0.52 - 0.97)	2.21 (-5.67 - 11.69)	1.23 (-7.79 - 18.81)
Insulin (uIU/mL)	1.55 (1.22)	1.30 (0.36)	40.01 (15.07)	27.64 (7.65)
HOMA	0.45 (0.34)	0.35 (0.12)	44.19 (16.11)	30.62 (9.74)

*Significantly different between groups ($P < 0.04$)

2.3.9 Plasma Inflammatory Markers

The concentrations of plasma inflammatory markers at baseline, after 8 weeks and the relative changes (absolute and percent) are shown in Tables 2.11 and 2.12. There were no differences in plasma inflammatory marker concentrations between the two groups at study entry or at the end of the supplementation period. Neither placebo nor fish oil had a significant effect on the plasma inflammatory markers measured, although fish oil tended to lower sICAM-1 concentrations. When data were analysed as % change from baseline concentration, the two groups were significantly different for sICAM-1 ($P = 0.05$), with an approximately 10% decrease from baseline in the fish oil group (Table 2.12). Using data from both groups and from both time points it was observed that CRP concentration was directly correlated with IL-6 concentration ($r = 0.740$, $P < 0.001$).

TABLE 2.11: Plasma Inflammatory Marker Concentrations at Baseline and After 8 Weeks Supplementation
Values are means (SEM) or median (10^{th}-90^{th} percentile)

Outcome	Fish oil (n = 9)		Placebo (n = 11)	
	Baseline (0 weeks)	8 weeks	Baseline (0 weeks)	8 weeks
sICAM-1 (ng/mL)	257 (28)	220 (20)	224 (16)	240 (15)
sVCAM-1 (ng/mL)	458.4 (61.1- 1375.2)	447.7 (82.5 - 1345.3)	648.7 (436.8 - 957.3)	635.9 (430.8 - 1023.9)
sE-selectin (ng/mL)	85.1 (16.1)	94.4 (20.5)	84.3 (13.2)	69.3 (9.3)
sP-selectin (ng/mL)	36.3 (10.3)	37.6 (10.2)	33.5 (3.9)	32.3 (3.8)
IL-6 (pg/mL)	1.4 (0.9 - 2.2)	1.3 (0.7 - 2.2)	1.2 (0.8 - 3.9)	1.1 (0.8 - 1.7)
CRP (mg/L)	1.9 (1.9 - 4.0)	1.9 (1.9 - 4.1)	1.9 (1.9 - 9.3)	1.9 (1.9 - 4.1)

Abbreviations: sICAM-1 = soluble intercellular adhesion molecule-1; sVCAM-1 = soluble vascular cellular adhesion molecule-1; sE = soluble endothelial; sP = soluble platelet; IL-6 = interleukin- 6; CRP = C-reactive protein.

TABLE 2.12: Change (Absolute and Percentage) in Plasma Inflammatory Marker Concentrations
Values are means (SEM) or median (10^{th} -90^{th} percentile)

Outcome	Change			
	Absolute		%	
	Fish oil	Placebo	Fish oil	Placebo
sICAM-1 (ng/mL)	32.05 (22.55)	15.95 (12.02)	-9.48* (6.86)	9.85 (6.14)
sVCAM-1 (ng/mL)	39.65 (30.86)	3.11 (46.07)	-2.10 (6.74)	2.30 (6.45)
sE-selectin (ng/mL)	9.34 (18.36)	14.95 (14.31)	18.10 (17.37)	-2.49 (16.26)
sP-selectin (ng/mL)	1.31 (3.00)	-1.17 (2.10)	17.17 (10.62)	4.34 (9.04)
IL-6 (pg/mL)	0.21	-0.03	17.62	-2.86
	(-1.21 - 1.30)	(-2.30 - 0.23)	(-44.0 - 104.44)	(-65.74 - 16.33)
CRP (mg/L)	0	0	0	0
	(-2.01 - 0.00)	(-7.19 - 0.56)	(-50.3 - 0)	(-72.28 - 14.36)

*Significantly different between groups (P = 0.05)
Abbreviations: sICAM-1 = soluble intercellular adhesion molecule-1; sVCAM-1 = soluble vascular cellular adhesion molecule-1; sE = soluble endothelial; sP = soluble platelet; IL-6 = interleukin-6; CRP = C-reactive protein.

2.3.10 Relationship between a Marker of Habitual Fatty Acid Intake and CVD Risk Factors

Adipose tissue fatty acid composition is believed to reflect the fatty acid composition of the habitual diet (Katan et al. 1997; Leaf et al. 1995). In the fasting state NEFAs are released from adipose tissue and the fatty acid composition of plasma NEFAs reflects that of adipose tissue. Thus, the fatty acid composition of NEFAs in the fasting state reflects that of the habitual diet. Here the % of individual fatty acids in the NEFA fraction at baseline was correlated with plasma concentrations of CVD risk factors in order to identify significant relationships. Table 2.13 shows the significant associations that were observed. In terms of inflammatory markers, it was found that sP-selectin was inversely correlated with myristic acid (r = -0.509, P = 0.009) and palmitic acid (r = -0.512, P = 0.02), whereas sE-selectin was negatively associated with palmitic acid (r = -0.517, P = 0.02). On the other hand, CRP was positively associated with α-linolenic acid (r = 0.445, P = 0.049) and 20:1n-9 (r = 0.493, P = 0.027). None of n-3 PUFAs was found to be associated with any

plasma lipid concentration, apart from DHA which was shown to be negatively related to HDL-c concentration (r = -0.492, P = 0.02). Oleic acid was negatively correlated with total cholesterol and LDL-c concentrations, and subsequently inversely associated with the total cholesterol: HDL-c ratio (r = -0.541, P = 0.014) and the total cholesterol: LDL-c ratio (r = -0.578, P = 0.008).

TABLE 2.13: Correlations between Fatty Acids in Plasma NEFAs and CVD Risk Factors

CVD risk factor	Fatty acid	r	P
Inflammatory markers			
sP-selectin	14:0	-0.569	0.009
	16:0	-0.512	0.021
sE-selectin	16:0	-0.517	0.020
sVCAM-1	16:1n-7	0.458	0.042
CRP	18:3n-3	0.445	0.049
	20:1n-9	0.493	0.027
Lipids			
Total cholesterol	16:0	0.514	0.020
	18:0	0.447	0.048
	18:1n-9	-0.511	0.021
LDL-c	16:0	0.474	0.035
	18:0	0.456	0.043
	18:1n-9	-0.518	0.019
HDL-c	22:6n-3	-0.492	0.028
TAGs	20:4n-6	0.671	0.001
	18:1n-9	-0.462	0.040
	18:0	0.484	0.031
Total cholesterol: HDL-c ratio	18:0	0.488	0.029
	18:1n-9	-0.541	0.014
	20:1n-9	0.521	0.019
	22:6n-3	0.449	0.047
Total cholesterol: LDL-c ratio	18:0	0.487	0.029
	18:1n-9	-0.578	0.008
	20:5n-3	0.462	0.040
	22:6n-3	0.492	0.042
Total NEFA	22:5n-3	-0.452	0.045

2.3.11 Relationship between a Marker of Inflammatory Cell Fatty Acid Composition and CVD Risk Factors

The fatty acid composition of plasma PC has been shown to be highly related to that of inflammatory cells (Kew et al. 2003). Thus, the fatty acid composition of plasma PC at baseline was related to CVD risk factors (Table 2.14). Figure 2.4 shows that arachidonic acid in plasma PC was positively correlated with sICAM-1 ($r = 0.604$, $P = 0.005$). On the other hand, the proportion of DHA in plasma PC was inversely correlated with sICAM-1 ($r = -0.710$, $P < 0.001$) (Figure 2.5) along with 22:1n-9 ($r = -0.512$, $P = 0.02$).

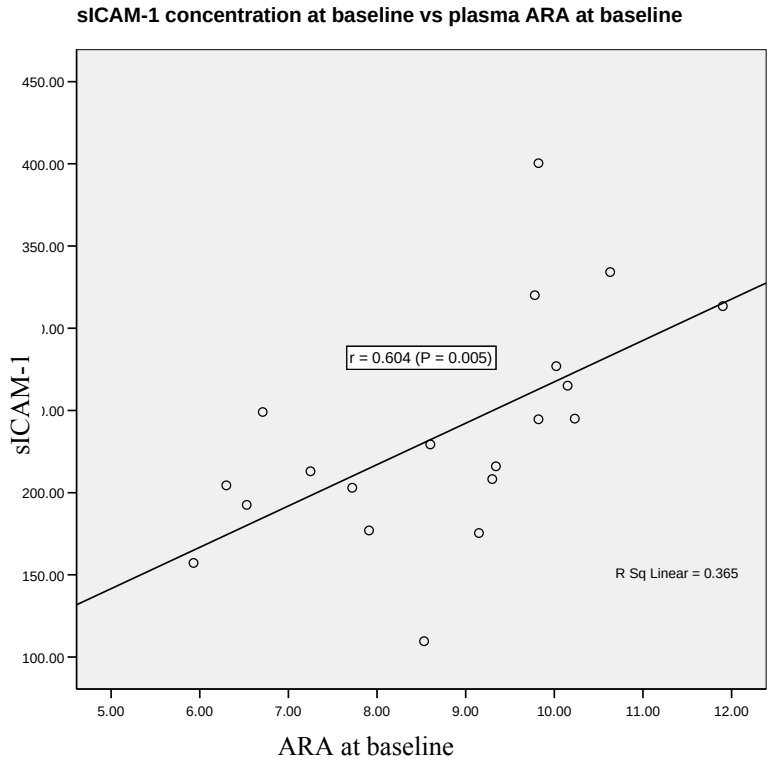


FIGURE 2.4: Proportion of ARA in Plasma PC vs. sICAM-1 Concentration

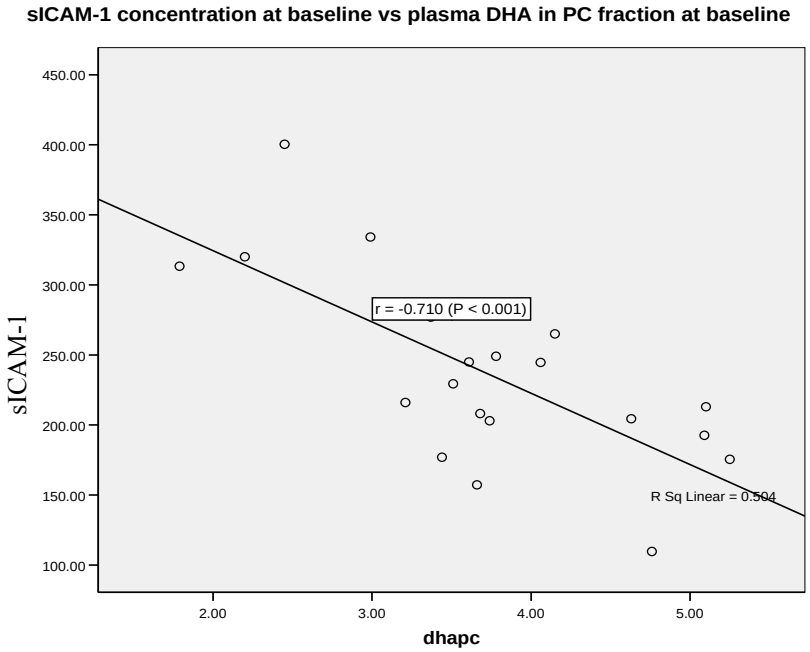


FIGURE 2.5: Proportion of DHA in Plasma PC vs. sICAM-1 Concentration

TABLE 2.14: Correlations between Fatty Acids in Plasma PC and CVD Risk Factors

CVD risk factor	Fatty acid	r	P
Inflammatory markers			
sE-selectin	20:0	0.604	0.005
sICAM-1	20:4n-6	0.604	0.005
	22:6n-3	-0.710	< 0.001
	22:1n-9	-0.512	0.020
Lipid profile			
Total cholesterol	22:6n-3	-0.529	0.016
LDL-c	18:0	0.518	0.019
	18:1n-9	-0.498	0.026
TAG	18:0	0.449	0.047
	18:2n-6	-0.494	0.027
	18:3n-6	0.623	0.003
LDL-c:HDL-c ratio	16:1n-7	0.484	0.030
	18:1n-9	-0.506	0.023
Total NEFA	20:5n-3	-0.488	0.029
Glucose	18:1n-9	-0.462	0.040
	18:3n-3	-0.450	0.047
HOMA	22:6n-3	0.446	0.049

2.3.12 Correlations between Changes in Individual Fatty Acids and Changes in Inflammatory Markers

If fatty acids are causally associated with inflammation, as determined by plasma inflammatory markers, then changes in status of those fatty acids should be associated with changes in levels of the inflammatory markers. Therefore, the change in the proportions of ARA, EPA and DHA in the different plasma pools was related to the change in inflammatory marker concentrations; pooled subjects were used in this analysis (Tables 2.15 to 2.17). There was an inverse relationship between the change in the level of plasma non-esterified ARA (NEFA fraction) and the change in sICAM-1 ($r = -0.588$, $P = 0.006$), and the changes in the level of plasma non-esterified EPA and the change in plasma sICAM-1 ($r = -0.543$, $P = 0.013$) and the change in plasma sVCAM-1 ($r = -0.463$, $P = 0.04$). Conversely, a positive association between the change in plasma non-esterified DHA and the change in plasma sICAM-1 ($r = 0.612$, $P = 0.04$) was observed. There were no significant correlations observed using the CE fraction (data not shown), although there were trends for the absolute and percentage changes in EPA in plasma CEs to be inversely related to the change in plasma sICAM-1 concentration ($r = -0.404$, $P = 0.077$ and $r = -0.312$, $P = 0.087$, respectively). Table 2.16 shows a negative correlation between change in DHA in plasma PC and change in sICAM-1 ($r = -0.675$, $P = 0.001$) and also clearly shown in Figure 2.6. There was a trend towards an inverse association between changes in plasma PC EPA and sICAM-1 ($r = -0.406$, $P = 0.076$). Surprisingly there were a significant positive association between changes in plasma PC EPA and IL-6 ($r = 0.451$, $P = 0.046$). An inverse association between percent change in ARA in plasma TAGs and percent change in sP-selectin was observed ($r = -0.455$, $P = 0.044$) (Table 2.17).

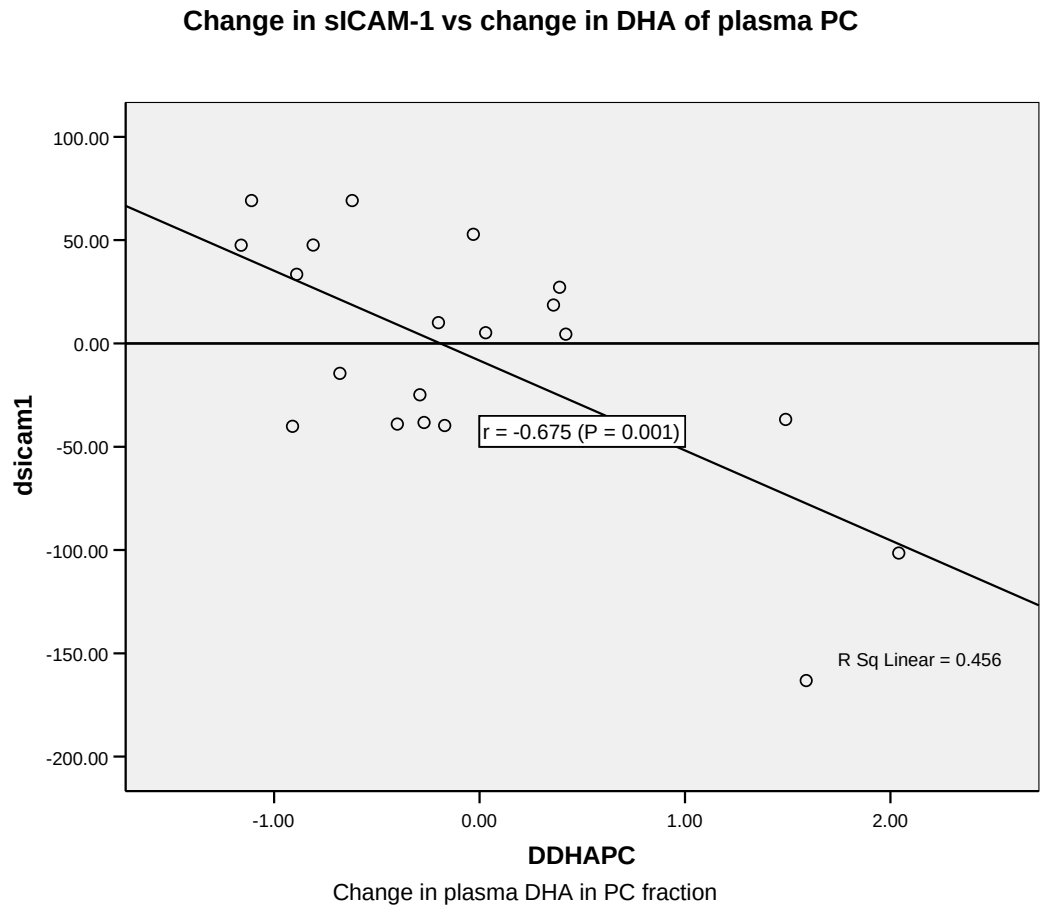


FIGURE 2.6: Change in the Proportion of DHA in Plasma PC vs. Change in sICAM-1 Concentration

TABLE 2.15: Correlation (r) between Changes in Individual PUFAs in Plasma NEFAs and Changes in Inflammatory Markers

Fatty acid (Absolute change)	Absolute change					
	sE-selectin	sP-selectin	sICAM-1	sVCAM-1	IL-6	CRP
ARA	–0.086	-0.053	-0.588*	-0.009	0.011	-0.079
EPA	0.085	-0.217	-0.543*	-0.463*	0.297	-0.093
DHA	-0.083	-0.205	-0.612*	-0.426	0.045	-0.030
Fatty acid (Percent change)	Percent change (%)					
	sE-selectin	sP-selectin	sICAM-1	sVCAM-1	IL-6	CRP
ARA	0.015	-0.032	-0.580*	-0.056	-0.008	0.005
EPA	0.175	0.118	-0.561*	-0.060	0.208	-0.126
DHA	-0.042	-0.038	-0.669*	-0.406	0.080	-0.100

*Significant at $P < 0.05$.

TABLE 2.16: Correlation (r) between Changes in Individual PUFAs in Plasma PC and Changes in Inflammatory Markers

Fatty acid (Absolute change)	Absolute change					
	sE-selectin	sP-selectin	sICAM-1	sVCAM-1	IL-6	CRP
AA	0.170	-0.193	-0.127	-0.263	0.108	0.188
EPA	0.025	-0.166	-0.406	-0.322	0.451*	0.153
DHA	-0.077	-0.248	-0.675***	-0.400	0.424	0.217
Fatty acid (Percent change)	Percent change (%)					
	sE-selectin	sP-selectin	sICAM-1	sVCAM-1	IL-6	CRP
AA	0.252 -	-0.345	-0.192	-0.337	0.132	0.156
EPA	0.064	-0.125	-0.131	-0.158	0.406	0.190
DHA	-0.068	-0.045	-0.474**	-0.070	0.184	0.236

*Significant at P = 0.046
**Significant at P = 0.008
***Significant at P = 0.001

TABLE 2.17: Correlation (r) between Changes in Individual PUFAs in Plasma TAGs and Changes in Inflammatory Markers

Fatty acid (Absolute change)	Absolute change					
	sE-selectin	sP-selectin	sICAM-1	sVCAM-1	IL-6	CRP
ARA	0.142	-0.259	0.100	0.168	0.125	-0.107
EPA	0.179	-0.091	-0.178	-0.239	0.268	0.129
DHA	0.174	-0.198	0.144	-0.255	0.070	0.069
Fatty acid (Percent change)	Percent change (%)					
	sE-selectin	sP-selectin	sICAM-1	sVCAM-1	IL-6	CRP
ARA	0.116	-0.455*	0.088	0.027	0.321	-0.033
EPA	0.436	0.356	-0.277	-0.164	0.182	0.083
DHA	0.056	-0.104	-0.351	-0.351	0.265	0.088

*Significant at P = 0.044

2.4 Discussion

This double-blind placebo-controlled study on the effect of a moderate dose (3 g/day) of a fish oil supplement providing 2.1 g/day of EPA plus DHA, mainly as EPA (1.8 g/day) was carried out for the duration of 8 weeks. The subjects were healthy male volunteers and they gave blood samples twice, at baseline before supplementation began and at the end of the study (after 8 weeks). The dose of LC n-3 PUFAs given was as the upper limit of the recommended range based upon the guideline range for oily fish intake among UK adults (SACN Report 2004). A MCFA-rich oil (providing 0.56 g/day of caprylic acid (8:0) and 1.96 g/day of capric acid (10:0)) was used as the placebo. MCFAs were used because these fatty acids are generally considered to be physiologically neutral and were not expected to change the blood (or cell) fatty acid profiles. This is because MCFAs enter the hepatic portal vein, rather than the systemic circulation, after absorption and so are directly targeted to the liver where they readily enter mitochondria and undergo rapid oxidation. Therefore the proportion of these fatty acids that make their way into the general circulation is very low. Indeed, there was no appearance of MCFAs in any of the plasma lipid fractions studied here. This agrees with the study by Tholstrup et al. (2004) which showed no appearance of 8:0 or 10:0 in plasma TAGs, PC or CEs after consumption of a diet containing increased amounts of these fatty acids. Thus, from this perspective MCFAs make a suitable placebo to study the effects of LC n-3 PUFAs.

The study was mainly aimed at identifying whether fish oil supplementation, providing LC n-3 PUFAs at the upper level of the current UK guideline range, but still achievable through a normal diet, has any beneficial effects on cardiovascular risk factors in apparently healthy, middle-aged male subjects. Thus, the main outcomes were based on the measurement of the blood lipid profile and inflammatory markers. In addition, the fatty acid composition in four plasma lipid fractions was measured.

2.4.1 Effect of Supplementation on Plasma Fatty Acid Composition

In this study, incorporation of EPA and DHA into plasma lipids was studied by investigating four different fractions, namely TAGs, NEFAs, CEs and PC. All four of these fractions represent transport pools for fatty acids in the bloodstream. NEFAs are released from adipose tissue as a result of hydrolysis of stored TAGs. Thus the fatty acid composition of the plasma NEFA fraction reflects the fatty acid composition in adipose tissue (Conquer & Holub 1998; Leaf et al. 1995) and these proportions are, in general, similar to the fatty acid composition of the habitual diet (Katan et al. 1997). Therefore analysis of this fraction can give information about the composition of dietary fatty acids. In addition, NEFAs are available for uptake by cells and tissues for use as fuels (Newsholme & Leech 1983) and may influence cell function (Kew et al. 2003). In the fasting state, fatty acids carried in the plasma TAGs fraction have been released from the liver and are being carried to peripheral cells and tissues. Plasma PC and CE fractions also represent transport of lipids including fatty acids. Because intact phospholipids and phospholipid fatty acids can exchange with cell membranes, the fatty acid composition of plasma PC is fairly similar to that of circulating cells (e.g. inflammatory cells; see Kew et al. 2003). Moreover, plasma PC originates mainly from liver, and so its composition may reflect tissue LC n-3 fatty acid levels, and also is a surrogate for cell membrane fatty acid composition. Plasma CEs, normally contain negligible amounts of LC n-3 fatty acids; thus this lipid class may also serve as useful marker for LC n-3 fatty acid intake (Harris 1989; Zock et al. 1997).

It was found that EPA was significantly increased in all four plasma lipid fractions studied, with the biggest increase (mean 368%) in the NEFA fraction. Differences in incorporation among the lipid fractions studied probably reflect the fact that this fatty acid is not esterified into different lipid classes to the same extent (Harris 1989). Plasma NEFA, PC and CE fractions all showed an average increase in EPA of about 320%. Previous studies reported increased incorporation of EPA in plasma phospholipids by approximately 400% with 3.6 to 4 g/d EPA supplementation (Woodman et al. 2002; Mori et al. 2000; Grimsgaard et al. 1997). The observed 363% increase in EPA in plasma PC when EPA at 1.8 g/d is given is consistent with the findings of Rees et al. (2006) obtained using different doses of EPA, and suggests a fairly linear dose-response incorporation of EPA into plasma phospholipids (Figure 2.7)

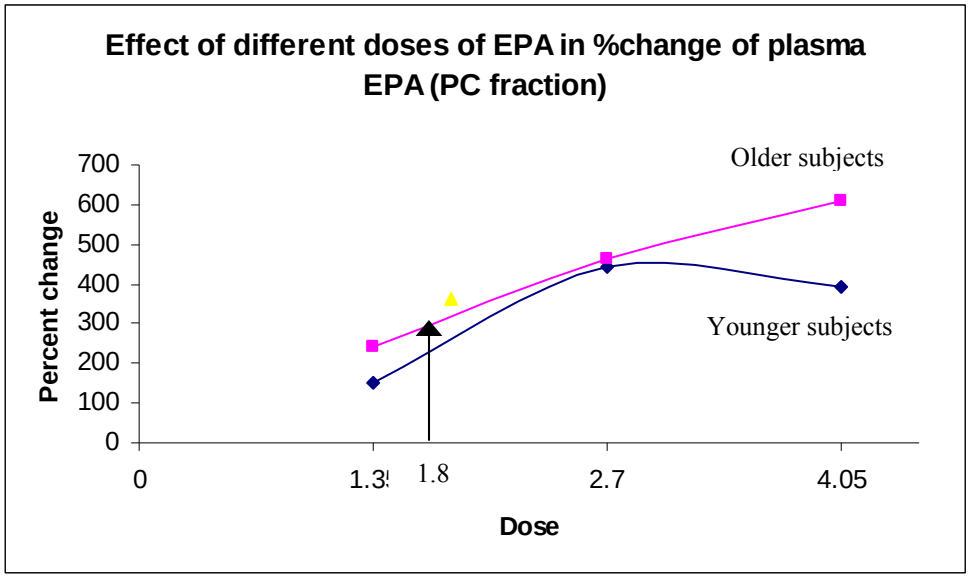


FIGURE 2.7: Effect of Different Doses of EPA on % Change of EPA in Plasma PC

Data for older and young subjects comes from Rees et al. (2006). The arrow shows a dose of 1.8 g/d EPA used in this study, indicating that the change in EPA seen at this dose agrees with that predicted by the Rees et al. (2006) data.

DHA was not significantly increased in any of the fractions studied, yielding average increases of 20 to 60%, depending upon the fraction studied. Previous studies supplementing with high amounts of DHA (> 3 g/d) reported significant incorporation of DHA (about a 150% increase) into plasma phospholipids (Woodman et al. 2003; Woodman et al. 2002, Mori et al. 2000; Halvorsen et al. 1997; Grimsgaard et al. 1997; Vidgren et al. 1997; Agren et al. 1996; Conquer & Holub 1996; Hamazaki et al. 1996). Thies et al. (2001) report a significant increase in plasma phospholipids DHA when DHA was given at a dose of 700 mg/day. However when DHA was given at a dose of 280 mg/day, as part of an EPA-rich fish oil preparation there was no significant effect on the proportion of DHA in plasma phospholipids (Thies et al. 2001). In the current study DHA was provided at 300 mg/day and the lack of increased DHA status observed is consistent with the findings of Thies et al. (2001) with low dose DHA. Thus, the lack of a significant increase in DHA status observed here probably simply reflects the relatively low amount of DHA in the supplement used.

The increase in the proportion of EPA in plasma TAGs and NEFAs was not accompanied by a significant decrease in proportion of any fatty acid in these fractions. However, in plasma CEs the proportion of LA and DGLA decreased by 7.6% and 12.4%, respectively (from 53.9% to 49.8% for LA and from 1.13% to 0.99% for DGLA) with the increase in

EPA being totally accounted for by these decreases. Likewise in plasma PC, the increase in EPA was accounted for by decrease in the proportion of LA (by 15%). ARA was not significantly affected by incorporation of EPA. In the recent study by Rees et al. (2006), EPA was shown to decrease the proportions of LA and DGLA in plasma phospholipids, but there was little effect on ARA. The findings of the current study largely agree with this. Thus, it seems that EPA more easily replaces LA (and DGLA), than ARA, in plasma phospholipids and CEs.

In general, n-3 PUFAs are proposed to decrease ARA-derived eicosanoid production by inflammatory cells, platelets and endothelial cells, which can subsequently result in inhibition of platelet aggregation, smooth muscle contraction and inflammation (British Nutrition Foundation 1992; 1999). These mechanisms result in anti-thrombotic and anti-inflammatory effects, which may be responsible for the protective effect of n-3 PUFAs against atherosclerosis and CVD (Calder & Yaqoob 2003). Indeed many fish oil supplementation studies reported that ARA levels in plasma and cell lipids are decreased by increased LC n-3 PUFA intake (Dewailly et al. 2001; Damsgaard et al. 2006; Yaqoob et al. 2003; Sperling et al. 1993). However, in the current study no significant reduction in ARA in plasma lipids was observed. In a study of postmenopausal women on HRT, Ciubotaru et al. (2003) found that after 5-week supplementation with fish oil providing 1.2 g EPA plus 1 g DHA/d, there was no significant effect on ARA in total plasma lipids. Thus, a modest LC n-3 PUFA intake, as used by Ciubotaru et al. (2003) and in the present study, may not alter ARA availability and subsequently influence the ARA pathway. If this is the case then relatively few functional effects may be observed with modest LC n-3 PUFA intakes. Interestingly, a study with high dose LC n-3 PUFAs (5.3 g EPA plus DHA/day) also did not significantly decrease the proportion of ARA in plasma phospholipids (Allard et al. 1997).

The present study indicates that modest increases in LC n-3 PUFA intake alter the fatty acid composition of plasma lipid fractions, but that there are differences in the effects seen according to the lipid fraction investigated.

2.4.2 BMI and Blood Pressure

BMI increased by 2% in subjects taking fish oil, but not in the placebo group. Since both groups consumed that same amount of oil, this difference is not due to consuming more oil or energy from oil. The difference may be due to a variation in diet or an alteration in metabolism in the fish oil group. No dietary or physical activity information was collected, so it is not possible to identify the reason for the increase in BMI.

Mean values for systolic and diastolic blood pressures were normal for both groups at baseline. No effect of fish oil on blood pressure was observed. In a meta-analysis of studies with hypertensive subjects who consumed an average 5.6 g/day of LC n-3 fatty acids, Morris et al. (1993) reported that there was a significant reduction in blood pressure of -3.4 (systolic) and -2.0 (diastolic) mm Hg. Moreover, Appel et al. (1993) found that blood pressure was decreased -5.5 (systolic) and -3.5 (diastolic) mm Hg in trials of untreated hypertensive subjects when they were given more than 3 g/day of LC n-3 fatty acids. The lack of effect on blood pressure in the current study may be because the dose of LC n-3 PUFAs used was too low. Furthermore, Mori et al. (2000) reported that DHA seems to be more effective compared with EPA in lowering blood pressure, and in the current study intake of DHA was low (0.3 g/d). In addition, it was also reported in a recent meta-analysis that the blood pressure lowering effects of fish oil tend to be greater in subjects that were older (> 45 years) or hypertensive (Geleijnse et al. 2002). The current study used subjects with a mean age < 45 y and they were mainly normotensive.

2.4.3 Plasma Lipid Profile

In the current study, supplementation with a MCFA-rich oil (as a placebo) significantly raised total cholesterol (by 17%) and LDL-c (8%). These findings are contrary to early studies which reported that saturated fatty acids with a chain length < 12 carbons failed to raise serum cholesterol (Katan et al. 1995). However, it was also documented that, compared with oleic acid, MCFAs (8:0 plus 10:0, as used in the current study) result in 11% higher plasma total cholesterol, 12% higher plasma LDL-c and 22% higher plasma TAGs in

healthy young men (Tholstrup et al. 2004). Thus, the findings of the current study agree in part with those of Tholstrup et al. (2004). Previously, there was little information about the effects of the MCFAs caprylic (8:0) and capric (10:0) on plasma lipids and lipoproteins (Kris-Etherton & Yu 1997). Some studies emphasized that generally their effects are similar to those of carbohydrates, even though there is limited evidence to support this conclusion (Grundy 1994). Indeed, Cater et al. (1995) described that MCFAs act similarly to palmitic acid as compared to oleic acid. In the current study, the additional MCFAs given to the subjects was only 2.5 g/day or equivalent to 0.7% of total calorie intake, and therefore it was assumed that the effects would be negligible. Most studies on the effects of individual fatty acids on plasma lipids were carried out at more than 4% of energy exchange. Since no dietary intake data were gathered in the current study, the increase in lipid profile might due to other factors, for instance, changes in dietary fat intake which was not monitored.

In terms of the effect of fish oil supplementation on the blood lipid profile, the results obtained were consistent with other studies showing that LC n-3 PUFAs do not lower the concentration of blood cholesterol, and indeed, that they have a slight cholesterol, LDL-c and HDL-c raising effect (Katan et al. 1995; Harris et al.1996). It was reported that at a dose of 4 g/day EPA plus DHA, the increases in total cholesterol and HDL-c were 5-10% and 1-3%, respectively (Harris 1997). In this study, total cholesterol and HDL-c were increased by 9% and 6%, respectively following supplementation with 2.1 g/d EPA plus DHA. Recently, no beneficial effect, in terms of changes in blood lipids in healthy individuals classified according to different levels of lipidemia, was seen following an increase in LC n-3 PUFA intake (corresponding to an intake of 2.5 to 3 times fish per week) (Castro et al. 2007). Since, there are many factors that may influence the cholesterol level in plasma, it is difficult to identify the precise dose response relationship between LC n-3 PUFAs and cholesterol concentrations.

There was no significant difference in the concentrations of plasma TAG between the fish oil and placebo groups. Fish oil supplementation did not exhibit the favourable TAG lowering effect as previously shown (Roche 1999; Harris 1996). Harris (1997) reported in a comprehensive review of human studies that approximately 4 g/day of LC n-3 PUFAs from fish oil decreased serum TAG concentration by 25%-30%, with accompanying increases in LDL-c of 5% to 10% and in HDL-c of 1% to 3% only. Again, most published studies

reporting significant reductions in TAG concentration use doses in the region of 4 g/day (Hamazaki et al. 1996; Grimsgaard et al. 1998; Mori et al. 2000; Woodman et al. 2002; Buckley et al. 2004). Moreover, a lower dose of 1.8 g/day EPA was reported to be ineffective at lowering TAG in patients with angina and elevated TAG levels (Yamamoto et al. 1995). The results of the current study are consistent with this lack of effect of modest doses of LC n-3 PUFAs. DHA is believed to be more important in TAG lowering compared with EPA (Mori et al. 2000). Even so, it has been documented that studies in which TAG were unchanged used relatively low doses of DHA (< 2 g/day DHA) (Conquer & Holub 1998), lower purity DHA (Conquer & Holub 1998; Hamazaki et al. 1996) and were carried out in subjects with normal TAG levels at baseline (Hamazaki et al. 1996). Again, reasons for a lack of TAG lowering in the current study may be too low an intake of LC n-3 PUFAs, not enough DHA or study of normotriglyceridemic subjects.

There was a trend towards increased plasma TAG concentration in both the fish oil and placebo groups, although this was not significant and not different between the groups. The increase in plasma TAG in the fish oil group may be due to other potential effect modifiers, such as dietary changes and BMI. BMI was significantly increased in the fish oil group. It is possible that the beneficial TAG lowering effect of fish oil might have been prevented by weight gain. The TAG-increasing effect of MCFAs that was observed by others (Uzawa et al. 1964; Hill et al. 1990; Tholstrup et al. 2004) is supported by the results of the present study, although the elevation seen was not significant. It has been suggested that a mechanism for the TAG-increasing effect of MCFAs is stimulation of insulin secretion and of anabolic-related processes (Hill et al. 1990).

The increased TAG level in subjects supplemented with fish oil might due to higher intake of carbohydrate from the diet, which subsequently can be converted to fatty acids and incorporated in TAGs (Becker & Hannun 2004). If this is the explanation, it can be hypothesized that ingestion of fish oil supplement was not responsible for the increased level of TAG in plasma but that this was due to unusual changes in the habitual diet of the subjects, particularly in carbohydrate intake. It has been reported that eucaloric low-fat and high-carbohydrate diet increased plasma TAG significantly (Kasim-Karakas et al. 2006).

Even though one of the mechanisms to explain how EPA and DHA might beneficially modulate cardiovascular risk has been proposed to be via TAG lowering, the role of TAGs in the pathogenesis of atherosclerosis is still controversial. This is due to an inconsistent association between circulating TAGs and coronary heart disease risk in prospective epidemiological studies (Stampfer et al. 1996; Kannel et al. 1985). Harris & Bulchandani (2006) in their review proposed that LC n-3 PUFAs reduce TAG levels via decreasing hepatic lipogenesis, but concluded that the mechanism of action in human remains unknown. Moreover, it has been suggested that the negative impact of raised TAGs in plasma is lower compared with elevated LDL-c: HDL-c ratios and that the risk is actually due to a strong inverse relationship between TAG and HDL-c concentrations.

2.4.4 Effect of Supplementation on Glucose, Insulin and HOMA

It was found that MCFAs significantly increased insulin and subsequently HOMA. Because blood glucose concentration did not change, this suggests induction of insulin resistance with MCFAs. In general, studies show that saturated fat significantly worsens insulin-sensitivity, while monounsaturated and polyunsaturated fatty acids improve it through modification of the composition of cell membranes (Riccardi et al. 2004). Recently Tholstrup et al. (2006) reported that, compared with the intake of high-oleic sunflower oil, MCFA intake resulted in higher plasma glucose; however insulin concentration did not differ significantly between the diets. There are few studies on MCFAs and fasting plasma glucose available, and the results of the present study are in accordance with these i.e. there is no effect of MCFAs on fasting blood glucose concentrations (Hill et al. 1990). On the other hand, Feskens et al. (1994) reported that PUFA intake was inversely associated with plasma insulin level. In the current study, positive effects of LC n-3 PUFAs on glucose and insulin sensitivity markers were not observed. Reported effects of LC n-3 PUFAs on insulin sensitivity are equivocal (Moore et al. 2006).

2.4.5 Inflammatory Markers

In the present study, the anticipated positive effects of LC n-3 PUFAs on plasma markers of inflammation were not observed, except there was a significant difference between the fish oil and placebo groups in terms of percent change from baseline for sICAM-1. It was demonstrated that in the fish oil group, sICAM-1 decreased by 9.5% compared with a 9.9% increase in the placebo group. Previously, the effect of LC n-3 PUFAs on markers of activated endothelium has been studied extensively. For example, in an *in vitro* study, De Caterina et al. (2000) found reduced cytokine-induced expression of VCAM-1, ICAM-1 and sE-selectin on cultured human endothelial cells exposed to DHA. Miles et al. (2001) reported no significant effects of 1.2 g EPA + DHA/day over 12 weeks on sICAM-1 or sE-selectin concentrations in 12 elderly healthy subjects, whereas sVCAM-1 was significantly reduced when compared with control subjects. Abe et al. (1998) demonstrated reduction of sICAM-1 after > 7 months supplementation with 4 g LC n-3 PUFAs/day in hypertriglyceridemic men. They also found that a reduction in sE-selectin was more pronounced in subjects with diabetes and a reduction in sVCAM-1 was observed only in subjects with diabetes as well. Berstad et al. (2003) reported that supplementation of 2.4 g of EPA plus DHA/day decreased sICAM-1 level significantly. Hjerkinn et al. (2005) found that supplementation with 2.4 g LC n-3 PUFAs daily and dietary counselling for 3 years reduced sICAM-1 and thrombomodulin indicating decreased endothelial activation. The finding of the current study with respect to sICAM-1 is in general accordance with previous studies (Berstad et al. 2003; Hjerkinn et al. 2005).

Even though there were no significant changes in CRP and IL-6 in the current study, CRP was strongly associated with IL-6 ($r = 0.740$, $P < 0.001$). Geelen et al. (2004) also reported that fish oil supplementation does not lower CRP concentrations in healthy subjects. Since CRP is synthesised by the liver and is regulated mainly by the cytokine IL-6 (Baumann & Gauldie 1990; Heinrich et al. 1990), this association was not unexpected. Whether CRP is simply a marker of inflammatory processes associated with atherosclerosis, or whether it plays an aetiological role in atherogenesis is still under debate (Ferns 2001). Although the pathophysiological role of CRP is unclear, it may be involved in complement activation and the regulation of tissue factor expression. Zwaka et al. (2001) suggested the involvement of CRP in monocyte chemotaxis, adhesion molecule upregulation and LDL uptake which can be associated with the development of atherosclerosis.

Again, a lack of pronounced significant effects of 2.1 g EPA plus DHA/day on plasma inflammatory markers in the current study may be due to too low intake of LC n-3 PUFA, especially DHA. Correlation analysis suggested that DHA rather than EPA is important in determining inflammatory marker concentrations

2.4.6 Does Habitual Fatty Acid Intake Influence CVD Outcomes?

The influence of habitual fatty acid intake on CVD outcomes was investigated based on the association between fatty acid composition in the NEFA fraction and CVD markers. NEFAs in plasma are mainly released from adipose tissue and subsequently used as a source of energy in the fasting condition (Newsholme & Leech 1983). It was assumed that the composition of fatty acids in adipose tissue, which can be determined in the fasting state using the NEFA fraction, is similar to the fatty acid composition of diet (Conquer & Holub 1998; Leaf et al. 1995).

LC n-3 PUFAs in the NEFA fraction were not found to be associated with lipid profile parameters, apart from DHA which was negatively associated to HDL-c concentration. Conquer & Holub (1998) reported that DHA supplementation, even at a high concentration (1.5 g DHA/day) had no significant effect on serum lipid and lipoprotein levels. It was also found that oleic acid (18:1n-9) was negatively correlated with total cholesterol, LDL-c and TAG and with the total cholesterol:LDL-c and total cholesterol: HDL-c ratios. This effect of oleic acid is consistent with reports from interventions with that fatty acid (Katan et al. 1995). However, in terms of the relationship between NEFAs and inflammatory markers, palmitoleic acid was inversely associated with sVCAM-1. This contrasts with findings of Yli-Jama et al. (2002) who reported an inverse linear association between serum non-esterified EPA, DHA and ARA and sVCAM-1 among elderly men at high risk for CHD (Hjerkinn et al. 2005). These authors did not report what the effect of oleic acid was. As far as I am aware, there are no other studies of this type, which restricts further discussion and comparison. There is a need for future research to determine the relationships between plasma NEFAs, since these are reflective of dietary fatty acid pattern, and CVD risk markers especially those related to inflammation.

2.4.7 Does Fatty Acid Composition of Inflammatory Cells Relate to CVD Outcomes?

The relationship between the fatty acids in plasma phospholipids and CVD outcomes was determined using the PC fraction at baseline. Plasma PC was used as a surrogate for inflammatory cell phospholipids, since there is a close relationship between the fatty acid composition of plasma PC and of inflammatory cells (Kew et al. 2003). ARA, a potentially potent of pro-inflammatory factor (Dwyer et al. 2004), was shown to be positively correlated with sICAM-1. Inflammatory cells typically contain a high proportion of ARA (~20%) which is usually the major substrate for eicosanoid synthesis. These eicosanoids are involved in modulating the intensity and duration of inflammatory responses (Lewis et al. 1990; Tilley et al. 2001). Yli-Jama et al. (2002) showed that non-esterified ARA was inversely associated with sVCAM-1 which might indicate an anti-inflammatory effect on the vascular endothelium. These contradictory results are difficult to explain, except that free-ARA and ARA in phospholipids might have different effects.

2.4.8 Correlation between Inflammatory Markers and Specific Fatty Acids

The role of LC n-3 PUFAs, particularly EPA and DHA on inflammatory responses have been studied in cell culture with results indicating that non-esterified DHA reduces the expression of adhesion molecules, particularly VCAM-1 (De Caterina et al. 1994; De Caterina et al. 1995; De Caterina et al. 2000; Weber et al. 1995). Miles et al. (2001) reported no significant effects of 1.2 g/day of n-3 PUFAs on sICAM-1 or sE-selectin, whereas sVCAM-1 was significantly decreased. In contrast, in the current study, it was found that there were no significant correlation between sVCAM-1 and n-3 PUFAs. Yli-Jama et al. (2002) reported in their human studies that the inverse correlation between sVCAM-1 was more prominent for DHA than EPA, with no association found for sICAM-1. Moreover, in their study there was a significant inverse linear association between serum non-esterified ARA and sICAM-1 level. However, after fish oil supplementation (2.1 g of EPA plus DHA per day for 18 months), there was a significant positive correlation between the change in serum non-esterified DHA and sVCAM-1 (Berstad et al. 2003). This is odd,

because it suggests that long term (18 months) supplementation with fish oil changed the relationship between the level of endothelial markers and fatty acids in the serum NEFA fraction. In other studies, it was found that supplementation with n-3 PUFAs significantly reduced sICAM-1 (Berstad et al. 2003; Hjerkinn et al. 2005), but correlation analyses did not provide any clues of possible relations between changes in the fatty acid profile and the reduction of sICAM-1 (Hjerkinn et al. 2005). The present study is the first time an inverse association between the levels of sICAM-1 and plasma LC n-3 PUFAs (DHA in PC fraction) has been documented. This finding strengthens the hypotheses from other studies that LC n-3 PUFAs, particularly DHA inhibits the inflammatory response in the vascular endothelium.

sICAM-1 concentration has been used to mark the inflammatory state (Adams et al. 1993), and several studies reported that patients with elevated sICAM-1 are at risk for future CVD events (Morisaki et al. 1997; Ridker et al. 1998). Therefore, a decrease in sICAM-1 concentration is likely to be clinically important. Therefore, it can be concluded that an intake of LC n-3 PUFAs at the upper end of current UK dietary recommendations is beneficial in modulating the inflammatory marker of sICAM-1 and subsequently has a potential to reduce progression of the atherosclerotic plaque and future CVD events.

2.5 Conclusion

The present study indicates that a dose of 2.1 g/d of EPA plus DHA is not sufficient to demonstrate maximal effects on CVD risk factors including inflammatory markers among fairly healthy young male subjects, although results show that percent change for the sICAM-1 decreased significantly compared with placebo. Correlation analyses showed a significant inverse relationship between the change in plasma DHA in PC fraction and change in sICAM-1 concentration. The expected positive effect of LC n-3 PUFAs on TAG levels was not seen. The reason for the observed trend to increased TAG among the subjects supplemented with fish oil remains unresolved. Results also agree with previous studies that non-esterified oleic acid has beneficial effect on cholesterol levels and DHA seems to be

more important than EPA since an inverse relationship between DHA and sICAM-1 was found in the present study. In healthy subjects, a higher dose of LC n-3 PUFAs will be needed to modify the CVD risk factor profile.

Chapter 3

The Effects of a Moderate Dose of Long Chain n-3 PUFAs on Cardiovascular Risk Factors, Particularly Plasma Lipids and Inflammatory Markers, in Patients Awaiting Carotid Endarterectomy

3.1 Introduction

3.1.1 Background

It has been widely reported that consumption of long chain n-3 polyunsaturated fatty acids (LC n-3 PUFAs) found mainly in oily fish protects against cardiovascular disease (CVD) morbidity and mortality (Daviglus et al. 1997; Albert et al. 1998; Hu et al. 2002; Iso et al. 2001; He et al. 2002). On this basis recommendations have been made to increase oily fish and LC n-3 PUFA consumption (SACN 2004). However, fish consumption is low in the UK population (SACN 2004) and it has proved difficult to convince people to change their diets to improve this (British Nutrition Foundation 2005; SACN 2004). An alternative source of LC n-3 PUFAs is fish oil capsules. Most fish oils contain about 35% of eicosapentaenoic acid (EPA) plus docosahexaenoic acid (DHA). Thus a single one g capsule of fish oil can provide only about 300 mg EPA+DHA. A pharmaceutical preparation of LC n-3 PUFAs in ethyl ester form called Omacor is available and this contains about 90% EPA plus DHA; Omacor is produced to a pharmaceutical standard

(Figure 3.1) because it is a licensed medication for secondary prevention of myocardial infarction (Harrison & Abhyankar 2005). The production process results in undetectable levels of environmental toxins (heavy metals, halogenated polycarbons and dioxins), less than 0.05% of *trans* fatty acids, < 90 mg of n-6, -7 and -9 fatty acids and markedly reduced potential for fishy taste or belching. Clinical studies using Omacor have demonstrated its efficacy to lower plasma

triacylglycerol (TAG) levels in various patient populations by 19% to 47% (Eritsland et al. 1996; Abe et al. 1996; Johansen et al. 1999; Grundt et al. 1995; Durrington et al. 2001; Davidson et al. 2007).

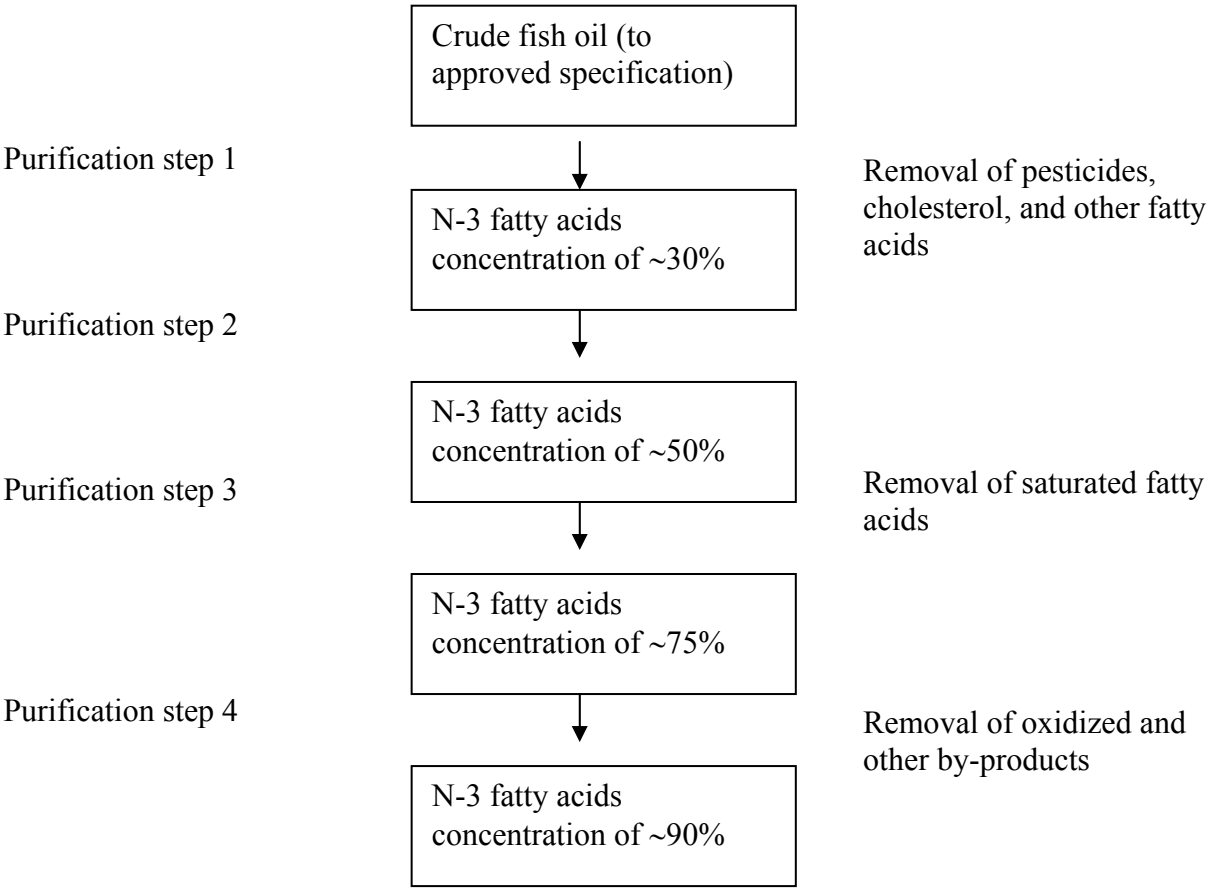


FIGURE 3.1: The Process of Omacor Preparation

In Chapter 2, the effect of a moderate dose of fish oil, providing 2.1 g/d EPA+DHA, on CVD risk factors, particularly plasma lipids and inflammatory markers, was investigated in healthy middle-aged males. The main conclusion was that moderate fish oil supplementation (2.1 g/d EPA plus DHA) is not sufficient to demonstrate maximal effects on CVD risk factors including inflammatory markers in this group. This may be because the total dose of LC n-3 PUFAs provided was too low, because not enough DHA was provided, or because relatively healthy subjects are fairly resistant to n-3 PUFA-induced improvements in the CVD risk factor profile. Therefore, in this chapter the effect of moderate dose n-3 PUFA supplementation (1.8 g/day) including a significant amount of DHA (0.675 g/day) in patients with advanced carotid atherosclerosis was investigated. It was thought that patients with disease and so, most likely, an altered risk factor profile might be more sensitive to the effects of LC n-3

PUFAs than healthy subjects. It was also thought that the higher amount of DHA used, compared with the study described in Chapter 2, would make effects more likely to occur. The total dose of LC n-3 PUFA was selected to be similar to that used in Chapter 2, to allow comparison, to be relevant to the guideline range for intake of LC n-3 PUFAs among UK adults (SACN Report 2004), and to allow comparison with results from a previous study of fish oil in carotid endarterectomy patients which used about this dose (Thies et al. 2003). No previous studies of Omacor in carotid endarterectomy patients have been performed. In accordance with the focus of the study described in Chapter 2, the main outcomes studied here were plasma lipid concentrations and inflammatory markers.

3.1.2 Aim of the Study

The aim of this study was to identify the effects of LC n-3 PUFAs (EPA plus DHA from Omacor) on selected cardiovascular risk factors, with a focus on plasma lipid concentrations and inflammatory markers. To address this aim, a double blind, randomised, placebo-controlled study of 1.8 g/d EPA plus DHA was conducted in patients awaiting carotid endarterectomy. The main outcomes were:

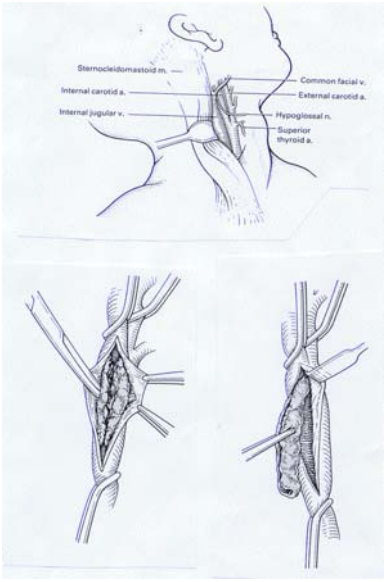
- The fatty acid composition of plasma phosphatidylcholine (PC)
- The fatty acid composition of plaque phosphatidylcholine (PC)
- The concentrations of plasma lipids
- The concentrations of plasma inflammatory markers
- The concentrations of plaque inflammatory markers (measured at the mRNA level)
- Blood pressure

In addition, comparison was made, for the first time, between inflammatory markers measured in atherosclerotic plaques and in plasma.

3.2 Methods

3.2.1 Study Design

Ethical permission for all protocols was obtained from the Southampton and South West Hampshire Local Research Ethics Committee. This study was carried out between March 2003 and December 2004 for the duration between 7 and 102 days before patients underwent surgical removal of the carotid plaque (Figure 3.2).



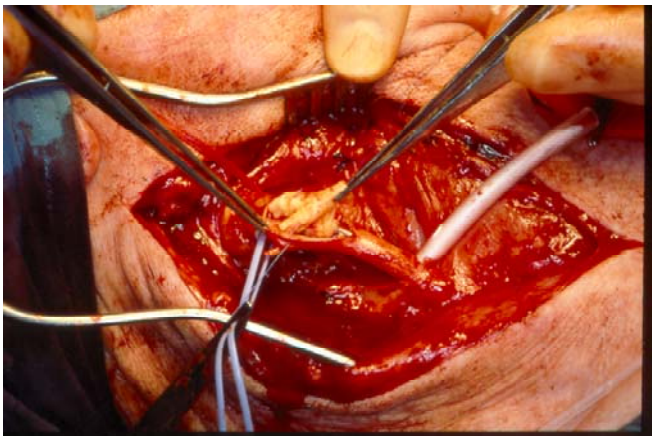


FIGURE 3.2: Surgical Removal of the Carotid Plaque

3.2.2 Recruitment of the Subjects

Patients were identified in the clinics of Professor C.P. Shearman, Southampton General Hospital and Mr. S. Payne, Princess Alexandra Hospital, Portsmouth. Patients were recruited by Mrs Jennifer Williams, Dr Abbie Cawood and Mrs Frances Knapper. Inclusion criteria were patients awaiting carotid endarterectomy, being > 18 y of age and being able to give written informed consent. Exclusion criteria were inability to give written informed consent, consuming fish oil supplements, eating > one oily fish meal per week, being pregnant or lactating, or participation in another trial. All patients gave their written informed consent after a detailed description of the study was provided prior to entering the study. Patients continued their usual medication throughout the

study period and they were advised not to change their current diet. A total of 121 subjects were recruited (n = 58 and n = 63 for Omacor and placebo, respectively); however only one hundred patients (n = 47 and n = 53 for Omacor and placebo, respectively) were used in the final analysis. Figure 3.3 shows the summary of the trial profile in this study. Patients were excluded from the study if they went to surgery within 7 days, others were lost to follow-up, and others reported that they could not comply. A further twelve patients who completed the study were excluded from the analysis because they were identified as protocol violators, including consuming insufficient capsules. Compliance was promoted by regular contact with patients and was monitored using capsule count and by analysis of the plasma fatty acid profile.

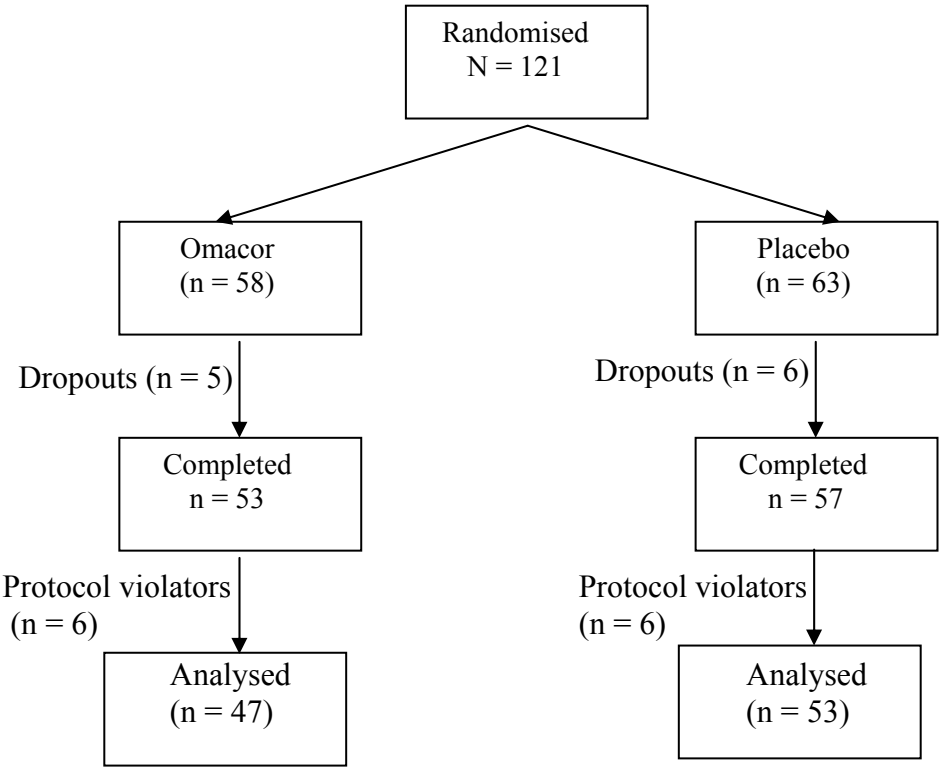


FIGURE 3.3: Summary of Trial Profile

3.2.3 Capsules

Capsules were provided in sealed containers and patients took two capsules/day (2 g of Omacor or olive oil as placebo) until surgery. The capsules were gelatine-coated and non-transparent. The amount of EPA and DHA provided was 810 mg and 675 mg/d

respectively, which is achievable in the diet with regular, but high, oily fish consumption. The amount of oleic acid provided by the olive oil placebo was 1.2 g/day; this amount is considered negligible since the adult UK consumption of oleic acid is 20-30 g/day (British Nutrition Foundation 1999).

3.2.4 Blood Samples and Measurements Made

At study entry and just prior to surgery a 20 mL fasting venous blood sample was taken from the forearm into tubes containing lithium-heparin. Blood samples were collected after a 12-hour overnight fast, put on ice and plasma separated by centrifugation at 3000 rpm for 10 minutes at 4^{o} C. Aliquots of plasma were kept frozen at -80^{o} C until analysis.

Weight and height were taken to the nearest 0.1 kg and 0.1 cm, respectively. Body mass index (BMI) was calculated as weight (in kg) divided by the square of the standing height (m^2). Two-blood pressure measurements were obtained from the non-dominant side arm and an additional reading was done if values from two consecutive measurements were more than 10 mm Hg apart. Subjects were lying down when blood pressure was measured using a blood pressure meter, Marquette®.

The plaque was collected at surgery, rinsed, sectioned and frozen at -80^{o} C until analysis. These plaque manipulations were performed by Dr Abbie Cawood.

3.2.5 Determination of Fatty Acid Composition of Plasma and Plaque Phosphatidylcholine

Plasma and plaque phosphatidylcholine (PC) fatty acid composition was determined as described for plasma in Section 2.2.7. This analysis was performed by Dr Abbie Cawood and Miss Ruth Young.

3.2.6 Measurement of TAG, and Total, LDL and HDL Cholesterol Concentrations in Plasma

Plasma TAG and total, LDL and HDL cholesterol concentrations were determined as described in Sections 2.2.8, 2.2.9, 2.2.10 and 2.2.11. These analyses were performed at the Chemical Pathology Laboratory in Southampton General Hospital by …….

3.2.7 Measurement of Inflammatory Marker Concentrations in Plasma

Plasma concentrations of IL-6, IL-10, sE-selectin, sICAM-1, sVCAM-1, MMP-2, MMP-9, CRP, TGF-β1, sCD40L, IP-10 and Mig were measured using commercial ELISA kits. sE-selectin, sICAM-1 and sVCAM-1 kits were from Biosource Europe, Nivelles, Belgium; IL-6, IL-10, MMP-2, MMP-9, sCD40L, IP-10, Mig and TGF-β1 kits were from R&D Systems, Minneapolis, USA; high sensitivity CRP kits were from Diagnostic System Laboratories, Texas, USA. For all assays, the manufacturer's instructions were followed and the absorbance was read on a plate reader using 450 nm as the primary wavelength and 610-650 nm as the reference wavelength. The sensitivity of the assays were: < 0.039 pg/mL (IL-6), < 0.5 ng/mL (IL-10), 0.5 ng/mL (sE-selectin), < 0.5 ng/mL (sICAM-1), 0.9 ng/mL (sVCAM-1), 0.16 ng/mL (MMP-2), < 0.156 ng/mL (MMP-9), < 4.61 pg/mL (TGF-β1), < 10.1 pg/mL (sCD40L), < 4.46 pg/mL (IP-10), < 11.3 pg/mL (Mig) and 1.6 ng/mL (CRP).

3.2.8 Measurement of Plaque mRNA Expression

Total cellular RNA was extracted from plaques using an RNAgents total RNA isolation system (Promega UK Ltd., Southampton). The purity and integrity of the RNA samples were assessed byA260/A280 spectrophotometric measurements. RNA samples were subsequently converted to cDNA by reverse transcription with reverse transcription system (Promega UK Ltd., Southampton). Real-time PCR was performed using an ABI prism 7700 Sequence Detection System (Applied Biosystems, Foster City, USA). PCR primers and probes (Table 3.1) were designed using Primer Express Program (version 2.0, Applied Biosystems). PCR products for the MMP1 were detected using the SYBR Green method and the others were detected using probes labelled with

reporter dye 6-carboxy-fluorescein (FAM) at the 5' and the quencher dye 6-carboxy-tetramethyl-rhodamine (TAMRA) at the 3' end. The 2–ΔΔCt method was used to analyze the results. The expression of each target gene was normalized to 36B4 value and applied to the subsequent statistical analysis. These measurements were performed by Dr Ren Ding.

TABLE 3.1: Primers and Probes for Real-time PCR

Primer/probe	Sequence
MMP1	Forward primer 5'-ACAGCCCAGTACTTATTCCCTTTG-3' reverse primer: 5'-GGGCTTGAAGCTGCTTACGA-3'
MMP3	Forward primer 5'-AAAGGATACAACAGGGACCAATTTA-3' reverse primer: 5'-CAGTGTTGGCTGAGTGAAAGAGA-3'
MMP7	Forward primer 5'-CGGGAGGCATGAGTGAGCT-3' reverse primer: 5'-GCATTTTTTGTTTCTGAGTCATAGAGATA-3'
MMP8	Forward primer 5'-CTCACAGGGAGAGGCAGATATCA-3' reverse primer: 5'-GATTCCATTGGGTCCATCAAA-3'
MMP9	Forward primer 5'-TGGCACCACCACAACATCAC-3' reverse primer: 5'-GCAAAGGCGTCGTCAATCA-3'
MMP12	Forward primer 5'-CCCACGTTTTTATAGGACCTACTTCT-3' reverse primer: 5'-GGGATAACCAGGGTCCATCAT-3'
MMP13	Forward primer 5'-ATGGCATTGCTGACATCATGA-3' reverse primer: 5'-GCCAGAGGGCCCATCAA-3'
TIMP1	Forward primer 5'-CCAGCGCCCAGAGAGACA-3' reverse primer: 5-AGCAACAACAGGATGCCAGAA-3'
TIMP2	Forward primer 5'-AGCCTGAACCACAGGTACCAGAT-3' reverse primer: 5'-AGGAGATGTAGCACGGGATCA-3'
IL6	Forward primer 5'-TCCAGGAGCCCAGCTATGAA-3' reverse primer: 5'-CCCAGGGAGAAGGCAACTG-3'
IL10	Forward primer 5'-CTACGGCGCTGTCATCGATT-3' reverse primer: 5'-TGGAGCTTATTATTAAAGGCATTCTTCA-3'
TNF-α	Forward primer 5'-ATCTTCTCGAACCCCGAGTGA-3' reverse primer: 5'-AGCTGCCCCTCAGCTTGA-3'
ICAM1	Forward primer 5'-GCAGACAGTGACCATCTACAGCTT-3' reverse primer: 5'-CTTCTGAGACCTCTGGCTTCGT-3'
PPAR-γ	Forward primer 5'-AAAGGCGAGGGCGATCTT-3' reverse primer: 5'-

	CCCATCATTAAGGAATTCATGTCA-3'
36B4	Forward primer 5'-CCATTCTATCATCAACGGGTACAA-3' reverse primer: 5'-AGCAAGTGGGAAGGTGTAATCC-3'
MMP3 probe	6FAM-TGCTCATGAAATTGGCCACTCCCTG-TAMRA
MMP7 probe	6FAM-CAGTGGGAACAGGCTCAGGACTATCTCAAGA-TAMRA
MMP8 probe	6FAM-TTGCTTTTTACCAAAGAGATCACGGTGACAAT-TAMRA
MMP9 probe	6FAM-ACTACTCGGAAGATTGCCGCGGG-TAMRA
MMP12 probe	6FAM-TGTAGATAACCAGTATTGGAGGTATGATGAAAGGAGACA-TAMRA
MMP13 probe	6FAM-TCTTTTGGAATTAAGGAGCATGGCGACTTCTA-TAMRA
TIMP1 probe	6FAM-AGAGAACCCACCATGGCCCCCTTT-TAMRA
TIMP2 probe	6FAM-CTGCGAGTGCAAGATCACGCGC-TAMRA
IL6 probe	6FAM-CCTTCTCCACAAGCGCCTTCGGT-TAMRA
IL10 probe	6FAM-AACAAGAGCAAGGCCGTGGAGCAG-TAMRA
ICAM1 probe	6FAM-CCGGCGCCCAACGTGATTCT-TAMRA
PPAR-γ probe	6FAM-ACAACAGACAAATCACCATTCGTTATCT-TAMRA
36B4 probe	6FAM-TCTCCACAGACAAGGCCAGGACTCGT-TAMRA

3.2.9 Statistical Analysis

The Kolmogorov-Smirnov and Shapiro-Wilk tests were applied to assess normality of data. Data for continuous variables that were normally distributed are presented as mean values and their standard errors (SEM) whilst non-normally distributed data are presented as medians and 10^{th} and 90^{th} percentiles. Comparison of normally distributed data between groups was performed using the unpaired Student's t-test and within a group using the paired Student's t-test. Non-normally distributed data were compared using the Wilcoxon signed ranks and Mann-Whitney U tests. Relationships between variables were evaluated using Pearson's correlation coefficient or Spearman's rank correlation. In all cases a value for $P \leq 0.05$ was taken to indicate a significant effect. SPSS version 14.02 (SPSS Inc., Chicago, IL) was used for all statistical analyses.

3.3 Results

3.3.1 Characteristics of the Subjects

Of the one hundred and twenty-one patients recruited, twenty-one patients were excluded for various reasons: some of them underwent surgery within 7 days, while some were unable to comply or were lost to follow-up. Characteristics of the included 100 patients were not significantly different between the groups at baseline (Table 3.2). Table 3.2 shows that patients' BMIs were slightly higher than normal (27.2 and 26.5 kg/m^2, for Omacor and placebo groups, respectively). Moreover, in general, patients were mildly hypertensive with mean systolic blood pressure of 155 mm Hg *vs* < 140 mm Hg (as normal reference), and many (> 50% of patients) had higher plasma LDL-c concentration than normal (< 2.6 mmol/L) whereas HDL-c concentration on the other hand, was slightly lower than normal (1.2-1.3 mmol/L *vs* > 1.6 mmol/L).

The median durations of supplementation were 26 and 29 days for Omacor and placebo, respectively, and ranged between 7 and 102 days. Based on the counting of the returned capsules, compliance was very high: 95.5% and 95.1% for Omacor and placebo groups, respectively.

Compliance amongst the 100 included patients was not significantly different between groups ($P = 0.808$). Based on the fatty acid analysis in plasma PC; EPA, DPA and DHA were significantly increased ($P \leq 0.001$) in the Omacor group (section 3.3.2) indicating compliance in that group.

TABLE 3.2: Baseline Characteristics of the Subjects
Unless otherwise indicated data are mean (SEM) or median (10^{th}-90^{th} percentile)

	Omacor (n = 47)	Placebo (n = 53)
Sex (n)		
Men	32	36
Women	15	17
Smoking status (n (%))		
Yes	8 (17.0)	8 (15.1)
No	8 (17.0)	11 (20.8)
Ex-smokers	31 (66.0)	34 (64.2)
Medication use (n (%))		
Aspirin	41 (87.2)	38 (71.7)
Anti-coagulant	13 (27.7)	5 (9.4)
Beta-blocker	17 (36.2)	16 (30.2)
Calcium	18 (38.3)	16 (30.2)
ACE-inhibitor	28 (59.6)	27 (50.9)
Statin	45 (95.7)	39(73.6)
Diuretics	26 (55.3)	25 (47.2)
Nitrates	13 (27.7)	8 (15.1)
Insulin	2 (4.3)	1 (1.9)
Age (y)	72.8 (1.5)	72.7 (1.2)
Duration of treatment (days)	21 (8-57)	22 (9-66)
BMI (kg/m^2)	27.2 (0.7)	26.5 (0.5)
Systolic blood pressure (mm Hg)	155.3 (4.1)	155.2 (3.0)
Diastolic blood pressure (mm Hg)	80.6 (2.0)	81.9 (1.8)
Total cholesterol (mmol/L)	4.8 (0.2)	4.9 (0.2)
LDL-c (mmol/L)	2.5 (1.6-4.3)	2.7 (1.5-4.4)
HDL-c (mmol/L)	1.3 (0.9- 2.4)	1.2 (0.9- 1.9)
Triglycerides (mmol/L)	1.3 (0.7-2.2)	1.3 (0.7-2.6)
Total cholesterol:HDL-c ratio	3.2 (2.4-4.8)	3.8 (2.6-5.0)
LDL-c:HDL-c ratio	1.8 (1.0-3.6)	2.24 (1.3-3.2)
sICAM-1 (ng/mL)	167.1 (73.2- 425.7)	216.1 (64.9- 444.7)
sVCAM-1(ng/mL)	673.3 (226.3-1578.4)	594.3 (271.8-1130.8)
sE-selectin (ng/mL)	46.0 (16.51-117.2)	45.7 (7.8-145.2)
IL-6 (pg/mL)	1.2 (0.4- 4.0)	1.2 (0.1- 4.8)
IL-10 (pg/mL)	1.5 (0-5.24)	0.5 (0-2.9)
MMP-2 (ng/mL)	192.2 (129.2-290.3)	191.2 (129.0-293.7)
MMP-9 (ng/mL)	167.2 (46.6-421.1)	138.0 (28.8-389.3)
TGF-β1 (ng/mL)	9308 (2394-19170)	9788 (3356-16844)
CRP (mg/L)	1.0 (1.0- 31.7)	1.0 (1.0-9.1)
sCD40L (pg/mL)	775.8 (243.2-2238.7)	774.0 (212.7-3139.9)
IP-10 (pg/mL)	111.3 (51.9-298.5)	105.8 (45.9-282.0)
Mig (pg/mL)	123.9 (45.9-417.7)	107.0 (31.4-252.9)

Abbreviations: BMI = Body mass index; LDL-c = low density lipoprotein-cholesterol; HDL-c = high density lipoprotein-cholesterol; sICAM-1 = soluble intercellular adhesion molecule-1; sVCAM-1 = soluble vascular cellular adhesion molecule-1; sE = soluble endothelial; IL-6 = interleukin-6; IL-10 = interleukin-10; MMP-2 = matrix metalloproteinase-2; MMP-9 = matrix metalloproteinase-9; TGF- β1 = transforming growth factor-β1; CR P= C-reactive protein.

3.3.2 Effect of Supplementation on the Fatty Acid Composition of Plasma PC

Table 3.3 shows the fatty acid composition of the plasma PC fraction. The most abundant fatty acids in this fraction were palmitic, linoleic, stearic and oleic comprising 31%, 19%, 14% and 11% of total fatty acids, respectively. There were no significant differences between groups at baseline for any fatty acid. The proportions of EPA, DPA and DHA were significantly increased ($P < 0.001$) in the Omacor group (Table 3.4, Figure 3.4). Linoleic (LA), γ-linolenic (ALA), dihomo-γ-linolenic (DGLA) and arachidonic acids were significantly decreased with Omacor (Table 3.4, Figure 3.4). There was a small increase in DPA and DHA in the placebo group (Figure 3.4).

TABLE 3.3: Fatty Acid Composition of Plasma PC (g/100 g Fatty Acid)
Values are means (SEM) or median (10^{th}-90^{th} percentile)

Fatty acid	Omacor (n = 47)				Placebo (n = 53)			
	Baseline		After treatment		Baseline		After treatment	
	Mean or median	SEM or 10^{th}-90^{th} percentile	Mean or median	SEM or 10^{th}-90^{th} percentile	Mean or median	SEM or 10^{th}-90^{th} percentile	Mean or median	SEM or 10^{th}-90^{th} percentile
14:0	0.4	0.2 - 0.5	0.3	0.2 - 0.6	0.4	0.2 - 0.5	0.4	0.2 - 0.6
16:0	30.8	29.0 - 34.4	31.8	29.2 - 34.4	31.0	27.9 - 34.1	31.3**	29.4 - 35.1
16:1n-7	0.6	0.3 - 1.2	0.6	0.4 - 1.1	0.6	0.3 - 1.0	0.7	0.4 - 1.1
18:0	14.5	12.1 - 17.4	14.5	12.2 - 16.9	14.5	12.6 - 16.6	13.9*	12.2 - 16.2
18:1n-9	11.2	9.0 - 13.8	10.4**[a]	8.2 - 12.8	11.3	9.8 - 14.5	11.4[b]	9.0 - 14.5
18:2n-6	19.1	0.5	16.5***[c]	0.4	19.9	0.4	18.0*[d]	0.4
18:3n-3	0.2	0 - 0.4	0.1	0 - 0.3	0.2	0	0.2	0
18:3n-6	0.3	0.1 - 0.6	0.2**	0.1 - 0.3	0.2	0.1 - 0.6	0.2	0.1 - 0.5
20:3n-6	3.3	2.3 - 4.5	2.6***[e]	1.7 - 3.7	3.1	2.3 - 4.5	3.3[f]	2.3 - 5.1
20:4n-6	9.9	6.3 - 14.2	9.1*	6.7 - 12.7	9.8	6.6 - 12.9	10.0	6.7 - 14.2
20:5n-3	1.2	0.7 - 2.1	3.5***[e]	2.1 - 4.4	1.2	0.8 - 1.7	1.2[f]	0.8 - 2.0
22:5n-3	1.0	0	1.3***[c]	0	1.0	0	1.1*[d]	0
22:6n-3	3.7	0.2	5.8***	0.2	3.9	0.2	4.2 *	0.2

*Significantly different from baseline ($P < 0.05$)
**Significantly different from baseline ($P < 0.01$)
***Significantly different from baseline ($P < 0.001$)
Values not sharing the same alphabetic superscript are significantly different between groups at this point; [ab]$P \leq 0.05$, [cd]$P \leq 0.01$, [ef] $P \leq 0.001$.

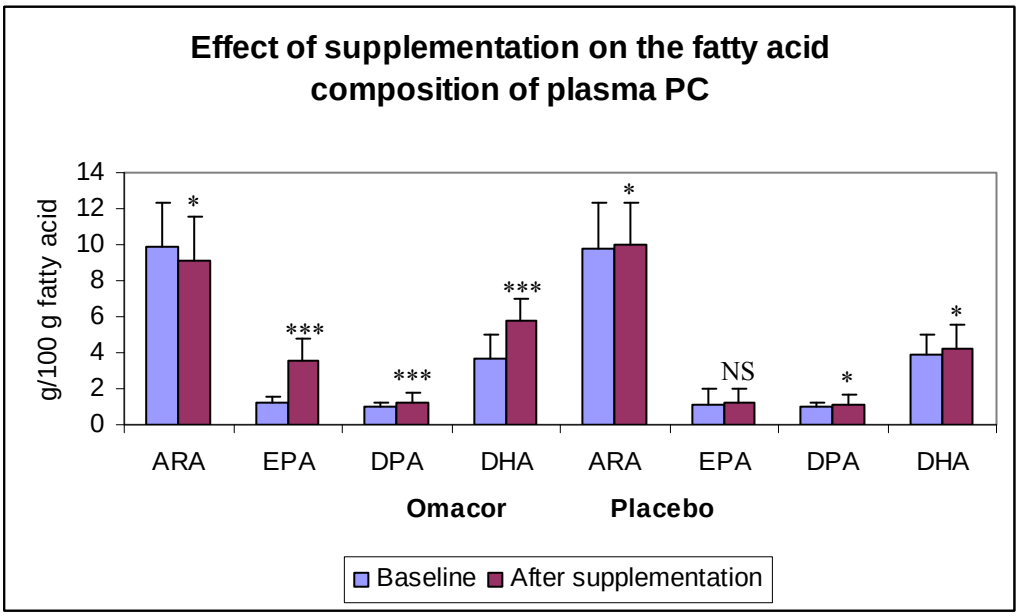


FIGURE 3.4: Effect of Supplementation on the Plasma PC n-6 and n-3 Fatty Acid Composition
*Significantly different from baseline ($P < 0.05$)
***Significantly different from baseline ($P < 0.001$)

Table 3.4 shows the percent change for the main n-6 and n-3 fatty acids present in plasma PC. In the Omacor group, it was found that LA, DGLA and ARA (n-6 fatty acids) were decreased significantly from baseline, 13%, 22% and 2%, respectively compared with the placebo ($P < 0.01$). On the other hand, the n-3 fatty acids increased significantly ($P < 0.001$) by 161% and 70% for EPA and DHA, respectively. As a result of these changes, the ratios of ARA: EPA and ARA:DHA in the Omacor group were significantly decreased from 8.98 to 3.14 and from 2.96 to 1.74, respectively ($P < 0.001$) (Figure 3.5) and no significant effect was found in the placebo group.

TABLE 3.4: Percent Change from Baseline for n-6 and n-3 Fatty Acids in Plasma PC
Values are mean (SEM) or median (10^{th}-90^{th} percentile)

Fatty acid	% change from baseline		P
	Omacor	Placebo	
LA, 18-2n-6	-13.0 (1.8)	-2.2 (3.2)	0.005
DGLA, 18:3n-6	-22.2 (-92.3-66.6)	0 (-58.4-126.5)	0.024
ARA, 20:4n-6	-2.1 (-21.4-14.9)	0 (-18.5-28.4)	0.006
EPA, 20:5n-3	160.9 (54.2-383.6)	0 (-38.2-56.4)	< 0.001
DPA, 22:5n-3	29.3 (5.6)	15.3 (5.8)	0.088
DHA, 22:5n-3	70.1 (8.5)	12.9 (5.0)	< 0.001

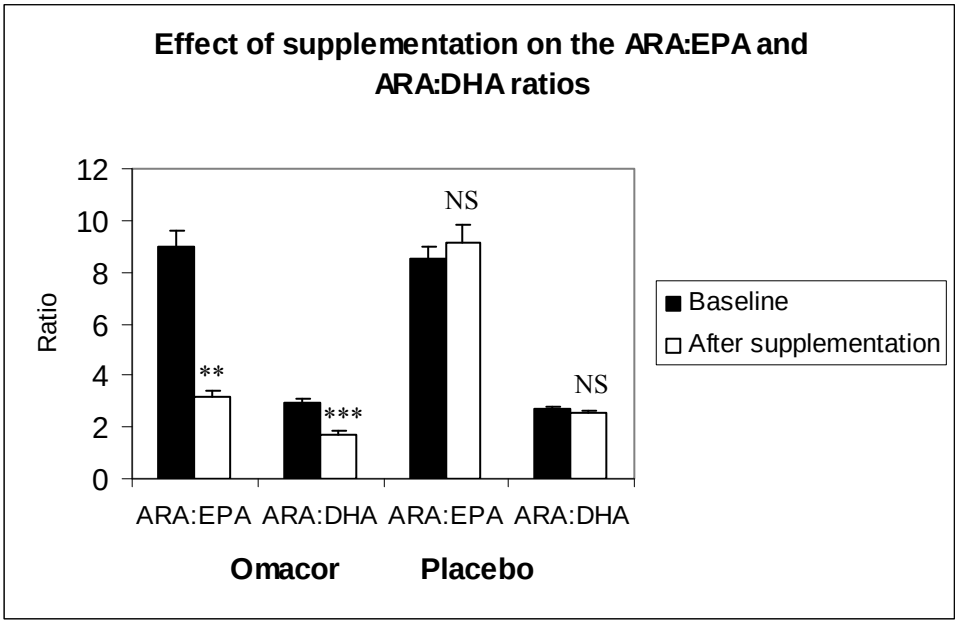


FIGURE 3.5: Effect of Supplementation on the ARA: EPA and ARA:DHA Ratios of Plasma PC
***Significantly different from baseline (P < 0.001)

3.3.3 Effect of Supplementation on the Fatty Acid Composition of Plaque PC

Table 3.5 shows the fatty acid composition of plaque PC. The most abundant fatty acids were palmitic, stearic and oleic comprising 36%, 23% and 11% of total fatty acids, respectively. Total LC n-3 PUFAs was approx. 6% of total fatty acids with most as DHA (about 60% of total LC n-3 PUFAs). There were no significant differences between groups for any fatty acid in plaque PC apart from EPA which was significantly higher in the Omacor group (P < 0.001). The ratio of ARA: EPA in the Omacor group was much lower than that in the placebo group (13.5 and 24.5, respectively).

TABLE 3.5: Fatty Acid Composition of Plaque PC (g/100 g Fatty Acid)
Values are means (SEM) or median (10^{th}-90^{th} percentile)

Fatty acid	Omacor (n = 47)		Placebo (n = 53)	
	Mean or median	SEM or 10^{th}-90^{th} percentile	Mean or median	SEM or 10^{th}-90^{th} percentile
14:0	0.7	0	0.7	0
16:0	35.6	31.0 - 39.7	35.1	11.0 - 38.6
16:1n-7	0.6	0.3 - 1.1	0.6	0.3 - 1.0
18:0	22.7	0.8	22.4	0.8
18:1n-9	11.4	8.7 - 14.8	11.8	9.0 - 15.1
18:2n-6	9.6	7.0 - 12.3	9.4	6.7 - 12.8
18:3n-3	0.1	0- 0.2	0.1	0- 0.2
18:3n-6	0.1	0- 0.2	0.1	0- 0.2
20:3n-3	1.8	1.1 - 2.8	1.8	1.2 - 2.8
20:4n-6	9.6	6.7- 14.4	10.0	6.4 - 13.6
20:5n-3	0.7***	0.3 - 1.6	0.4	0.2 - 0.7
22:5n-3	1.0	0.7 - 1.9	1.0	0.5 - 1.5
22:6n-3	2.6	0.1	2.3	0.1

***Significantly different from placebo group ($P < 0.001$)

3.3.4 Effect of Supplementation on BMI, Blood Pressure and Plasma Lipid Profile

Table 3.6 shows the BMI, blood pressure and plasma lipid profile at baseline and after supplementation. Interestingly, it was found that there were significant reductions in systolic blood pressure, from 156 mm Hg to 143 mm Hg ($P = 0.014$) and from 155 mm Hg to 142 mm Hg ($P = 0.001$) with Omacor and placebo supplementation, respectively. The decrease in diastolic blood pressure was more apparent, from 81 mm Hg to 72 mm Hg in Omacor group and 82 mm Hg to 74 mm Hg in placebo group, respectively (both $P < 0.001$). Plasma TAG and total cholesterol concentrations were significantly decreased in both groups (both $P < 0.001$) and HDL-c was also found to decrease significantly (both $P = 0.05$). LDL-c was significantly decreased with Omacor ($P = 0.015$), but not in the placebo group. There were no significant effects on total cholesterol:HDL-c and LDL-c:HDL-c ratios in either group.

Similar trends were also found when the data was analysed by gender (Table 3.7 and Table 3.8) except for LDL-c which was not significantly decreased in females in the Omacor group, but was significantly decreased ($P = 0.022$) in females in the placebo group.

TABLE 3.6: Blood Pressure and Plasma Lipid Concentrations at Baseline and After Supplementation
Data are means (SEM) or median (10^{th}-90^{th} percentile)

Outcome	Omacor (n = 47)		Placebo (n = 53)	
	Before	After	Baseline	After
BMI (kg/m^2)	27.3 (0.7)	27.0	26.5 (0.5)	26.4 (0.5)
Systolic blood pressure (mm Hg)	155.9 (4.1)	142.7* (3.5)	155.2 (3.0)	142.0** (2.6)
Diastolic blood pressure (mm Hg)	80.6 (2.0)	72.4** (1.5)	82.0 (1.8)	73.8** (1.6)
TAG (mmol/L)	1.3 (0.7-2.2)	1.0** (0.6-1.6)	1.3 (0.7-2.6)	1.1* (0.6-2.4)
% change TAG	-20.0 (-52.4-19.0)		-9.1 (-52.9-60.4)	
Total cholesterol (mmol/L)	4.8 (0.2)	4.3** (0.2)	4.9 (0.2)	4.3** (0.2)
% change TC	-12.7 (-22.0-6.8)		-8.8 (-32.3-9.0)	
HDL-c (mmol/L)	1.3 (0.9-2.4)	1.3** (0.9-2.1)	1.2 (0.9-1.9)	1.1** (0.7-1.8)
% change HDL-c	-10.81 (-25.1-7.1)		-9.94 (-30.9-13.3)	
LDL-c (mmol/L)	2.5 (1.6-4.3)	2.5* (1.6-3.7)	2.8 (1.5-4.4)	2.5 (1.5-3.9)*
% change LDL-c	-5.4 (-26.7-15.1)		-7.1 (-42.5-28.6)	
Total cholesterol:HDL-c ratio	3.2 (2.4-4.8)	3.4 (2.5-5.1)	3.8 (2.6-5.0)	3.9 (2.4-5.4)
LDL-c:HDL-c ratio	1.8 (1.0-3.6)	2.0 (1.2-3.4)	2.2 (1.3-3.2)	2.4 (1.3-3.7)

*Significantly different from baseline ($P \leq 0.05$)
**Significantly different from baseline ($P < 0.01$)

TABLE 3.7: Blood Pressure and Plasma Lipid Concentrations at Baseline and After Supplementation in Male Patients
Data are means (SEM) or median (10^{th}-90^{th} percentile)

Outcome	Omacor (n = 32)		Placebo (n = 36)	
	Baseline	After	Baseline	After
BMI (kg/m^2)	26.8 (0.8)	26.8** (0.9)	26.9 (0.6)	26.8* (0.6)
Systolic blood pressure (mm Hg)	154.7 (4.6)	135.0** (3.6)	156.8 (3.5)	143.3*** (2.8)
Diastolic blood pressure (mm Hg)	83.0 (58.2-98.0)	70.0** (56.4-86.6)	86.0 (65.7-99.7)	75.0 (61.4-90.6)
TAG (mmol/L)	1.2 (0.7-2.4)	1.0** (0.6-1.9)	1.3 (0.7-2.6)	1.1*** (0.6-2.4)
% change TAG	-20.0 (-53.7-27.7)		-7.0 (-51.7-64.8)	
Total cholesterol (mmol/L)	4.7 (0.2)	4.2*** (0.2)	4.6 (0.2)	4.3 (0.2)
% change TC	-12.9 (-23.93-9.1)		-8.6 (-29.7-13.2)	
HDL-c (mmol/L)	1.3 (0.9-2.3)	1.1*** (0.8-2.1)	1.1 (0.9-1.7)	1.0*** (0.7-1.4)
% change HDL-c	-13.67 (-28.2-11.2)		-10.7 (-32.1-16.5)	
LDL-c (mmol/L)	2.5 (1.6-4.1)	2.4* (1.5-3.7)	2.4 (1.4-4.4)	2.4 (1.5-3.9)
% change LDL-c	-5.0 (-28.6-30.6)		-7.1 (-35.0-32.8)	
Total cholesterol:HDL-c ratio	3.6 (0.2)	3.7 (0.2)	4.0 (0.2)	4.2 (0.2)
LDL-c:HDL-c ratio	1.9 (1.0-3.4)	2.0 (1.2-3.4)	2.3 (1.2-3.4)	2.6* (1.4-4.0)

*Significantly different from baseline ($P \leq 0.05$)
**Significantly different from baseline ($P \leq 0.01$)
***Significantly different from baseline ($P \leq 0.001$)

TABLE 3.8: Blood Pressure and Plasma Lipid Concentrations at Baseline and After Supplementation in Female Patients
Data are means (SEM) or median (10^{th}-90^{th} percentile)

Outcome	Omacor (n = 15)		Placebo (n = 17)	
	Before	After	Baseline	After
BMI (kg/m^2)	28.0 (1.4)	27.5 (1.3)	25.5 (1.1)	25.5 (1.1)
Systolic blood pressure (mm Hg)	156.6 (8.5)	158.5 (6.1)	151.9 (6.0)	139.1 (5.7)
Diastolic blood pressure (mm Hg)	81 (60.2-102.2)	75 (61.2-90.0)	78 (61.6-96.2)	70** (57.2-80.8)
TAG (mmol/L)	1.3 (0.1)	1.0* (0.1)	1.1 (0.7-2.7)	0.9 (0.6-1.9)
% change TAG	-20.5 (5.8)		-18.7 (7.9)	
Total cholesterol (mmol/L)	5.0 (3.7-7.5)	4.5** (3.3-6.5)	5.2 (4.1-7.0)	4.7** (2.9-5.7)
% change TC	-8.9 (2.6)		-14.2 (4.3)	
HDL-c (mmol/L)	1.6 (0.1)	1.4* (0.1)	1.6 (0.1)	1.4* (0.1)
% change HDL-c	-9.7 (-24.7-11.3)		-9.9 (-27.1-8.2)	
LDL-c (mmol/L)	3.0 (0.3)	2.7 (0.2)	3.2 (0.2)	2.64* (0.2)
% change LDL-c	-7.01 (3.4)		-14.4 (5.7)	
Total cholesterol:HDL-c ratio	3.0 (1.8-5.0)	3.3 (1.9-5.4)	3.4 (0.2)	3.2 (0.3)
LDL-c:HDL-c ratio	1.7 (0.9-4.3)	2.0 (0.8-3.6)	2.1 (0.2)	2.0 (0.2)

*Significantly different from baseline ($P \leq 0.05$)
**Significantly different from baseline ($P < 0.01$)
***Significantly different from baseline ($P < 0.001$)

3.3.5 Plasma Inflammatory Markers

The concentrations of plasma inflammatory markers at baseline and after treatment (between 7 and 102 days) are shown in Table 3.9. Supplementation with Omacor decreased sE-selectin significantly ($P = 0.006$) from 46.0 ng/mL at baseline to 30.2 ng/mL after supplementation, sVCAM-1 from 673.3 ng/mL to 544.3 ng/mL ($P < 0.001$) and MMP-2 from 192.2 ng/mL to 165.7 ng/mL ($P < 0.001$) by 23%, 25% and 17%, respectively. However, it was also found that in the placebo group, the same effect was observed although this was less significant for sE-selectin ($P = 0.035$), sVCAM-1 ($P = 0.005$) and MMP-2 ($P < 0.001$), respectively. The anti-inflammatory mediator TGF-β1 was found to decrease significantly ($P = 0.046$) in the Omacor group. In terms of percent change, the decrease in the Omacor group was more pronounced compared with that in the placebo group. Omacor supplementation had no significant effect on plasma sICAM-1, IL-6, MMP-9, sCD40L, IP-10 and Mig concentrations, even though trends toward decreases were observed.

When the data were analysed by gender (Tables 3.10 and 3.11), sE-selectin, sVCAM-1 and IL-6 concentrations were significantly decreased in male patients with Omacor supplementation and only MMP-2 was found to be significantly decreased in both supplementation groups. On the other hand, in female patients, sVCAM-1 and MMP-2 were decreased in both groups.

TABLE 3.9: Plasma Inflammatory Markers before and after Supplementation
Data are median (10^{th}-90^{th} percentile)

Outcome	Omacor (n = 47)		Placebo (n = 53)	
	Baseline	After	Baseline	After
CRP (mg/L)	1.0 (1.0- 31.7)	1.0 (1.0-20.0)	1.0 (1.0- 9.1)	1.0 (1.0-23.0)
Change vs % change	0 (-14.6-8.2) vs 0 (-69.1-224.1)%		0 (-3.3-10.6) vs 0 (-60.9-231.1)%	
sE-selectin (ng/mL)	46.0 (16.5-117.2)	30.2* (8.7-120.3)	45.7 (7.8-145.2)	47.7* (8.2-137.5)
Change *vs* % change	-12.1 (-44.8-29.8) vs -22.9 (-68.1-44.8)%		-9.0 (-42.1-31.0) vs -18.8 (-58.1-123.1)%	
sICAM-1 (ng/mL)	167.1 (73.2-425.7)	146.3 (69.2-383.3)	216.1 (64.9-444.7)	202.0 (74.2-345.5)
Change *vs* % change	-9.0 (-117.7-82.4) vs -5.7 (-54.9-56.1)%		-9.14 (-120.37-92.99) vs -3.52 (-49.05-80.86)%	
sVCAM-1 (ng/mL)	673.3 (226.3-1578)	544.3*** (252.4-1146)	594.3 (271.8-1130.8)	488.9** (236.3-932.5)
Change *vs* % change	-186.7 (-575.8-133.8) vs -25.4 (-46.5-28.5%		-113.3 (-452.7-242.8) vs -18.7 (-48.2-76.3)%	
IL-6 (pg/mL)	1.2(0.2-4.0)	1.0 (0.2-4.1)	1.2 (0.1-4.8)	0.9 (0-3.7)
Change *vs* % change	-0.2 (-1.4-1.0) vs -26.2 (-80.6-113.0)%		-0.1 (-2.3-2.2) vs -10.9(-89.7-229.7)%	
IL-10 (pg/mL)	1.5 (0-5.2)	0.9 (0-6.2)	0.5 (0-2.9)	0.8 (0-3.5)
Change *vs* % change	0 (-1.8-2.3) vs -18.8 (-96.4-307.1)%		0 (-1.4-1.5) vs -27.0 (-100.0-138.2)%	
MMP-2 (ng/mL)	192.2 (129.2-290.3)	165.7*** (100.8-248.4)	191.2 (129.0-293.7)	155.3*** (71.8-233.2)
Change *vs* % change	-29.1 (-85.6-21.8) vs -16.5 (-32.6-15.3)%		-26.7 (-89.0-13.8) vs -14.9 (-46.7-12.4)%	
MMP-9 (ng/mL)	167.2 (46.6-421.1)	163.3 (32.0-558.3)	138.0 (28.8-389.3)	152.1 (34.2-403.2)
Change *vs* % change	5.2 (-178.13-201.42) vs 3.35 (-66.99-249.12)%		27.6 (-212.4-203.4) vs 21.62 (-72.80-143.94) %	
TGF-β1 (ng/mL)	9308 (2394.40-19170.40)	7908.00* (2956.00-13690.40)	9788 (3356-16844)	7516 (3484-17388)
Change *vs* % change	-1640 (-9584-4728) vs -22.4 (-66.6-93.7)%		-840 (-8096-6424) vs -9.4(-57.4-104.9)%	
sCD40-L (ng/mL)	775.8 (243.2-2238.7)	655.4 (178.8-1677.4)	774.0 (193.2-2905.7)	659.3 (247.8-2285.1)
Change vs % change	-93.6 (-1214.2-764.2) vs -22.9 (-79.7-220.7)%		-114.9 (-1056.8-688.4) vs -12.8 (-74.8-222.1)%	
IP-10 (pg/mL)	103.9 (51.6-273.3)	100.2 (55.4-353.1)	102.8 (47.8-289.4)	114.9 (35.9-295.9)
Change vs % change	2.6 (-78.4-130.7) vs 2.91 (-45.8-97.1)%		-17.0 (-74.4-110.4) vs -11.1 (-55.9-158.0)%	
Mig (pg/mL)	119.3 (398-360.7)	107.2 (30.60-320.6)	107.4 (30.6-294.3)	97.40 (16.9-327.2
Change vs % change	-20.7 (-63.18-47.8) vs -13.2 (-43.92-35.7)%		-18.1 (-97.6-56.6) vs -19.0 (-83.1-121.6)%	

*Significantly different from baseline ($P < 0.05$)
**Significantly different from baseline ($P < 0.01$)
***Significantly different from baseline ($P < 0.001$)

TABLE 3.10: Plasma Inflammatory Markers before and after Supplementation in Male Patients
Data are means (SEM) or median (10^{th}-90^{th} percentile)

Outcome	Omacor (n = 32)		Placebo (n = 36)	
	Before	After	Before	After
CRP (mg/L)	1.0 (1.0-24.9)	1.0 (1.0-15.7)	1.5 (1.0-13.0)	1.0 (1.0-24.5)
Change vs % change	0 (-13.6-2.8) vs 0 (-87.5-139.7)%		0 (-2.8-6.8) vs 0 0 (-64.7-193.9)%	
sE-selectin (ng/mL)	65.0 (20.4-134.3)	38.3** (16.0-128.8)	57.7 (5.8-149.2)	53.5* (9.2-148.3)
Change *vs* % change	-12.2 (-53.9-13.8) vs -23.0 (-60.4-31.8)%		-10.7 (-44.0-30.6) vs -19.2 (-55.1-114.4)%	
sICAM-1 (ng/mL)	177.8 (73.15-459.1)	146.7 (63.3-386.3)	231.9 (27.2)	213.1 (25.5)
Change *vs* % change	-20.0 (13.6) vs -5.7 (6.5)%		-18.82 (14.0) vs 1.21 (6.5)%	
sVCAM-1 (ng/mL)	671.1 (184.6-1633.4)	598.9** (73.5-1238.7)	665.3 (56.9)	590.3 (50.6)
Change *vs* % change	-147.1 (45.6) vs -22.6 (-71.2-37.2)%		-75.0 (66.3) vs -9.7 (-75.2-82.5)%	
IL-6 (pg/mL)	1.3 (0.2-5.0)	0.8* (0.1-4.7)	2.7 (0.7)	1.71 (0.3)
Change *vs* % change	-0.3 (0.2) vs -27.0 (-79.5-88.5)%		-1.0 (0.7) vs -24.4 (-95.1-244.7)%	
IL-10 (pg/mL)	1.59 (0.00-5.24)	1.01 (0.00-5.8)	0.4 (0-4.3)	0.5 (0-4.9)
Change *vs* % change	0.00 (-6.91-2.36) vs -20.42 (-100.00-245.25)%		0 (-10.4-1.6) vs -84.2 (-100.0-360.9)%	
MMP-2 (ng/mL)	199.4 (13.2)	168.0*** (10.4)	185.8 (11.5)	154.5** (10.5)
Change *vs* % change	-31.3 (5.9) vs -14.5 (-38.4-10.8)%		-31.3 (8.8) vs -14.0 (-57.2-16.8)%	
MMP-9 (ng/mL)	197.2 (28.7-454.9)	158.15 (0.00-545.72)	152.3 (1.5-402.2)	152.1 (22.6-410.6)
Change *vs* % change	-10.2 (27.9) vs -9.9 (-92.1-130.4)%		5.8 (25.3) vs 17.7 (-83.9-135.3)%	
TGF-β1 (ng/mL)	9012 (2949-17076)	7636 (3426-13256)	8872 (2723 -16760)	7612 (193-15733)
Change *vs* % change	-1680 (-11468-4512) vs -22.4 (-80.9-74.7)%		-860 (-10104-6084) vs -14.6 (-97.7-105.3)%	
sCD40-L (ng/mL)	720.4 (155.5-2084.8)	576.0 (67.1-1230.6)	617.8 (123.5-1537.7)	633.2 (143.9-1813.8)
Change vs % change	-176.0 (-1230.3-418.9) vs -36.2 (-85.0-128.6)%		0.7 (-980.6-702.1) vs -2.3 (-76.0-246.4)%	
IP-10 (pg/mL)	110.4 (38.3-257.7)	98.8 (28.4-315.0)	105.8 (38.9-304.7)	103.4 (18.8-315.3)
Change vs % change	2.2 (-74.54-102.0) vs 2.8 (-46.8-72.1)%		-20.9 (-86.5-52.9) vs -23.2 (-56.3-63.3)%	
Mig (pg/mL)	108.3 (13.1-317.2)	96.8 (6.7-317.7)	103.2 (10.1-302.4)	86.8 (2.5-424.2)
Change vs % change	-21.3 (-54.7-93.4) vs -13.0 (-51.0-35.6)%		-29.3 (-125.7-63.9) vs -23.4 (-86.3-156.0)%	

*Significantly different from baseline ($P < 0.05$)
**Significantly different from baseline ($P < 0.01$)
***Significantly different from baseline ($P < 0.001$)

TABLE 3.11: Plasma Inflammatory Markers before and after Supplementation in Female Patients
Data are means (SEM) or median (10^{th}-90^{th} percentile)

Outcome	Omacor (n = 15)		Placebo (n = 17)	
	Before	After	Before	After
CRP (mg/L)	2.5 (1.0-38.0)	3.0 (1.0-24.5)	1.0 (1.0-9.1)	2.2 (1.0-30.1)
Change vs % change	0 (-17.8-18.4) vs 0 (-57.8-682.6)%		0 (-3.5-26.3) vs 0 (-55.0-2126.0)%	
sE-selectin (ng/mL)	28.5 (5.9-101.1)	21.5 (1.7-123.7)	39.1 (11.5-150.3)	35.8 (7.6-159.4)
Change *vs* % change	-0.4 (6.0) vs -19.3 (-90.5-99.2%)		-2.0 (8.9) vs -8.6 (-69.1-320.4)%	
sICAM-1 (ng/mL)	141.3 (49.9-357.7)	134.7 (52.5-373.0)	594.3 (248.8-977.2)	433.5** (203.6-669.1)
Change *vs* % change	-11.7 (17.8) vs -4.7 (-69.6-304.3)%		-0.8 (35.6) vs 9.0 (-78.4-135.9)%	
sVCAM-1 (ng/mL)	734.2 (367.0-1859.7)	517.7** (296.41-1310.71)	586.6 (62.6)	442.0** (41.4)
Change *vs* % change	-233.5 (56.6) vs -37.8 (-47.1-18.4)%		-144.6 (37.8) vs -26.6 (-47.5-44.0)%	
IL-6 (pg/mL)	1.1 (0.2-8.2)	1.3 (0-3.0)	1.1 (0.2-3.9)	1.3 (0-3.2)
Change *vs* % change	0.2 (-6.1-1.2) vs 23.8 (-88.2-184.1)%		0.1 (-1.3-2.2) vs 10.2 (-93.0-1352.5)%	
IL-10 (pg/mL)	0 (0-3.9)	0.4 (0-8.6)	0 (0-4.3)	0.4 (0-3.4)
Change *vs* % change	0 (-1.3-5.3) vs -17.2 (-100.0-15.9)%		0 (-2.3-0.7) vs -15.7 (-100.0-304.1)%	
MMP-2 (ng/mL)	207.2 (13.8)	172.7* (13.5)	204.1 (19.7)	166.11** (16.8)
Change *vs* % change	-34.5 (12.3) vs -18.4 (-39.6-37.7)%		-38.0 (8.8) vs -17.7 (-46.0-8.5)%	
MMP-9 (ng/mL)	129.0 (24.1-454.9)	199.4 (44.3-560.1)	129.0 (34.5-375.3)	199.4 (47.6-422.9)
Change *vs* % change	69.6 (37.1) vs 32.3 (-54.5-2728.4)%		47.7 (34.0) vs 38.0 (-58.88-203.7)%	
TGF-β1 (ng/mL)	11596 (496.8-54647.2)	10076 (2108-21508)	10796 (1366-17580)	7228 (2294-28365)
Change *vs* % change	-1120 (-9340-8331) vs -19.7 (-72.6-138.9)%		-560 (-10408-12240) vs -7.2 (-63.2-101.7)%	
sCD401 (ng/mL)	916.7 (198.3-2645.6)	1118.0 (310.7-3021.6)	1325.2 (334.3-5278.2)	791.7 (226.0-3868.3)
Change vs % change	159.7 (-1388.04-1936.5) vs 22.0 (9-70.5-381.6)%		500.6 (-3993.6-2251.6) vs -38.4 (-85.1-274.3)%	
IP-10 (pg/mL)	94.3 (53.9-491.2)	109.5 (60.8-474.4)	97.6 (42.9-295.2)	151.4 (47.9-330.1)
Change vs % change	8.70 (-83.8-209.9) vs 11.2 (-50.2-193.0)%		12.7 (-72.7-169.3) vs 14.1 (-52.3-263.9)%	
Mig (pg/mL)	178.2 (43.8-570.8)	155.7 (37.2-428.8)	116.5 (55.5-232.1)	132.0 (38.0-347.7)
Change vs % change	-14.6 (-154.2-33.5) vs -6.1 (-35.9-78.7)%		5.30 (-80.6-95.7) vs -17.5 (-67.3-58.5)%	

*Significantly different from baseline ($P < 0.05$)
**Significantly different from baseline ($P < 0.01$)

3.3.6 Relationship between a Marker of Inflammatory Cell Fatty Acid Composition and CVD Risk Factors

Kew et al. (2003) reported that the fatty acid composition of plasma PC was highly related to that of inflammatory cells. Thus, in order to see if fatty acid status was related to inflammation in these patients, the fatty acid composition of plasma PC at baseline was related to plasma inflammatory marker concentrations at that time point (Table 3.12). It was found that oleic acid was significantly but slightly positively correlated with sE-selectin ($r = 0.199$, $P = 0.047$), ALA (18:3n-3) was inversely correlated with sVCAM-1 ($r = -0.367$, $P < 0.001$), and palmitoleic acid was negatively correlated with MMP-2 ($r = -0.223$, $P = 0.026$). DGLA was positively correlated with IL-6 ($r = 0.340$, $P = 0.001$) and an inverse association was found between sICAM-1 and myristic acid ($r = -0.198$, $P = 0.049$).

TABLE 3.12: Correlation between Fatty Acids in Plasma PC and Plasma Inflammatory Markers

Inflammatory marker	Fatty acid	r	P
sE-selectin	18:1n-9	0.199*	0.047
sICAM-1	14:0	-0.198*	0.049
sVCAM-1	18:3n-3	-0.367**	0.001
IL-6	20:3n-6	0.340**	0.001
MMP-2	16:1n-7	-0.223*	0.026

3.3.7 Correlation between Changes in Plasma PC Fatty Acids and Changes in Inflammatory Markers

If fatty acids are causally associated with inflammation, as determined by plasma inflammatory markers, changes in status of those fatty acids should be associated with changes in levels of the inflammatory markers. In this analysis, pooled subjects were used to study whether the change in the proportion of ARA, EPA, DPA and DHA in the plasma PC fraction related to the change in inflammatory marker concentrations. There were no significant associations with ARA or EPA. Table 3.13 shows the association between changes in DPA and DHA in plasma PC fraction and changes in the inflammatory markers of sE-selectin and MMP-9. These positive associations suggest that DPA and DHA may act in a pro-inflammatory, not an anti-inflammatory, manner. There were no other significant associations with these fatty acids.

TABLE 3.13: Correlation between Changes in DPA and DHA and Changes in Plasma Inflammatory Markers

Inflammatory marker	Fatty acid	r	P
sE-selectin			
Change	DPA	0.213	0.033
% change		0.501	< 0.001
MMP-9			
% change	DPA	0.217	0.031
	DHA	0.415	< 0.001

3.3.8 mRNA Expression of Inflammatory Markers in Plaque

The level of mRNA encoding a range of inflammatory markers, including some of those measured in plasma above, in plaque is shown in Table 3.14. Expression of mRNA for MMP-7, MMP-9, MMP-12, TIMP-2 and IL-6 were significantly lower following Omacor supplementation compared with the placebo group ($P < 0.05$). However, ICAM-1 mRNA was significantly higher in the Omacor group as compared to the placebo ($P = 0.014$).

TABLE 3.14: mRNA Expression of Inflammatory Markers in Plaques from Patients Supplemented with Omacor or Placebo
Values are median (10^{th}-90^{th} percentile)

CT36 plaque fraction	Omacor	Placebo	P
TNF-α	0.009 (0-0.142)	0.030 (0.002-0.256)	0.145
PPAR-γ	0.001 (0-0.005)	0.002 (0-0.005)	0.055
MMP-1	0.003 (0-0.026)	0.005 (0.001-0.034)	0.146
MMP-3	0 (0-0.002)	0 (0-0.002)	0.886
MMP-7	0.005 (0-0.188)	0.019 (0.001-0.434)	0.005
MMP-8	0.002 (0-0.010)	0.003 (0-0.024)	0.126
MMP-9	0.033 (0.001-1.684)	0.225 (0.009-2.425)	0.005
MMP-12	0.009 (0-0.254)	0.044 (0.002-0.794)	0.004
MMP-13	0.001 (0-0.011)	0.001 (0-0.024)	0.149
TIMP-1	0.648 (0.005-9.020)	1.248 (0.177-11.479)	0.053
TIMP-2	0.044 (0-0.688)	0.151 (0.013-0.973)	0.014
IL-10	0.002 (0-0.011)	0.003 (0.001-0.019)	0.218
IL-6	0.019 (0.001-0.900)	0.026 (0.050-0.203)	0.039
ICAM-1	0.556 (0.065-2.553)	0.448 (0.080-1.542)	0.014

3.3.9 Correlation between mRNA Expression of Plaque Inflammatory Markers and Fatty Acid Composition in Plaque

In section 3.3.2 it was demonstrated that Omacor treatment increases LC n-3 PUFAs in circulating PC while in section 3.3.3 it was demonstrated that Omacor results in higher EPA in plaque PC. If these fatty acids are causally related to inflammatory processes within the plaque, then a relationship between fatty acids in plaque and mRNA expression of inflammatory markers should be apparent. Table 3.15 shows correlations between inflammatory mRNA expression in the plaque and fatty acids in plaque PC using data from both groups of patients.

Significant inverse associations between EPA and MMP-7, MMP-9, MMP-12, TIMP-1, TIMP-2, IL-6 and ICAM-1 mRNAs were observed (Table 3.15), suggesting that EPA acts in anti-inflammatory manner in advanced plaques. Only MMP-3 mRNA was significantly negatively associated with DPA. There were no significant associations between mRNA expression of inflammatory marker and DHA in plaque PC. Surprisingly ARA in plaque PC was inversely correlated with PPAR-γ, MMP-1, MMP-3, MMP-7, MMP-9, MMP-12, TIMP-1 and ICAM-1 mRNAs (Table 3.15).

TABLE 3.15: Inverse Relationship between Plaque mRNA Expression and Fatty Acids in Plaque PC

Fatty acid	mRNA	r	P
ARA	PPAR-γ	-0.254	0.022
	MMP-1	-0.239	0.018
	MMP-3	-0.242	0.017
	MMP-7	-0.225	0.027
	MMP-9	-0.247	0.014
	MMP-12	-0.207	0.043
	TIMP-1	-0.257	0.011
	ICAM-1	-0.235	0.021
EPA	MMP-7	-0.212	0.038
	MMP-9	-0.237	0.019
	MMP-12	-0.226	0.027
	TIMP-1	-0.252	0.012
	TIMP-2	-0.311	0.002
	IL-6	-0.240	0.024
	ICAM-1	-0.282	0.005
DPA	MMP-3	-0.243	0.016

3.4 Discussion

This double-blind, placebo-controlled study on the effect of moderate dose (2 g/day) Omacor (providing 1.8 g/day of EPA plus DHA) was carried out for the duration between 7 and 102 days. The subjects were patients awaiting carotid endarterectomy and they were required to give their blood samples twice, at baseline before supplementation began and at the end of the study (before the carotid endarterectomy procedure). Omacor was used in the GISSI trial (GISSI 1999), one of the most important and largest studies advocating the beneficial effects of LC n-3 PUFAs towards cardiovascular mortality (Bays 2006). In the GISSI study, involving more than 11,000 high-risk patients, Omacor significantly reduced overall mortality, cardiovascular mortality and sudden death (GISSI 1999; Marchioli et al. 2002). Since Omacor is produced by an extensive purification and concentration process, each capsule has approximately 90% n-3 fatty acids primarily as EPA and DHA, and the amount of EPA and DHA used in the present study were 45% and 37.5% of fatty acids, respectively. In this study, olive oil was used as a placebo providing approximately 1.2 g of monounsaturated oleic acid and this amount is considered negligible as compared to the average adult UK intake.

3.4.1 Effect of Omacor Supplementation on Plasma PC Fatty Acid Composition

In the present study, incorporation of EPA and DHA into plasma lipids was investigated using the PC fraction. This is because the fatty acid composition of plasma PC is similar to that of cells and tissues (Kew et al. 2003) and therefore may reflect a marker of functional significance. EPA and DHA were significantly increased in the Omacor group, by 161% and 70%, respectively. Previous studies reported increased EPA incorporation in plasma phospholipids by 390% with 3.6 to 4 g/d EPA supplementation (Woodman et al. 2002; Mori et al. 2000; Grimsgaard et al. 1997). In the present study, the dose of EPA provided was only 810 mg/d. Figure 3.6 plots the % change in EPA content of plasma PC seen in the current study alongside that seen in the study described in Chapter 2 as well as the data from Rees et al. (2006) for plasma PC. It is clear that the change in EPA observed here fits

well on this dose-response curve and is in accordance with what is seen in other studies using other doses of EPA.

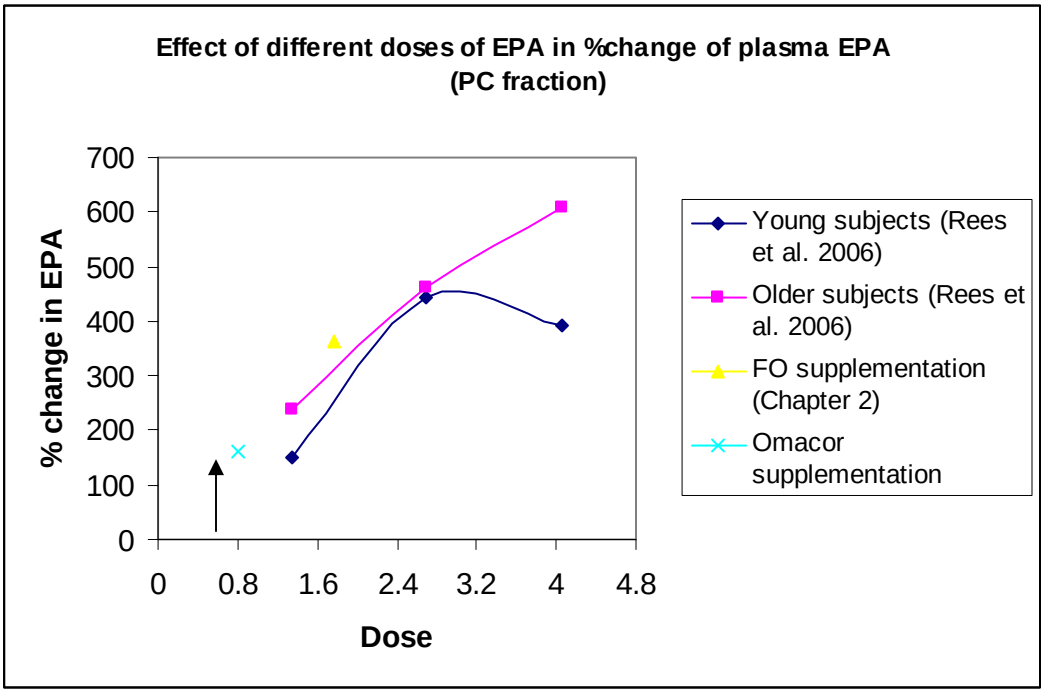


FIGURE 3.6: Effect of Different Doses of EPA on % Change of EPA in Plasma PC
Data for older and young subjects comes from Rees et al. (2006). The arrow shows a dose of 0.81 g/d EPA used in this study, indicating that the change in EPA seen at this dose agrees with that predicted by the Rees et al. (2006) data and also in accordance with data from Chapter 2.

The increase in the proportion of EPA in plasma PC was accompanied by a significant decrease in proportion of n-6 fatty acids DGLA (22%), LA (13%) and ARA (2%). The present findings are in agreement with Rees et al. (2006), whereby EPA was reported to decrease the proportion of DGLA and LA in plasma phospholipids, but with only a small effect on ARA. Again, similar to the findings of my first study (Chapter 2), it seems that EPA consistently more easily replaces DGLA and LA, than ARA, in plasma phospholipids.

Interestingly, in the present study, DHA was also significantly increased in plasma PC in patients taking Omacor. Previous studies supplementing with only high doses of DHA (>3 g/d) reported significant incorporation of DHA (about a 150% increase) into plasma PC (Woodman et al. 2003; Woodman et al. 2002; Mori et al. 2000; Halvorsen et al. 1997; Vidgren et al. 1997). Figure 3.7 plots the % change in DHA content of plasma PC seen in

the current study alongside that seen in the study described in Chapter 2 as well as the data from Rees et al. (2006) for plasma PC.

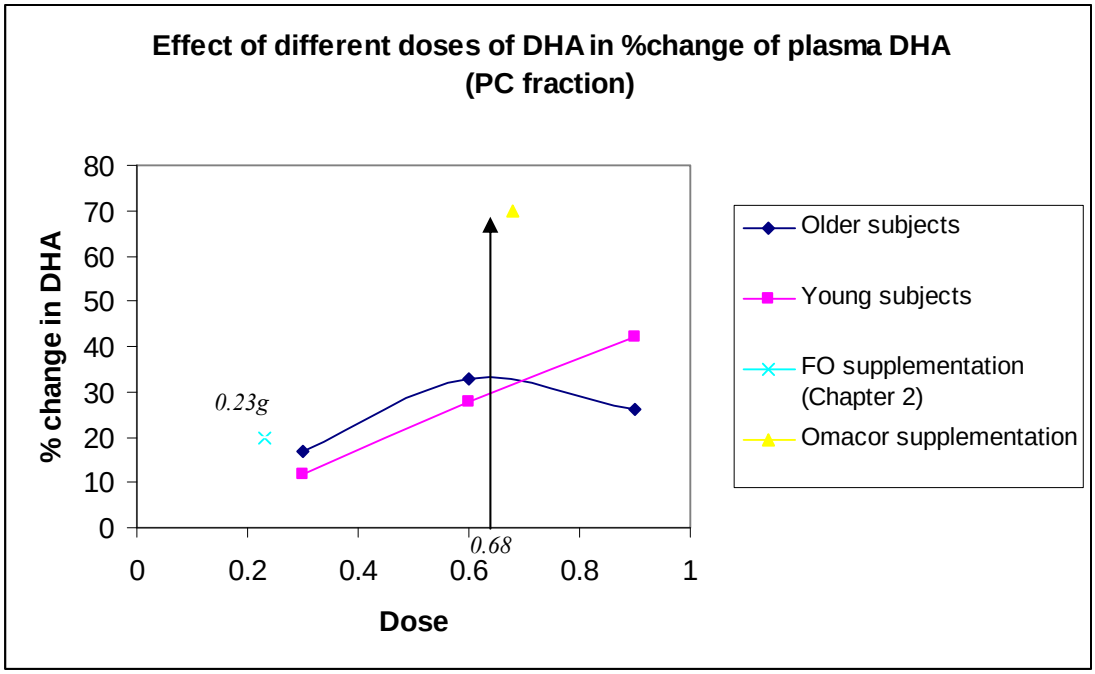


FIGURE 3.7: Effect of Different Doses of DHA on % Change of DHA in Plasma PC
Data for older and young subjects comes from Rees et al. (2006). The arrow shows a dose of 0.68 g/d DHA used in this study, indicating that the change in DHA seen at this dose was at a greater extent compared with that predicted by the Rees et al. (2006) data and data from Chapter 2.

It seems that the change in DHA observed here does not fit well on this dose-response curve and is not in accordance with what is seen in other studies using other doses of DHA. This might be due to the varying ratios of EPA to DHA used in different studies. Here the ratio of EPA to DHA was 1.21 and the contribution of DHA to total LC n-3 PUFAs was 37.5%. In the study described in Chapter 2 these values were 7.69 and 10.9%, respectively, while in the study of Rees et al. (2006) they were 4.5 and 18%, respectively. Thus, one explanation for the better incorporation of DHA seen in the current study is that there is less competition from EPA for incorporation into plasma phospholipids than occurred in the other studies. To investigate this further the contribution of DHA from the supplement can be "normalised" as follows:

Normalised amount of DHA = (Amount of DHA provided in g/d) x (DHA as proportion of EPA+DHA)

This gives "normalised" amounts of DHA (in g/day) of 0.306 for the current study, 0.023 for the study described in Chapter 2 and 0.054, 0.108 and 0.162 for the three doses used in

the study of Rees et al. (2006). These normalised intakes of DHA are plotted against the % change in DHA observed in the different studies in Figure 3.8.

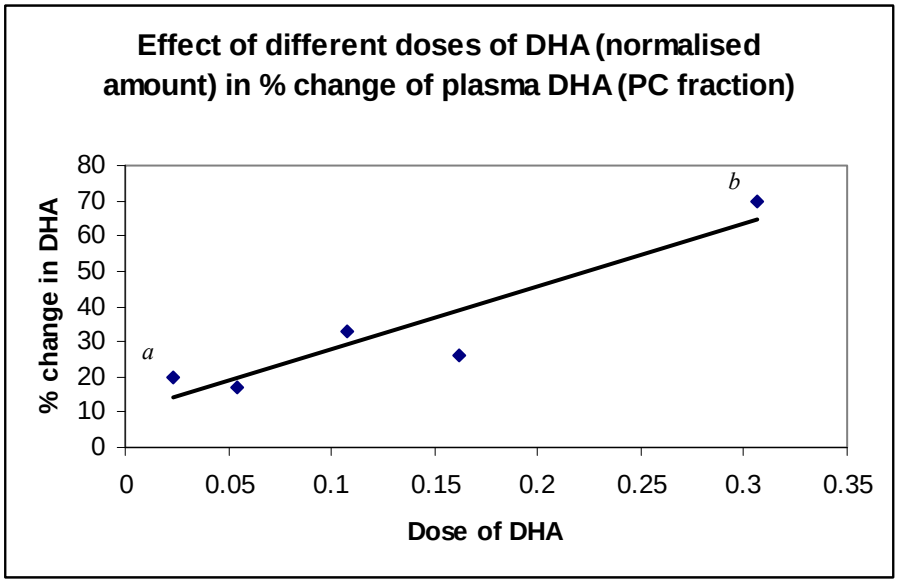


FIGURE 3.8: Effect of DHA (Normalised Amount) on % Change of DHA in Plasma PC. *a* represents value from the study in Chapter 2, whereas *b* represents value from the present study. The other three points are calculated from Rees et al. (2006).

It is clear from this figure that the change in DHA observed in the current study fits well on this dose-response curve and is in accordance with what is seen in other studies using other doses of DHA, once the competition of DHA with EPA is taken into consideration.

It has been reported that statin therapy *per se* results in unfavourable changes in plasma fatty acids, whereby the ARA:EPA and ARA:DHA ratios are increased (Harris et al. 2004). The patients studied here were almost all taking statins. However, it was possible to compare the fatty acid composition of plasma PC between these patients awaiting carotid endarterectomy and the healthy middle aged subjects studied in Chapter 2; data at study entry was compared. The only difference seen was a higher proportion of ARA in the patients ($P = 0.031$). The ARA:EPA and ARA:DHA ratios in the patients were slightly higher, but not significantly so ($P > 0.05$), than those seen in the healthier subjects studied in Chapter 2: 8.98 vs. 7.34 and 2.96 vs. 2.01, for patients and healthy subjects, respectively.

In the present study, it was found that following Omacor supplementation, the ratios of ARA:EPA and ARA:DHA were significantly reduced, from 8.42 to 2.58 and 2.68 vs. 1.58,

respectively; however no effect was observed in the placebo group, 8.35 vs. 8.54 and 2.48 vs. 2.41, respectively. Thus, increased intake of LC n-3 PUFAs among patients taking statins induces a similar fatty acid composition change in plasma PC to that seen in subjects not taking statins and overrides the effect of statins on the ARA:EPA and ARA:DHA ratios.

3.4.2 Blood Pressure and Plasma Lipid Concentrations

Systolic and diastolic blood pressures were found to decrease significantly in both groups. The reason for this is not clear but may involve some change in lifestyle induced by the knowledge that surgery was required. An antihypertensive effect of n-3 PUFAs has been described in the literature (Morris et al. 1993; Howe 1997), the effect being related to the initial extent of hypertension and being greater when a higher dose on n-3 PUFA is administered. However, here the effect of Omacor was similar to the effect of placebo, ruling out any n-3 PUFA-specific effect on blood pressure. The lack of an effect in the Omacor group may be because of an insufficient dose of LC n-3 PUFA.

It has been reported that fish oil increases total blood cholesterol by 5-10% and decreases blood TAG by 20 to 30% (Harris 1997). In the current study it was shown that total cholesterol was significantly decreased by 13% in the Omacor group compared with 9% in the placebo group. HDL-c was also significantly decreased in both groups. On the other hand, TAG concentrations were decreased by 20% in the Omacor group as compared to that 9% in the placebo group. Meyer et al. (2007) in their studies using high doses of DHA in statin-treated hyperlipidaemic subjects reported decreased TAG by 27% with 8 g/day DHA-rich fish oil. Mori and Woodman (2006) recently confirmed the effect of a high intake (4g/d) of EPA vs. DHA producing convincing evidence that EPA and DHA are equally effective at reducing serum TAG, but only DHA may raise HDL-c, specifically HDL_2 fraction as well as LDL. Thus, in this present study, the decrease in HDL-c could be due to not enough DHA being present. Contrary to a general tendency towards slightly increased LDL-c concentration with fish oil supplementation (Katan et al. 1995; Harris et al. 1996), LDL-c was found to decrease significantly in the Omacor group. Even though total cholesterol was already well controlled by statin treatment (mean initial levels were 4.8

mmol/L), since 84% of the patients were on statins, there was a further reduction in total cholesterol with Omacor, 13% compared with 9% with olive oil-placebo treatment.

In the present study, both groups exhibited significant TAG lowering. The percent reduction in the Omacor group was greater than in the placebo group (20% vs. 9%). N-3 PUFA supplementation studies typically show reductions in TAG of 25-30% in those with normal triglyceride levels (Harris 1997). Buckley et al. (2004) reported 7% TAG lowering with placebo and they also used olive oil as placebo. Thus, olive oil may also have a beneficial TAG-lowering effect, even though to a lesser extent compared to n-3 PUFAs. The hypotriglyceridaemic effect (20% lowering of fasting TAG) of n-3 PUFA supplementation seen in the present study suggests that it may be a useful adjunct to lipid-lowering therapy with statins.

It is believed that the combination of Omacor and statins exhibits a greater lipid-lowering effect than statins alone, and this is demonstrated in this study. Omacor is indicated as an adjunct to diet to reduce very high (≥ 5.65 mmol/L) plasma TAG in adult patients and has been shown to be effective in reducing plasma TAG concentrations when used in combination with statins (Durrington et al. 2001). A recent study by Meyer et al. (2007) demonstrated that DHA-rich fish oil supplementation (2.16 g/day) can improve plasma lipids in a dose-dependent manner in patients taking statins. Moreover, Davidson and colleagues (2007) also found that combination of Omacor plus simvastatin and dietary counselling improved non-HDL-c and other lipid and lipoprotein parameters to a greater extend than simvastatin alone. Over the past decades, no other drugs have been proven to be as effective in cholesterol-lowering as statins. However statin therapy *per se* results in unfavourable changes in plasma fatty acids whereby it has been reported that ARA:EPA and ARA:DHA ratios are increased (Harris et al. 2004). Drug combinations are usually prescribed for treatment of hyperlipidemia and these drugs are well known for adverse side effects. For example, fibrates cause liver and muscle toxicity (Pierce et al. 1990; Schectman & Hiatt 1996) and niacin results in hepatic toxicity (Rader et al. 1992) and myopathy (Norman et al. 1988). Therefore it is suggested that fish oil supplementation in combination with statin treatment can be an alternative approach to eliminate harmful adverse effects due to combined therapy.

3.4.3 Effect of Supplementation on Plasma Inflammatory Markers

Contrary to the previous study (Chapter 2), positive effects of LC n-3 PUFAs on plasma inflammatory markers were observed in the present study. It was shown that in the Omacor group, sE-selectin and sVCAM-1 decreased significantly by 23% and 25%, respectively. In the study described in Chapter 2 there was no effect of fish oil supplementation in healthy middle-aged men on these molecules. In the present study reductions in sE-selectin and IL-6 with Omacor were observed in male, but not female, patients. The role of IL-6 as a better predictor for CVD risk in older adults has been examined (Harris 1999; Jenny et al. 2002; Cesari et al. 2003). Lee et al. (2006) reported no significant effect in plasma IL-6 in post-MI patients allocated to 1 g/day Omacor. However, cell culture studies by venous endothelial cells demonstrate that EPA and DHA can inhibit IL-6 production (De Caterina et al. 1994; Khalfoun et al. 1997). Supplementation with fish oil in healthy volunteers has been reported to decrease the IL-6 production by mononuclear cells (Meydani et al. 1993; Caughey et al. 1996; Trebble et al. 2003; Wallace et al. 2003). Reduction in plasma IL-6 with increased n-3 PUFA intake has been observed in population-based studies (Paschos et al. 2004; Lopez-Garcia et al. 2004; Esposito et al. 2004).

sVCAM-1 was significantly decreased by Omacor in both male and female patients. Previously, Miles et al. (2001) found no significant effects of 1.2 g EPA + DHA/day on plasma sICAM-1 or sE-selectin in elderly healthy subjects, although sVCAM-1 was significantly decreased when compared with control subjects. In the present study, sICAM-1 was not decreased following Omacor supplementation and this finding is in accordance with Miles et al. (2001). However, it has been reported that supplementation with 2.4 g of EPA plus DHA/day decreased plasma sICAM-1 significantly (Berstad et al. 2003; Hjerkinn et al. 2005). Thus, a lack of effect of n-3 PUFAs on sICAM-1 seen here and by Miles et al. (2001) may be due to insufficient dose. The effect of n-3 PUFAs on the expression of sVCAM-1 is inconsistent. Thies et al. (2001) reported reduced sVCAM-1 in healthy subjects with 1 g EPA plus DHA daily. Eschen et al. (2004), however found that sVCAM-1 was only reduced with high dose n-3 PUFAs (6.6 g/day) in female healthy subjects, whereas Cazzola et al. (2006) and Miles et al. (2001) reported this effect in older subjects with high dose EPA of 4.05 g and 1.2 g EPA plus DHA daily, respectively. Likewise, it was

also reported that the effects of n-3 PUFAs on sVCAM-1 may be time-dependent whereby in hypertriglyceridemic patients, six-week supplements with 4 g/d of n-3 PUFAs increased the level of sE-selectin, but had no effect on sVCAM-1 or sICAM-1 (Abe et al. 1998). Further continuation of supplementation for more than six months, however, led to a significant decrease in both sE-selectin and sICAM-1, respectively. Interestingly, in the present study, the effect of Omacor supplementation was more pronounced in male patients as compared to that of females.

This study also demonstrated that no beneficial effects with Omacor supplementation on IL-10, CRP, sCD40L, IP-10 and Mig. Balk et al. (2006) reported in their systematic review that the effect of n-3 PUFAs on CRP is inconsistent and non-significant. It has been argued that among many serum markers of CVD risk, a role for CRP is the most supported by research and clinical application (Ferranti & Rifai 2007).

Human and animal studies, as well as tissue culture work show that there are discrepancies on the effects of fish oil supplementation on inflammatory markers. Characteristics of the subjects (normal or healthy, hyperlipidemic or patient with heart disease), dosage, duration of the intervention will vary the outcomes. Several studies show that supplementation with fish oil (in higher dosage > 4 g/day EPA plus DHA) increases the inflammatory markers of TNF-α, E-selectin and VCAM-1 (Torstensen et al. 2000; Seljeflot et al. 1998; Holm 2001; Eschen et al. 2004) even though n-3 fatty acids are anti-inflammatory (Calder 2003; Yaqoob 2003) and also have been shown to decrease adhesion molecules (Hughes et al. 1996; Miles et al. 2001). Moreover, irrespective of the previous dietary pattern (for example diet rich in corn oil or n-3 PUFAs), it was found that higher dosage (> 5 g n-3 PUFAs/day) increased sE-selectin and sVCAM-1 (Johansen et al. 1999). These unexpected results suggest that supplementation with highly concentrated n-3 PUFAs to patients with coronary heart disease may decrease the homeostatic activity of the endothelium, and subsequently increase the inflammatory activity. Blok et al. (1997), on the other hand reported in a prospective trial that fish oil supplementation did not affect the levels of plasma cytokines. The reason for the apparent discrepancy is not clear. It is well known, however, that PUFAs (especially at higher dosage) are prone to peroxidation which further generates free radicals and oxidised LDL which are cytotoxic to cells (Steinberg et al. 1989). Yet, in other studies

(Nenseter et al. 1992; Lussier-cacan et al. 1993; Bonaname et al. 1996) using moderate dose (< 3 g of n-3 PUFAs/day), conflicting results have been reported as well.

IL-6 and TNF-α are generally considered as proinflammatory cytokines and the potent anti-inflammatory properties of IL-10 and TGF-β are well known (Pestka et al. 2004; Wahl & Chen 2003). Anti-inflammatory cytokines such as TGF-β have been shown to prevent vascular inflammation and to promote plaque stabilization in mice models (Gourdy et al. 2007). In the present study, however, Omacor significantly decreased TGF-β concentration. Thus this effect of Omacor is unlikely to be of benefit. The mechanism responsible for the anti-atherogenic effects of TGF-β, particularly reduction in the intimal inflammatory process and promotion of plaque stabilization were recently further analyzed (Tedgui & Mallat 2006; Singh & Ramji 2006). Fish oil supplementation has been demonstrated to elevate TGF-β levels in pregnant women (Krauss-Etschmann et al. 2007).

IP-10 and Mig are reported to be present in arterial plaque and immunohistochemical staining of human atherosclerotic lesions revealed that these chemokines are expressed in endothelial cells overlying the plaque, suggesting that IP-10 and Mig are likely play an important role in T cell recruitment to sites of inflammation (Mach et al. 1999). IP-10 was increased in a small group of coronary artery disease patients with restenosis compared with other coronary artery disease patients (Kawamura et al. 2003) and a positive correlation between IP-10 and ICAM-1 has been observed (Rothenbacher et al. 2006). In the present study, no effects of Omacor on IP-10 and Mig were observed.

One of the earliest events in the formation of atherosclerotic plaque is the adherence of circulatory monocytes to the vascular endothelium which subsequently enhances the secretion of a wide variety of growth factors in a series of complex cell-cell interactions (Hansson et al. 1989), such as inflammatory cytokines and matrix metalloproteinases (MMPs). MMPs are known to be expressed in human atherosclerotic plaques by both smooth muscle cells and foam cells, which have been demonstrated by *in situ* zymography and *in situ* hybridization (Henney et al. 1991; Galis et al. 1994) and may thus contribute to plaque vulnerability (Brown et al. 1995) as well as *de novo* atherosclerotic remodelling (Pasterkamp et al. 2000). Increased expressions of MMP-2 and MMP-9 that degrade the

major component of basement membrane have been demonstrated within plaque (Pasterkamp et al. 2000; Zaltsman et al. 1999). MMP-9, which is also known as gelatinase 13 or 92-kDa type 10 collagenase, is one of the MMPs found to be highly expressed in the vulnerable regions of atherosclerotic plaque. Even though, it has been documented that MMP-9 is undetectable in blood of 80% of healthy individuals (Sundstrom et al. 2004), in this study, MMP-9 was detected in blood of all patients.

Prior to the expression of adhesion molecules, CD40L on activated platelets and T-lymphocytes activates MMPs and thereby promotes plaque rupture. On the other hand, sCD40L is not only as a marker of immune activation but also may be involved in pathogenic processes in angina patients. Indeed, sCD40L has been implicated in acute coronary syndrome (Aukrust et al. 1999) and elevated sCD40L predicts an increased risk of future CVD events in healthy subjects (Schonbech et al. 2001). In this present study, no beneficial effects of Omacor on MMP-2, MMP-9 and sCD40L were found. Our findings agree with Aarsetoey et al. (2006) which also reported the lack of benefit with Omacor intervention on sCD40L and MMP-9. In their studies, the placebo corn oil group also exhibited significant decreases in sCD40L and non-significant decreases in MMP-9. Of note, in the present study, MMP-2 was reduced significantly in both groups.

The beneficial effects of Omacor, such as lipid-lowering, reduced platelet aggregation, antithrombotic and fibrolytic activities, reduced blood viscosity and anti-inflammatory properties have been demonstrated at high doses (Bhatnagar & Durrington 2003). In the present study, even though the dose administered was only 1.8 g/day EPA plus DHA, combination of Omacor and statins improved lipid profiles and certain inflammatory markers to a greater extent than statins alone, as represented in the placebo group. Therefore, again, the beneficial effect of Omacor as a useful adjunct to statins therapy has been demonstrated, not only in terms of lipid-lowering, but also in certain plasma inflammatory markers, particularly in male patients.

3.4.4 The Relationship between Fatty Acid Intake and CVD Outcomes

The influence of fatty acid intake and CVD outcomes was investigated based on the association between fatty acid composition in the PC fraction and CVD markers. Plasma PC represents transport of lipids in the bloodstream since exchange of phospholipids occurs with cell membranes (Kew et al. 2003). Moreover, plasma PC that originates mainly from liver may reflect tissue n-3 fatty acids levels and further may serve as a marker to document n-3 fatty acid intake and also is a surrogate for cell membrane fatty acid composition (Harris 1989).

The effects of n-3 PUFAs, particularly EPA and DHA on inflammatory responses have been investigated in *in vitro* (De Caterina et al. 1994; De Caterina et al. 1995; Weber et al. 1995; De Caterina et al. 2000) with results favouring DHA as a more potent anti-inflammatory fatty acid. In the present study, only ALA and DHA were found to be inversely associated with inflammatory markers sVCAM-1 and IL-6, respectively. Yli-Jama et al. (2002) reported an inverse linear association between sVCAM-1 and non-esterified DHA. Even though ARA has been documented, and portrayed, as a potent pro-inflammatory factor (Dwyer et al. 2004), in the present study, no associations were found. Yli-Jama et al. (2002) reported anti-inflammatory effect of ARA, however with sVCAM-1.

3.4.5 Correlation between Plasma Inflammatory Markers and Specific Fatty Acids

Section 2.4.8 has further discussed the role of LC n-3 PUFAs, particularly EPA and DHA on inflammatory responses. In the present study, only sE-selectin and MMP-9 showed a relationship with DPA and DHA, however with a direct association, indicating a pro-inflammatory response. Therefore, even though in the present study beneficial effects of Omacor have been demonstrated, it is difficult to state with certainty that EPA and DHA are causally responsible for these changes since there were no significant relations between these fatty acids and inflammatory markers studied. Of note, the role of fish oil supplementation to exert an anti-inflammatory response is well reported, even though the mechanism by which this action is working is still under extensive investigation.

3.4.6 Effect of Supplementation on the mRNA Expression of Plaque Inflammatory Markers

MMP-7 mRNA concentrations were significantly lower in the Omacor group than in the placebo group. Based on an animal study using apoE/MMP-7 double knock-out mice, it was found that MMP-7 has no effect on plaque growth or stability, although it is associated with reduced smooth muscle cell content in plaques (Johnson et al. 2005). Nilsson et al. (2006) reported that the plasma concentration of MMP-7 was increased in patients with stable and unstable CAD. Conversely, for MMP-8, the regions of advance atherosclerotic lesions displayed an increase of MMP-8 mRNA expression level in comparison with healthy tissues (Herman et al. 2001). In the present study however, there was no significant effect of Omacor on MMP-8 mRNA expression.

It has been reported that plasma MMP-9 had significantly higher concentrations and tissue inhibitor of metalloproteinase (TIMP-1) levels were lower in subjects with previous acute myocardial infarction (AMI) (Furenes et al. 2007). On the other hand, Tuomainen et al. (2007) documented that TIMP-1 concentrations alone had no predictive value. Moreover, it was found that n-3 PUFAs supplementation did not alter the levels of MMP-9 and TIMP-1 (Furenes et al. 2007). In the present study, MMP-9 mRNA levels were significantly lower in plaques in the Omacor group compared with the placebo group.

The decreases in plasma IL-6 in the Omacor group was also in accordance with lower IL-6 mRNA expression in plaque as compared to the placebo. Conversely, even though plasma sICAM-1 was not significantly decreased with Omacor supplementation, ICAM-1 mRNA expression in plaque was shown to decrease significantly. On the other hand, there was no significant effect on plaque VCAM-1 mRNA expression, even though plasma sVCAM-1 showed a significant decrease. To date, no similar studies which measure ICAM-1 or VCAM-1 mRNA levels in plaque were found with which to make comparison. However, it has been reviewed and documented that expression of ICAM-1 has been consistently observed in atherosclerotic plaque (Blankenberg et al. 2003). Therefore, the findings in the present study suggest that the association between plasma inflammatory markers and mRNA expression of plaque inflammatory markers is not consistent. It is believed that

changes in gene expression levels will lead to changes in protein level as well. However, this may not necessarily hold true.

3.5 Conclusion

In this study it was not possible to ascertain the positive effect of fish oil supplementation (Omacor) in patients awaiting carotid endarcterectomy on blood pressure and lipid profile since the results revealed that both placebo and Omacor groups showed significant decreases in TAG, total cholesterol and LDL-cholesterol. In terms of inflammatory markers, it was found that Omacor significantly decreased sE-selectin (23%) and sVCAM-1 (25%); even though in the placebo group, sVCAM-1 was also reduced but to a lesser extent, by 19%. However, the anti-inflammatory TGF-β1 was decreased significantly by 22% in the Omacor group. Interestingly, when the data were analyzed by gender, it was found that in males receiving Omacor, sE-selectin (-23%), sVCAM-1 (-23%), IL-6 (-27%) were reduced significantly. In the females receiving Omacor, only sVCAM-1 was found to be decreased significantly (-38%). Thus, the present study supports some anti-inflammatory actions of moderate dose of Omacor in carotid endarterectomy patients, especially males. Correlation analysis was carried out to study the association between mRNA expression of inflammatory markers in plaque and in plasma concentrations for MMP-9, IL-10, IL-6 and ICAM-1. Results, however demonstrated that there were no significant relationships between these inflammatory markers even though it was found that in the Omacor group, the mRNA expression of MMP-9, IL-6 and ICAM-1 in plaque was significantly different (lower) than in the placebo group. ARA has been shown to exert an anti-inflammatory response because mRNA expression of PPAR-γ, MMP-1, MMP-3, MMP-7, MMP-9, MMP-12, TIMP-1 and ICAM-1 were consistently inversely correlated with ARA. The beneficial effect of EPA as shown in plaque inflammatory expression was demonstrated by mRNA expression of MMP-7, MMP-12, TIMP-1 and IL-6. Therefore, I conclude that soluble inflammatory markers which are measured in blood cannot be used to reflect the expression of these molecules at the cell surface, i.e. in the vasculature or the plaque.

Chapter 4

The Effects of Fatty Acids on the Expression of Adhesion Molecules in Cultured Human Endothelial Cells

4.1 Introduction

Endothelial cells constitute the main barrier of exchange and contact between the blood and the tissue; they play a critical role in inflammatory events that influence the vessel wall (section 1.1). In relation to atherosclerosis development, adhesion of leukocytes to endothelial cells is a complex and crucial process involving adhesion molecules on both the leukocytes and endothelial cells. Important amongst these receptors are VCAM-1 (CD106), ICAM-1 (CD54) and E-selectin (CD62E) which play key roles in the tethering, activation, adhesion and transmigration of the leukocytes into the intimal space underlying the endothelium. As a result of the adhesion molecule mediated leukocyte-endothelium interactions there is an upregulation of production of inflammatory mediators. Unlike E-selectin, ICAM-1 and VCAM-1 are reported to be constitutively expressed by endothelial cells (Prober et al. 1988; Bevilacqua et al. 1989). Nevertheless, endothelial expression of all three of these adhesion molecules can be up-regulated by cytokines or other inflammatory stimuli. For example, the expression of these adhesion molecules is markedly up-regulated by pro-inflammatory cytokines, such as IL-1β and TNF-α (Prober et al. 1988; Bevilacqua et al. 1989; Prober et al. 1987) and the enhanced expression is dependent on mRNA and protein synthesis (Bevilacqua et al. 1989; Dustin et al. 1986).

In Chapter 2, I described findings from an intervention trial with fish oil in healthy, middle-aged men, which identified a small reduction in soluble ICAM-1 concentration in the plasma in subjects consuming 2.1 g/day of eicosapentaenoic acid (EPA) plus docosahexaenoic acid (DHA). The small effect on sICAM-1 and the lack of effect on other

biomarkers studied and the results of correlation analysis suggest that DHA is more important than EPA with regard to these outcomes. Furthermore, the findings suggest a need for further detailed examination of the differential effects of EPA and DHA.

In Chapter 3 I described findings from another intervention trial with n-3 fatty acids, this time in patients with established atherosclerotic disease. In this study, patients received either 1.8 g/day EPA plus DHA in the form of Omacor or 1.2 g/day oleic acid from olive oil as placebo. It was found that Omacor significantly decreased sE-selectin and sVCAM-1, but not sICAM-1, concentrations in plasma. The findings of these two studies indicate a need to further study effects of fatty acids on soluble adhesion molecules, in order to identify more clearly whether these are affected by fatty acids and, if so, the mechanisms involved. Correlation analyses between inflammatory markers in plaque (mRNA level) and in the bloodstream (circulating proteins) showed little relationship. From this, I concluded that soluble inflammatory markers which are measured in blood cannot be used to reflect the expression of these molecules at the cell surface, i.e. on the endothelium or in plaque. Thus studies at the cellular level are required. Thus, in this and the following chapter, I have used *in vitro* models to examine the direct effects of fatty acids on the expression of three adhesion molecules (VCAM-1, ICAM-1 and E-selectin) at two different levels, namely, protein (using ELISA) and mRNA (using real-time Reverse Transcriptase Polymerase Chain Reaction (PCR)), respectively. In this chapter, I will focus on findings on protein expression, and the mRNA expression results will be presented in Chapter 5.

4.1.1 Background of the Study

The present study is inspired by the pioneering work of De Caterina et al. (1994) using *in vitro* experiments using human saphenous vein endothelial cells (HSaVECs) activated by cytokines. Using these cells it was reported that EPA has no effect on endothelial adhesion molecule expression (De Caterina et al. 1994; De Caterina & Libby 1996; De Caterina et al. 1998). It was then demonstrated that EPA at higher concentrations (> 65 μM, compared

with 10-25 μM used by De Caterina and colleagues with HSaVECs), attenuated the expression of ICAM-1, VCAM-1 and E-selectin on human umbilical vein endothelial cells (HUVECs) (Collie-Duguid & Wahle 1996; Khalfoun et al. 1996). It is not clear whether EPA at these higher concentrations may attenuate the expression of adhesion molecules on HSaVECs since there are no studies investigating the effect of this fatty acid at high concentrations. DHA, on the other hand, was able to decrease ICAM-1, VCAM-1 and E-selectin expression on cytokine-stimulated HSaVECs or HUVECs (Collie-Duguid & Wahle 1996; Khalfoun et al. 1996; De Caterina et al. 1994; De Caterina & Libby 1996; De Caterina et al. 1998) and subsequently decreased leukocyte adhesion. Thus, DHA has been determined to be the most potent fatty acid inhibitor of adhesion molecules (De Caterina et al. 1995). Conversely however, the combination of EPA and DHA attenuated the expression of these three endothelial adhesion molecules (Nohe et al. 2002; Collie-Duguid et al. 1996) and reduced L-selectin and LFA-1 on peripheral blood lymphocytes (Khalfoun et al. 1996). Based on *in vitro* studies using HSaVECs, De Caterina et al. (2004) concluded that a minimum of a single double bond is required to inhibit adhesion molecule expression (therefore saturated fatty acids are inactive); the effectiveness does not depend on the chain length and the degree of effectiveness increases correspondingly with unsaturation (double bond insertion).

In atherosclerosis, occlusion of the blood vessels occurs mainly in the arteries, not the veins. To date, there were no studies on the effects of LC n-3 PUFAs on adhesion molecule expression performed using either arterial or aortic endothelial cells. Therefore, it is very important to perform such studies to enable comparison with data from the previous models which were using venous endothelial cells (HSaVECs and HUVECs). I have investigated the effects of individual fatty acids, including EPA, docosapentaenoic acid (DPA), DHA, arachidonic acid (ARA), oleic acid (OA) and stearic acid (SA) on adhesion molecule expression in different cell types (human saphenous vein endothelial cells - HSaVECs, human umbilical vein endothelial cells - HUVECs, human umbilical artery endothelial cells - HUAECs, human coronary artery endothelial cells - HCAECs and human aortic endothelial cells - HAOECs).

4.2 Aim of this Study

The aim of this study was to identify the effects of individual fatty acids on endothelial cells adhesion molecule expression. To investigate this aim, different endothelial cell types were used under similar experimental conditions. The objectives of the experiments were:

- To optimize conditions for LPS stimulation in five different endothelial cell types (HUVECs, HUAECs, HCAECs, HSaVECs and HAOECs) of adhesion molecules expression (LPS concentration and incubation time);

- To optimize conditions for fatty acid pre-incubation (time and concentration);

- To compare the effects of individual fatty acids (EPA, DPA, DHA, ARA, OA and SA) on VCAM-1 protein expression in each endothelial cell type;

- To compare the effects of individual fatty acids on ICAM-1 expression in each endothelial cell type;

- To compare the effects of individual fatty acids on E-selectin expression in each endothelial cell type

4.3 Methods

4.3.1 Reagents

Sodium salts of EPA, DPA, DHA, ARA, OA, and SA were from Sigma-Aldrich, Poole and each dissolved in ethanol to prepare 100 mM fatty acid stock solution at room temperature or at 37^{o} C, to overcome fatty acid insolubility in aqueous solution. LPS (from *E.coli*, cell culture tested) was from Sigma-Aldrich, Poole and was dissolved in growth media to prepare 1 mg/mL stock solution. Endothelial cell growth media were from

PromoCell, Heidelberg and ECACC, Salisbury (only for HAOECs). Cell detaching kit (HepesBSS: 30 mM Hepes, D-glucose, NaCl, KCl, Na Phosphate, Phenol red), trypsin/EDTA solution and soybean trypsin neutralizing solution from soybean (0.05%) were from PromoCell, Heidelberg. The antibiotics penicillin and streptomycin were from PAA Laboratories, Somerset.

4.3.2 Endothelial Cell Culture

Endothelial cells (HUVECs, HUAECs, HSaVECs and HCAECs) were from PromoCell, Heidelberg. HAOECs were from European Collection of Cell Culture (ECACC), Salisbury. The cells were thawed and subcultured following the supplier's instructions. Prior to use in experiments cells at passage four were grown in 96-well plates until confluence.

4.3.3 Optimization of LPS Concentration and Incubation Time

Confluent cultures were seeded after 3-5 minutes exposure to trypsin-EDTA and neutralized by trypsin neutralizing solution (Promocell, Heidelberg). Cells were grown at 1-2 x 10^4 cells per well in 0.2 mL culture medium in 96-well flat-bottomed plates to confluence. Then, cells were stimulated with LPS at different concentrations (1, 10 and 100 µg/mL) and for different durations (6, 12, 24, 48, 72 and 96 hours). At the end of the incubation, cells were incubated in 100 µL fresh, cold 2% formaldehyde in PBS and incubated at 4^o C for 20 minutes and the excess was drained off. After drying for about 2 hours at room temperature, plates were sealed with parafilm and frozen at -20^o C until analysis.

4.3.4 Detection of Adhesion Molecule Expression

The expression of adhesion molecule expression in endothelial cells was determined by ELISA. Prior to ELISA assays, the plate was removed from freezer and was allowed to acclimatize for at least 1 hour at room temperature before removing the parafilm. The plate

was washed three times with wash buffer (0.05% Tween® 20 in PBS, pH 7.4). Cells were then fixed with 100 µL per well of biotinylated anti-human VCAM-1 (CD106) antibody (200 ng/mL), biotinylated anti-human ICAM-1 (CD54) antibody (100 ng/mL) and biotinylated anti-human E-selectin (CD62E) antibody (0.5 µg/mL), respectively for 2 hours at room temperature. Following aspiration and then washing steps, 100 µL of steptavidin horse radish peroxidase (1:200 dilution) was added to each well and further incubated for 30 min at room temperature. After aspiration and washing steps again, 100 µL of substrate solution (1:1 mixture of hydrogen peroxide and tetramethylbenzidine) was added to each well and incubated for 20-30 min at room temperature. The development of colour was stopped by the addition of stop solution (1 M sulfuric acid) and the absorbance was determined on a microplate reader at 450 nm.

4.3.5 Statistical Analysis

Results are presented as mean ± SEM. Data were analyzed using SPSS Version 16.0 (SPSS Inc., Chicago). Multiple comparisons were carried out by one-way analysis of variance (ANOVA) and significant findings were further studied using the least significant difference (LSC) post-hoc comparison.

4.4 Results

4.4.1 Optimization of LPS Concentration

The influence of different LPS concentrations and of duration of LPS exposure upon expression of three adhesion molecules (VCAM-1, ICAM-1, E-selectin) in five endothelial cell types was examined. The aim was to identify optimal conditions (LPS concentration, duration of culture) for use in experiments with fatty acids.

4.4.1.1 Optimization of LPS Concentration in HUVECs

Figures 4.1- 4.3 show the expression of VCAM-1, ICAM-1 and E-selectin in HUVECs following LPS stimulation for 6, 12, 24 and 48 hrs, respectively. At 6 hours incubation time, no significant changes were observed for E-selectin and VCAM-1 (except for increased VCAM-1 at the highest LPS concentration of 100 µg/mL), even though there was significant induction of ICAM-1 at this early time point. LPS stimulation for 12 hours significantly induced the expression of all three adhesion molecules studied, although induction of E-selectin was weaker than of VCAM-1 or ICAM-1. Expression of these adhesion molecules was increased by an average of 424%, 143% and 11%, respectively, by 12 hrs exposure to 1 µg/mL LPS (compared with 12 hours incubation in the absence of LPS). A similar effect was observed at higher LPS concentrations and at 24 hrs LPS stimulation, but by 48 hrs expression of VCAM-1 and E-selectin declined. On the basis of these findings, 12 hours incubation at 1 µg/mL LPS was selected as the condition with which to study the effect of fatty acids on adhesion molecule expression by HUVECs.

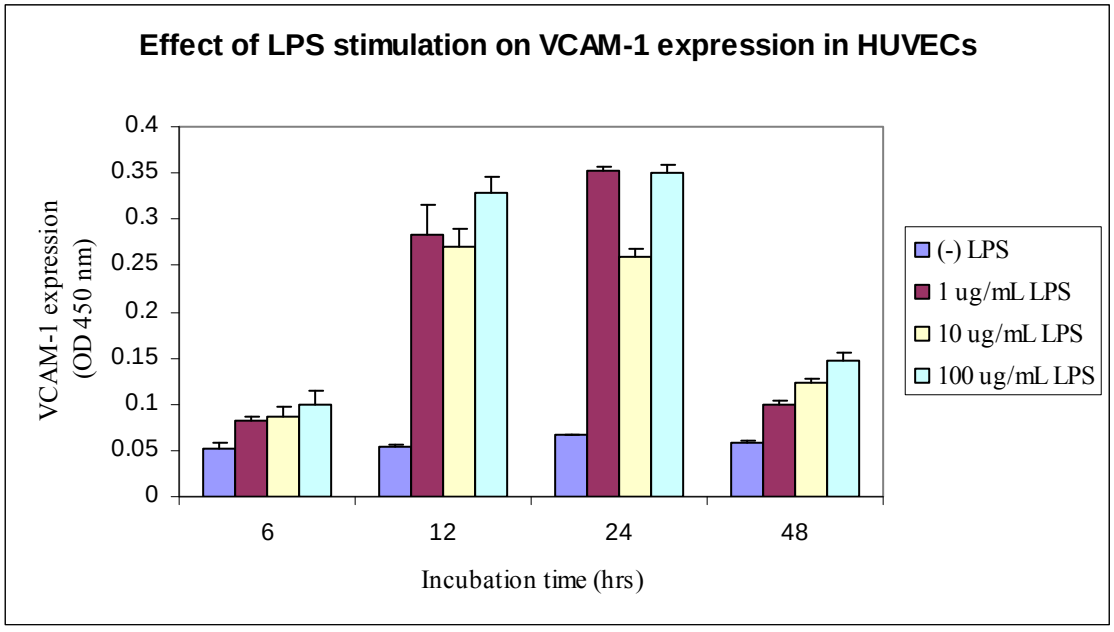


FIGURE 4.1: Effects of LPS Stimulation on VCAM-1 Expression in HUVECs

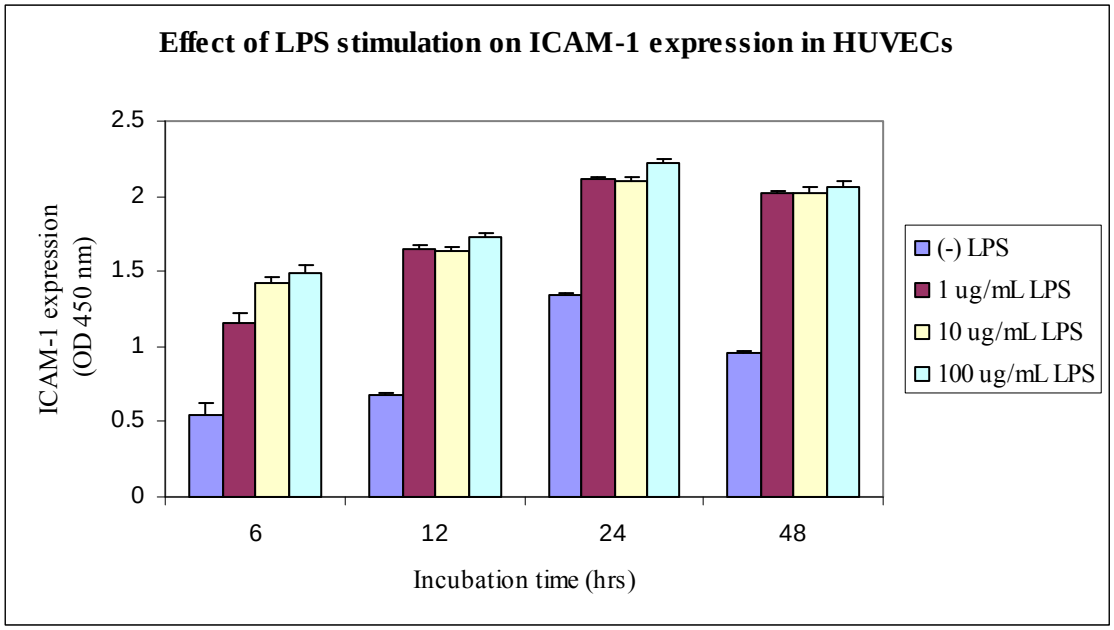


FIGURE 4.2: Effects of LPS Stimulation on ICAM-1 Expression in HUVECs

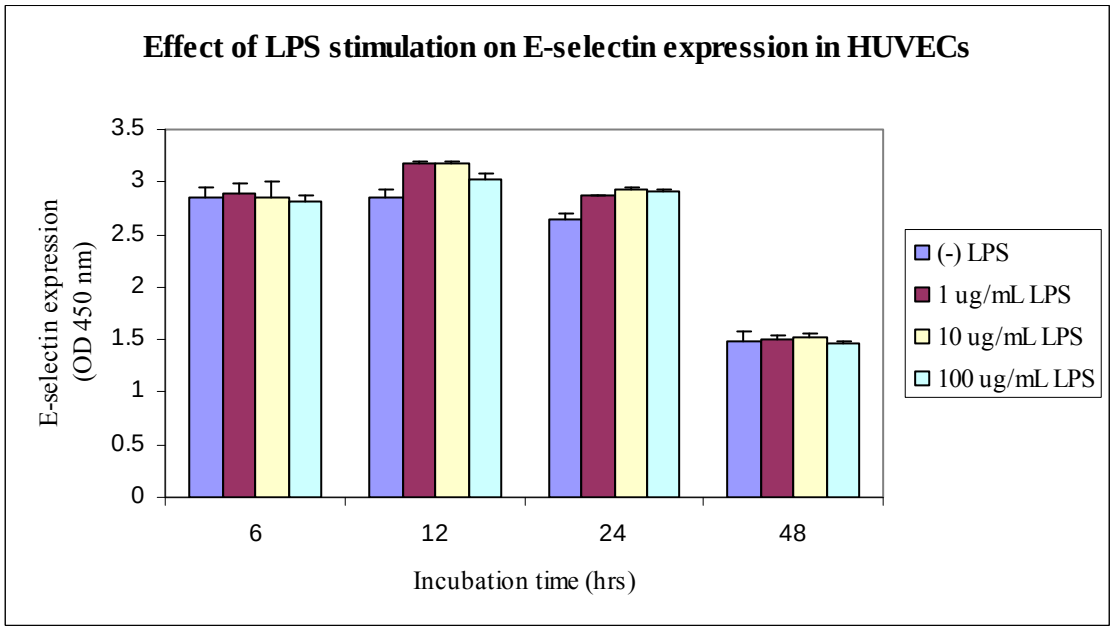


FIGURE 4.3: Effects of LPS Stimulation on E-selectin Expression in HUVECs

4.4.1.2 Optimization of LPS Concentration in HUAECs

Figures 4.4-4.6 show the expression of VCAM-1, ICAM-1 and E-selectin in HUAECs following LPS stimulation for 6, 12, 24 and 48 hrs, respectively. Six hrs incubation time

was sufficient to induce significant increases in expression of all three adhesion molecules. However greater expression of each was observed at 12 and 24 hrs with reduced expression of VCAM-1 and E-selectin occurring at 48 hrs. Expression of the adhesion molecules was increased by an average of 359% (VCAM-1), 62% (ICAM-1) and 29% (E-selectin), respectively, by 12 hrs exposure to 1 µg/mL LPS (compared with 12 hours incubation in the absence of LPS). On the basis of these findings, 12 hours incubation at 1 µg/mL LPS was selected as the condition with which to study the effect of fatty acids on adhesion molecule expression by HUAECs.

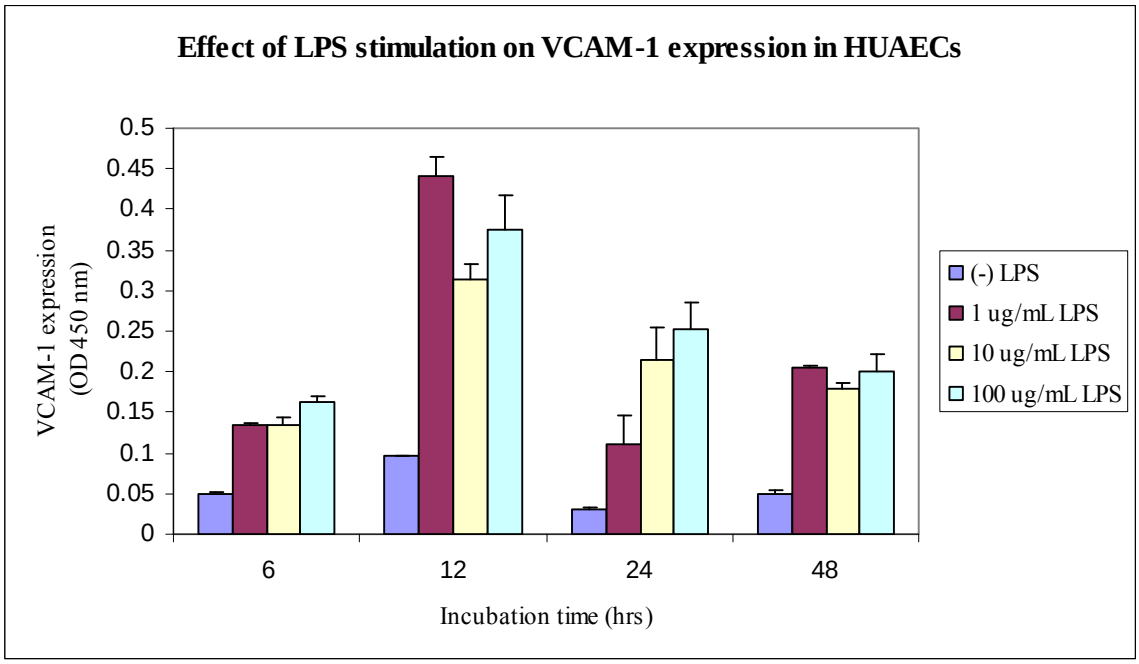


FIGURE 4.4: Effects of LPS Stimulation on VCAM-1 Expression in HUAECs

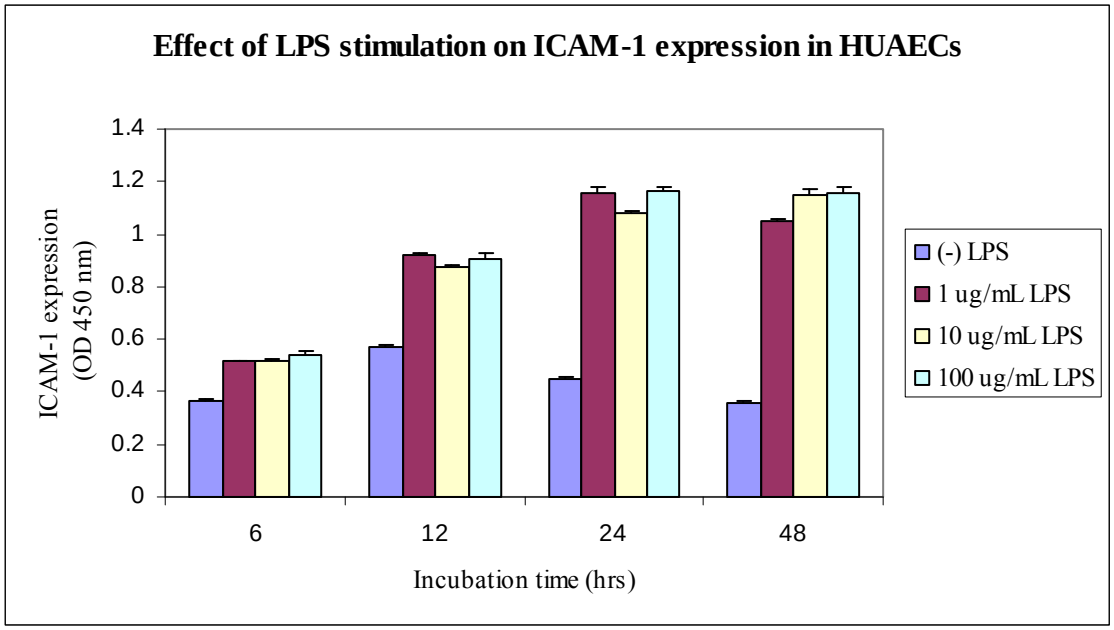


FIGURE 4.5: Effects of LPS Stimulation on ICAM-1 Expression in HUAECs

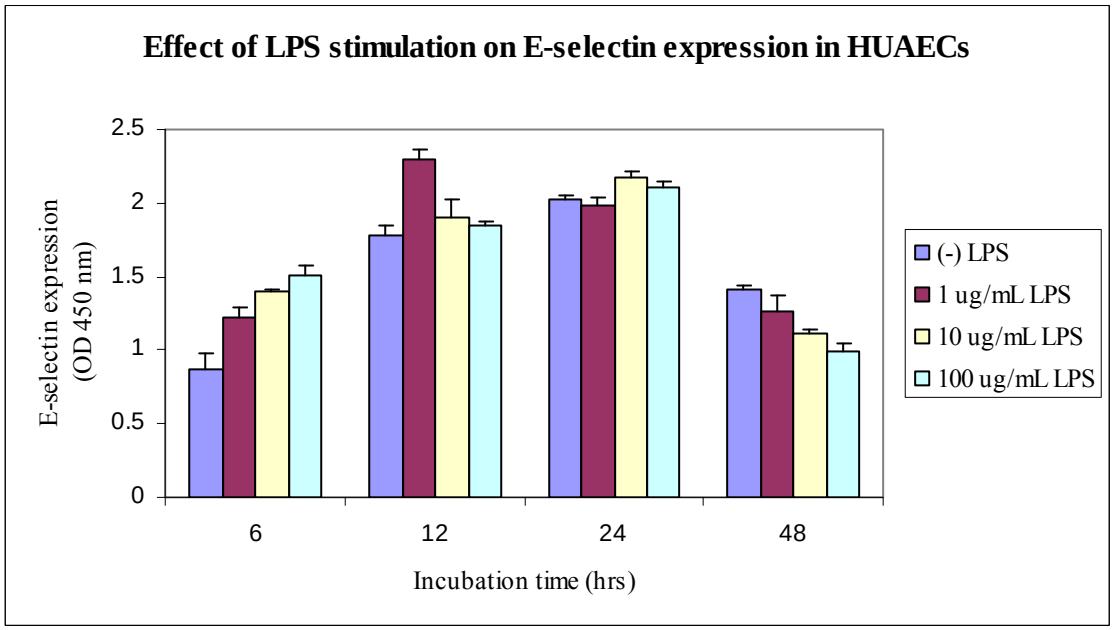


FIGURE 4.6: Effects of LPS Stimulation on E-selectin Expression in HUAECs

4.4.1.3 Optimization of LPS Concentration in HCAECs

Figures 4.7-4.9 show the expression of VCAM-1, ICAM-1 and E-selectin in HCAECs following LPS stimulation for 6, 12, 24, 48, 72 and 96 hrs, respectively. Six hrs incubation time was sufficient to induce significant increases in expression of all three adhesion

molecules. Indeed near maximal expression was seen at this time point. The effect of LPS was dose-dependent at the earlier time points. Expression of the adhesion molecules was increased by an average of 495% (VCAM-1), 134% (ICAM-1) and 46% (E-selectin), respectively, by 12 hrs exposure to 1 μg/mL LPS (compared with 12 hours incubation in the absence of LPS). Expression of VCAM-1 and ICAM-1 was maintained up to 96 hours but expression of E-selectin declined at 24 hrs of culture and thereafter. On the basis of these findings, 12 hours incubation at 1 μg/mL LPS was selected as the condition with which to study the effect of fatty acids on adhesion molecule expression by HCAECs.

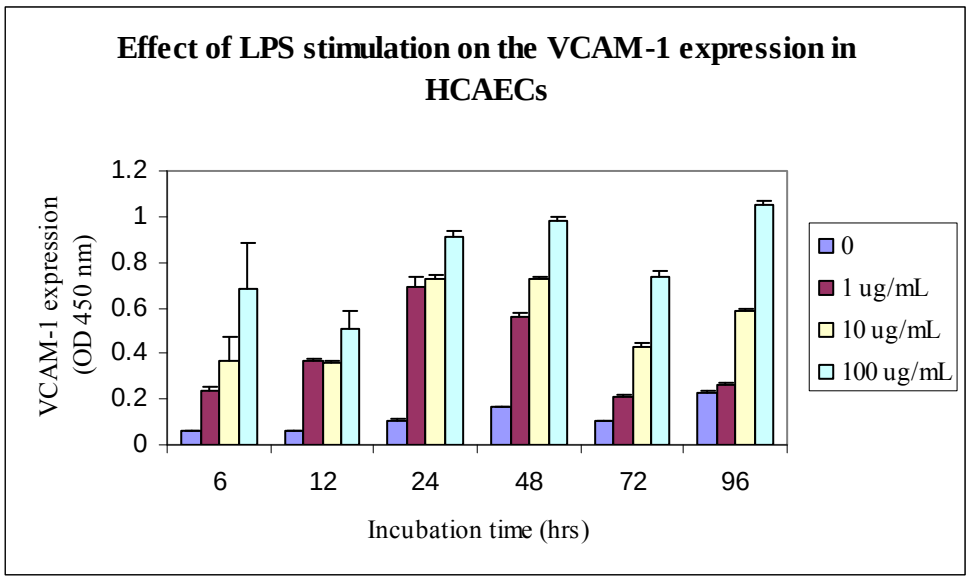


FIGURE 4.7: Effects of LPS Stimulation on VCAM-1 Expression in HCAECs

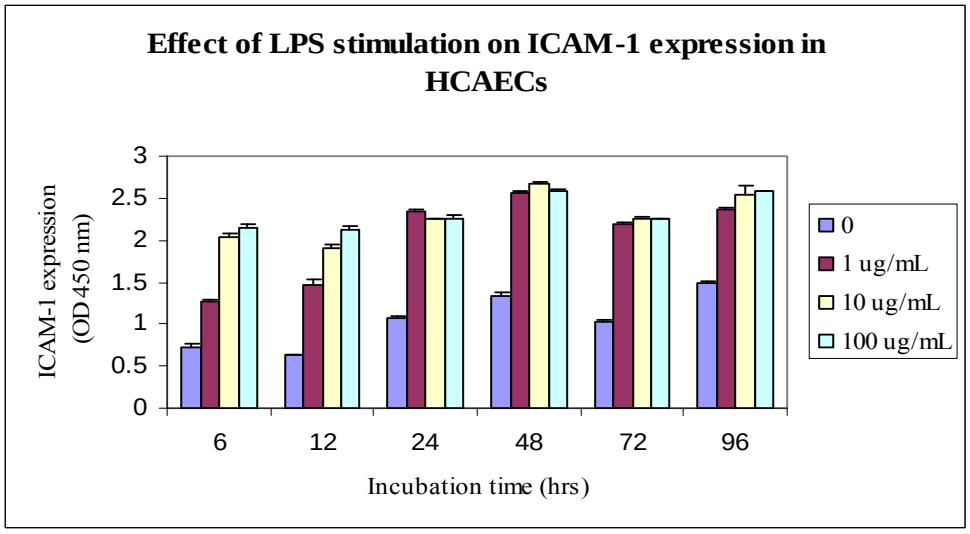


FIGURE 4.8: Effects of LPS Stimulation on ICAM-1 Expression in HCAECs

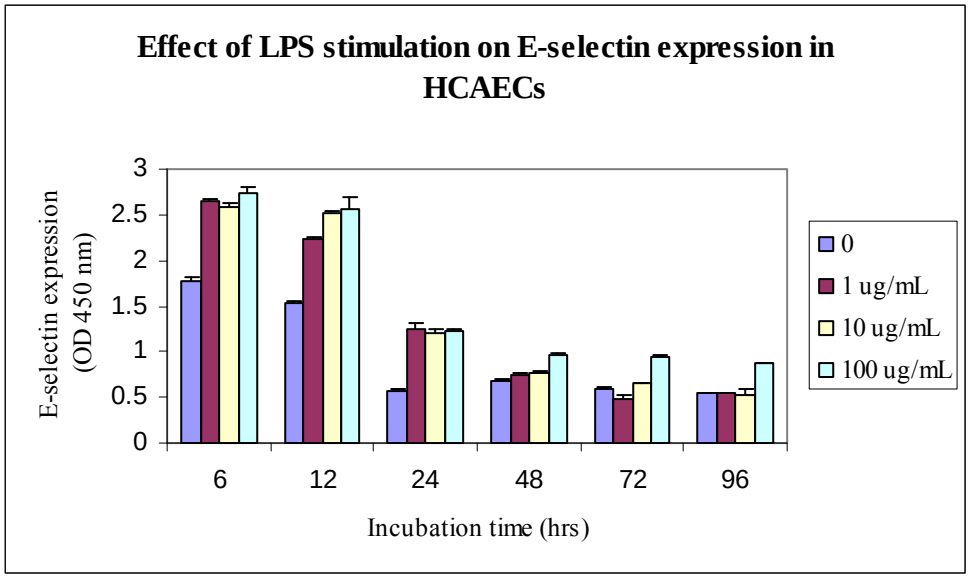


FIGURE 4.9: Effects of LPS Stimulation on E-selectin Expression in HCAECs

4.4.1.4 Optimization of LPS Concentration in HSaVECs

Figures 4.10-4.12 show the expression of VCAM-1, ICAM-1 and E-selectin in HSaVECs following LPS stimulation for 6, 12, 24, 48, 72 and 96 hrs, respectively. Six hrs incubation time was sufficient to induce an increase in expression of VCAM-1, with smaller increases in ICAM-1 and E-selectin. Greater expression of VCAM-1 was seen at 12 and 24 hrs of culture, while longer incubation times (48 or 72 hrs) were required to observe higher ICAM-1 expression. E-selectin expression declined beyond 12 hrs of culture, although LPS was still effective. Expression of the adhesion molecules was increased by an average of 309% (VCAM-1), 12% (ICAM-1) and 51% (E-selectin), respectively, by 12 hrs exposure to 1 μg/mL LPS (compared with 12 hours incubation in the absence of LPS). On the basis of these findings, 12 hours incubation at 1 μg/mL LPS was selected as the condition with which to study the effect of fatty acids on adhesion molecule expression by HUAECs.

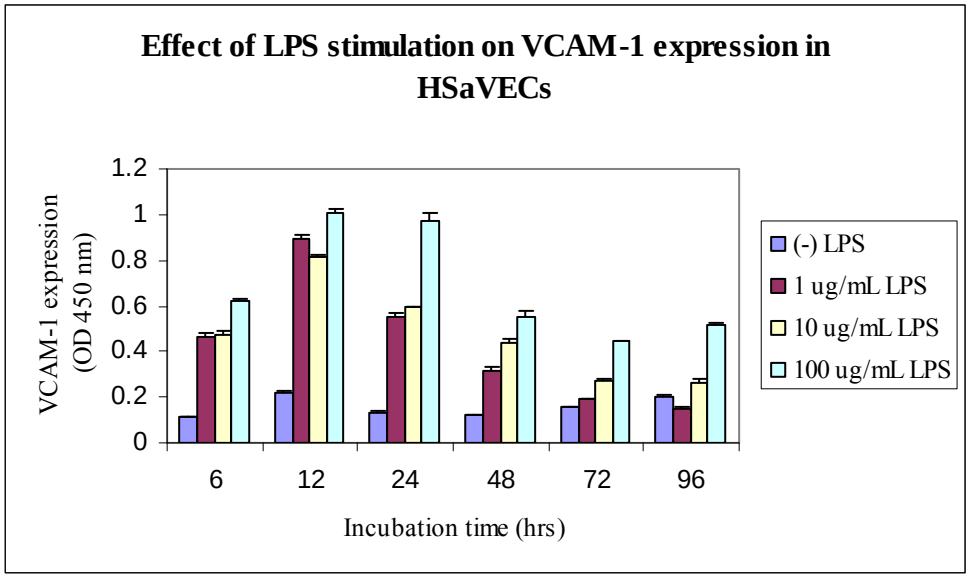


FIGURE 4.10: Effects of LPS Stimulation on VCAM-1 Expression in HSaVECs

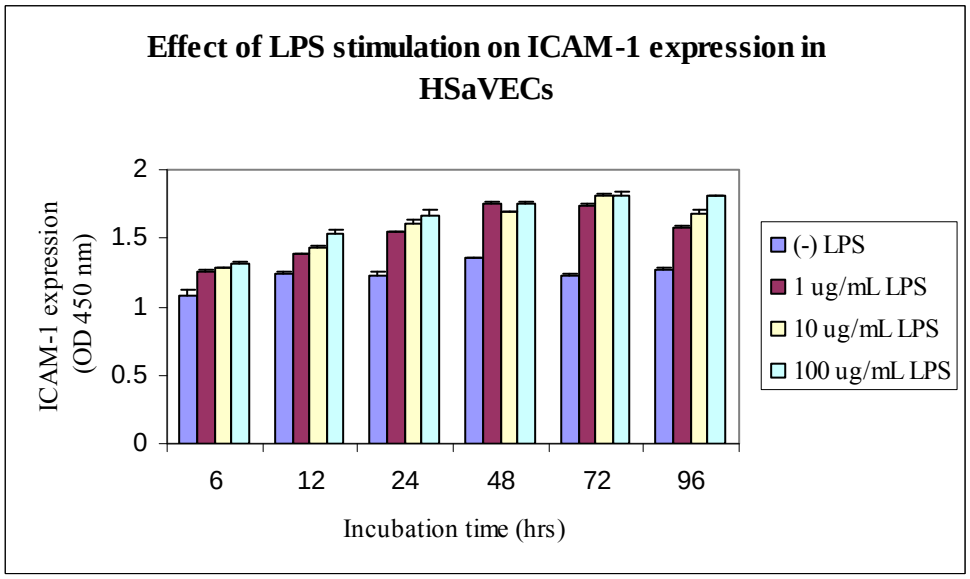


FIGURE 4.11: Effects of LPS Stimulation on ICAM-1 Expression in HSaVECs

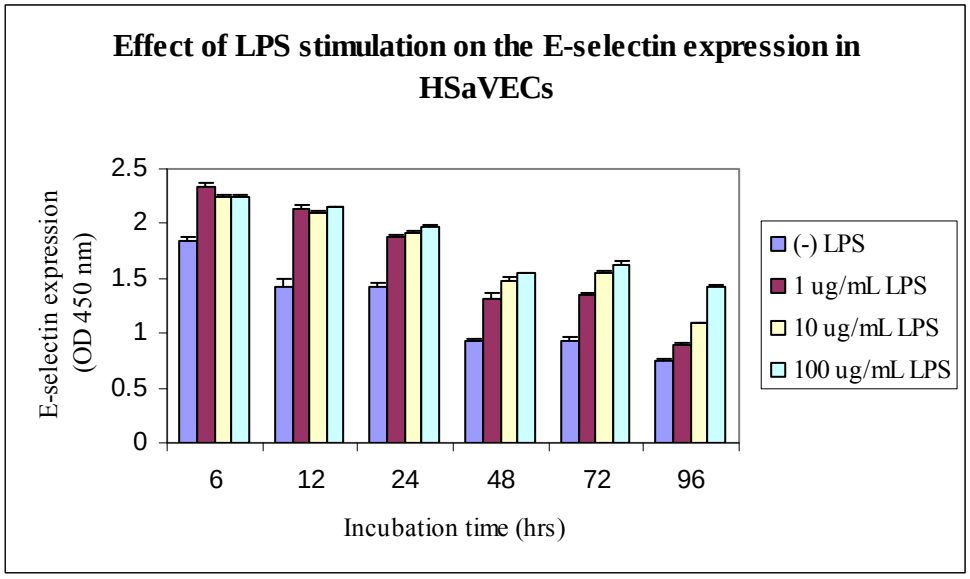


FIGURE 4.12: Effects of LPS Stimulation on E-selectin Expression in HSaVECs

4.4.1.5 Optimization of LPS Concentration in HAOECs

Figures 4.13- 4.15 show the expression of VCAM-1, ICAM-1 and E-selectin in HAOECs following LPS stimulation for 6, 12, 24, 48 and 72 hrs, respectively. Six hrs incubation time was sufficient to induce significant, and near maximal, increases in expression of all three adhesion molecules, although for HAOECs LPS at 1 μg/mL was ineffective. Expression of the adhesion molecules was increased by an average of 202% (VCAM-1), 47% (ICAM-1) and 54% (E-selectin), respectively, by 12 hrs exposure to 10 μg/mL LPS (compared with 12 hours incubation in the absence of LPS). On the basis of these findings, 12 hours incubation at 10 ug/mL LPS was selected as the condition with which to study the effect of fatty acids on adhesion molecule expression by HAOECs.

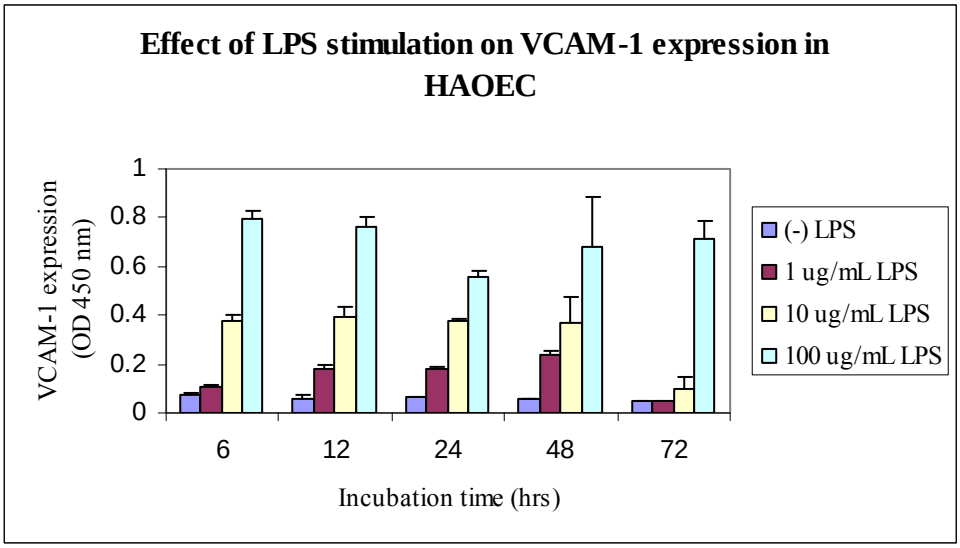


FIGURE 4.13: Effects of LPS Stimulation on VCAM-1 Expression in HAOECs

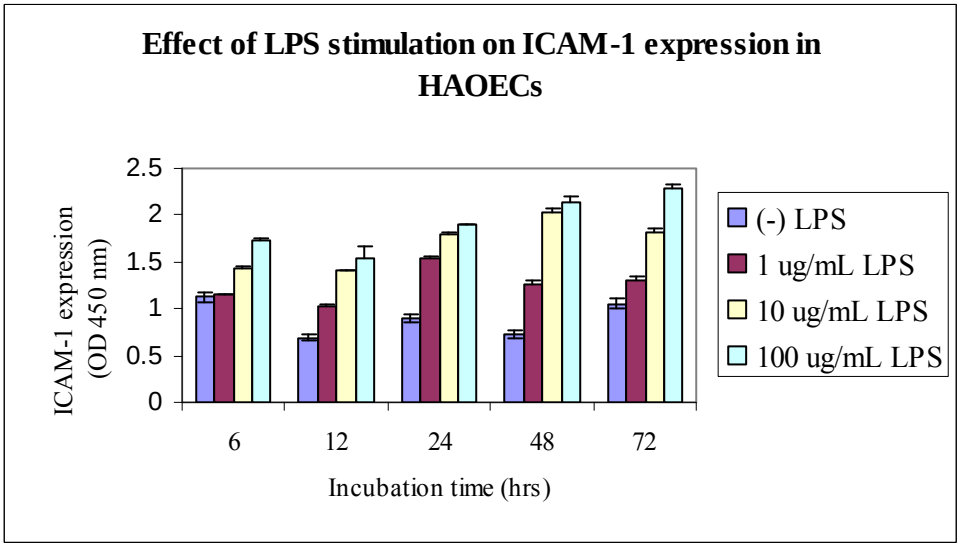


FIGURE 4.14: Effects of LPS Stimulation on ICAM-1 Expression in HAOECs

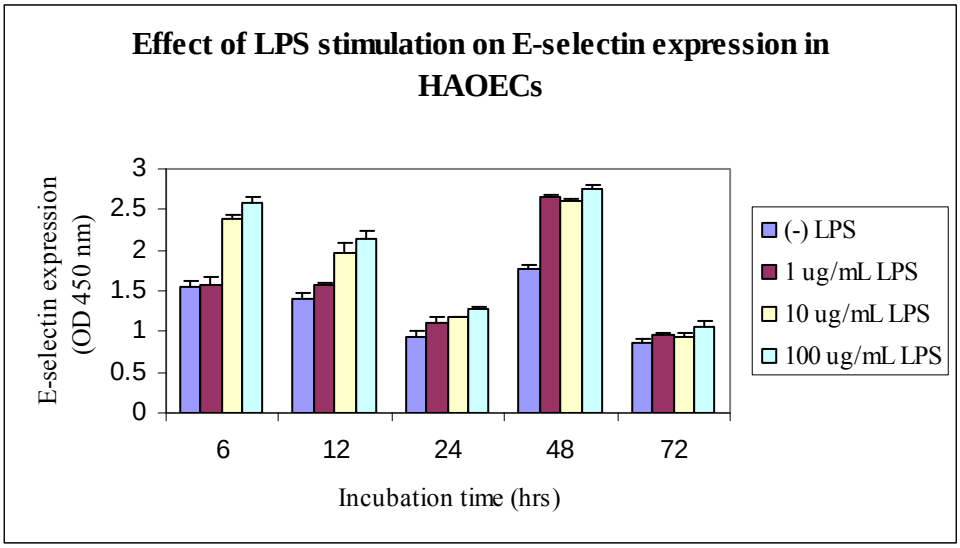


FIGURE 4.15: Effects of LPS Stimulation on E-selectin Expression in HAOECs

4.4.2 Optimization of Fatty Acid Pre-incubation Time and Concentration

In this set of experiments, two endothelial cell types were used, namely HUVECs and HAOECs. This is because from the LPS stimulation results (section 4.4.1), similar conditions were found for all cell types apart from HAOECs. Therefore, for these latter cells specific conditions, different from those used with the other four cell types, needed to be used. The aim of these experiments was to identify a fatty acid concentration and pre-incubation time to be used in experiments where several fatty acids would be compared. DHA was used for these studies, since it was expected to influence adhesion molecule expression.

4.4.2.1 Optimization of Fatty Acid Pre-incubation in HUVECs

HUVECs were pre-incubated with DHA at 0, 25, 50 and 100 μM for 24, 48 and 80 hrs. Then the cells were stimulated with LPS at 1 μg/mL for 12 hrs. Figures 4.16-4.18 show the effects of DHA preincubation on LPS-induced expression of VCAM-1, ICAM-1 and E-selectin. Exposure to DHA (50 or 100 μM) for 48 or 80 hrs was able to abolish the effect of LPS on VCAM-1 expression (Figure 4.16). Exposure to DHA (50 or 100 μM) for 48 hrs was able to reduce the effect of LPS on ICAM-1 expression while longer exposure to DHA

(80 hrs) abolished LPS-induced ICAM-1 exposure (Figure 4.17). DHA had little effect on LPS-induced E-selectin expression, except that this was increased at the shortest exposure (24 hrs) to 100 μM DHA (Figure 4.18). On the basis of this experiment, a fatty acid concentration of 50 μM and a pre-incubation time of 48 hrs was selected to compare the effects of different fatty acids.

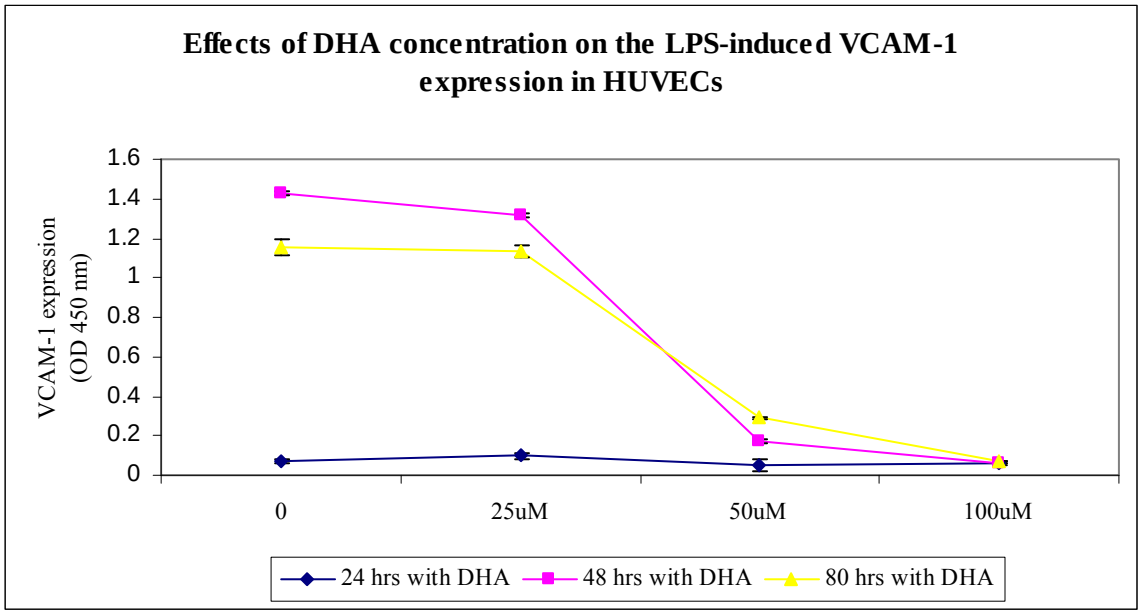


FIGURE 4.16: Effect of Fatty Acid Pre-incubation on VCAM-1 Expression in HUVECs

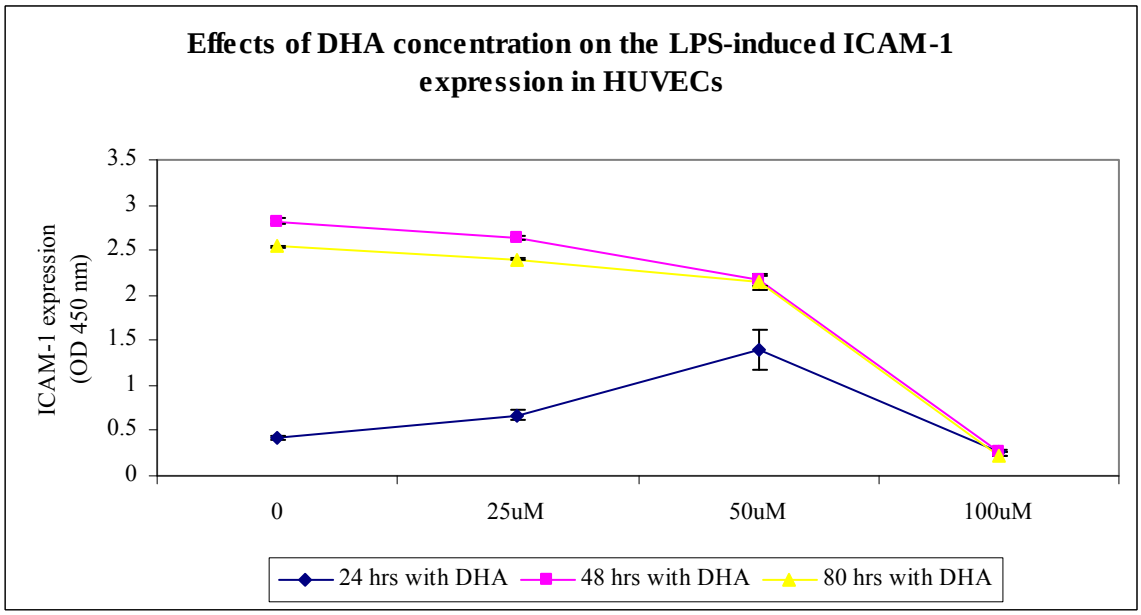


FIGURE 4.17: Effect of Fatty Acid Pre-incubation on ICAM-1 Expression in HUVECs

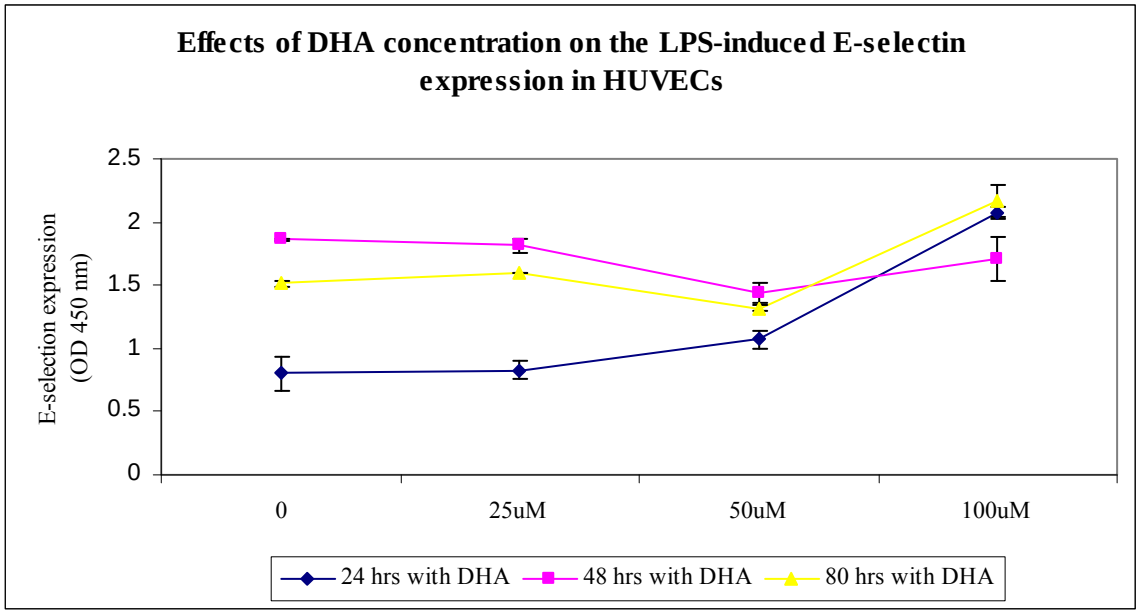


FIGURE 4.18: Effect of Fatty Acid Pre-incubation on E-selectin Expression in HUVECs

4.4.2.2 Optimization of Fatty Acid Pre-incubation in HAOECs

HAOECs were pre-incubated with DHA at 0, 25, 50 and 100 μM for 24, 48 and 80 hrs. Then the cells were stimulated with LPS at 1 μg/mL for 12 hrs. Figures 4.19-4.21 show the effects of DHA preincubation on LPS-induced expression of VCAM-1, ICAM-1 and E-selectin. Exposure to DHA (50 or 100 μM) for 24, 48 or 80 hrs abolished the effect of LPS on VCAM-1 and ICAM-1 expression (Figures 4.19 and 4.20). DHA (50 or 100 μM) increased LPS-induced E-selectin expression (Figure 4.21). On the basis of this experiment, a fatty acid concentration of 50 μM and a pre-incubation time of 48 hrs was selected to compare the effects of different fatty acids.

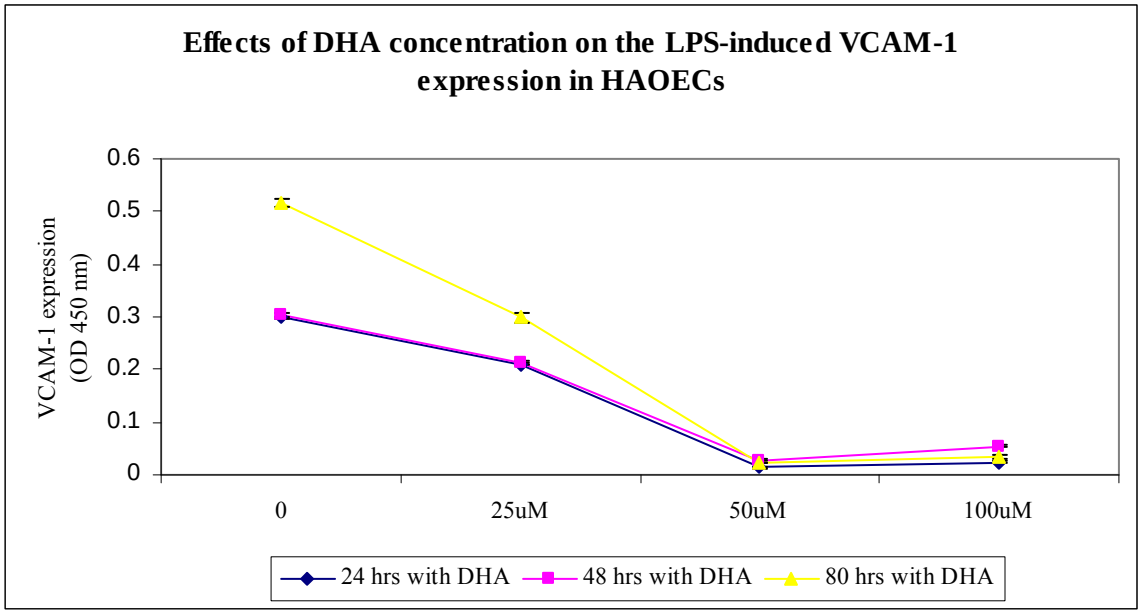


FIGURE 4.19: Effect of Fatty Acid Pre-incubation on VCAM-1 Expression in HAOECs

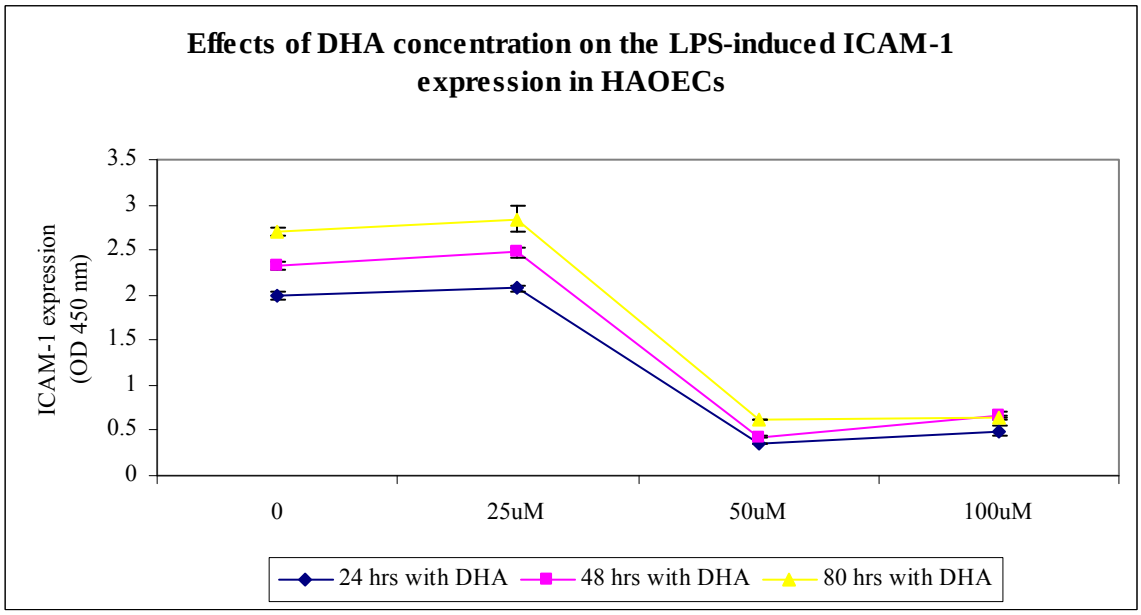


FIGURE 4.20: Effect of Fatty Acid Pre-incubation on ICAM-1 Expression in HAOECs

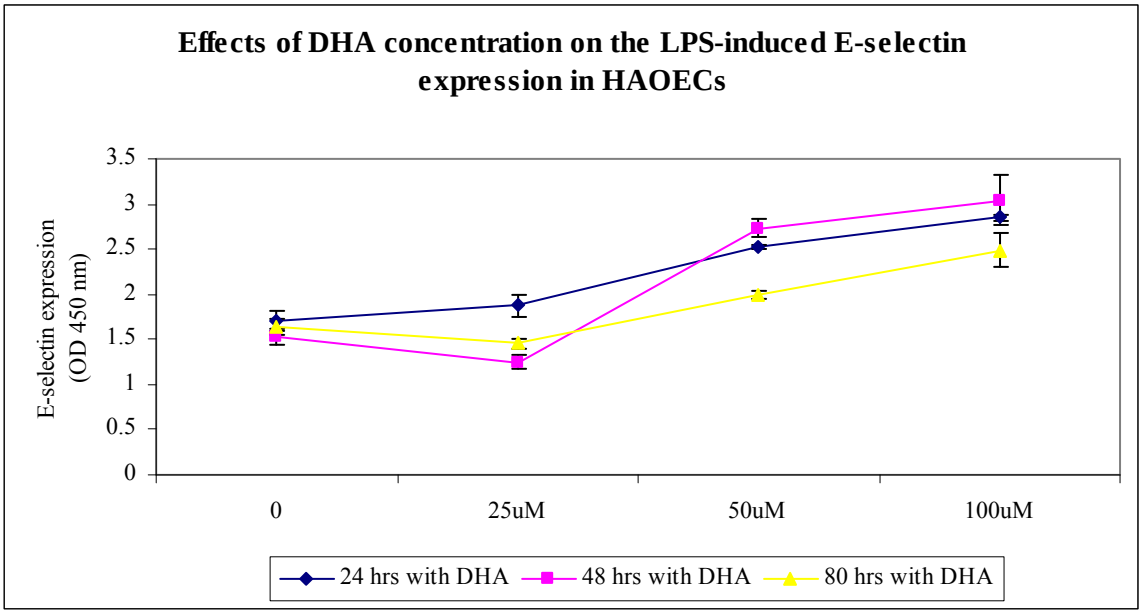


FIGURE 4.21: Effect of Fatty Acid Pre-incubation on E-selectin Expression in HAOECs

4.4.3 Effects of Fatty Acids on Adhesion Molecule Expression

A comparison of the effects of fatty acids on adhesion molecule expression was carried out under optimal conditions of 48 hrs pre-incubation with fatty acid at 50 μM and subsequent stimulation with 1 μg/mL or, for HAOECs, 10 μg/mL LPS for a further 12 hrs. Expression of adhesion molecules was determined by ELISA as described in Section 4.3.4. To assess whether any fatty acid *per se* exhibits any effects on adhesion molecule expression, parallel comparative experiments without LPS stimulation were conducted. Six fatty acids were used: EPA, DPA, DHA, ARA, OA and SA.

4.4.3.1 Effects of Fatty Acids on Adhesion Molecule Expression in HUVECs

Figures 4.22-4.24 show the effects of six fatty acids on VCAM-1, ICAM-1 and E-selectin expression on unstimulated and LPS-stimulated HUVECs. It was observed that LPS significantly up-regulated adhesion molecule expression in HUVECs, particularly VCAM-1. None of the fatty acids except DHA demonstrated any significant effect on adhesion molecule expression in the absence of LPS stimulation ($P > 0.05$). DHA induced expression

of ICAM-1 by 73% ($P = 0.01$) and of E-selectin by 22% ($P < 0.001$) on unstimulated HUVECs.

Under the optimized experimental conditions, all fatty acids studied significantly attenuated VCAM-1 expression in HUVECs ($P < 0.05$) (Figure 4.22). DHA exerted the greatest inhibitory effect (average 85%), followed by ARA (71%), EPA (63%) and DPA (54%). OA and SA exhibited a similar degree of inhibition (34%) of LPS-induced expression of VCAM-1.

The observed effects of fatty acids on LPS-induced ICAM-1 and E-selectin expression were less marked than the effects on VCAM-1 expression (Figure 4.23 and 4.24, respectively). ARA, DHA, EPA, and DPA inhibited ICAM-1 expression significantly ($P < 0.05$) by an average of 30%, 23%, 23% and 12%, respectively. Neither OA nor SA showed any significant effects for ICAM-1 expression ($P > 0.05$). EPA, ARA and DHA significantly attenuated LPS-induced E-selectin expression by 16%, 15% and 6%, respectively. DPA did not affect E-selectin expression while both OA and SA significantly increased E-selectin expression in HUVECs ($P < 0.001$), by 13% and 16%, respectively.

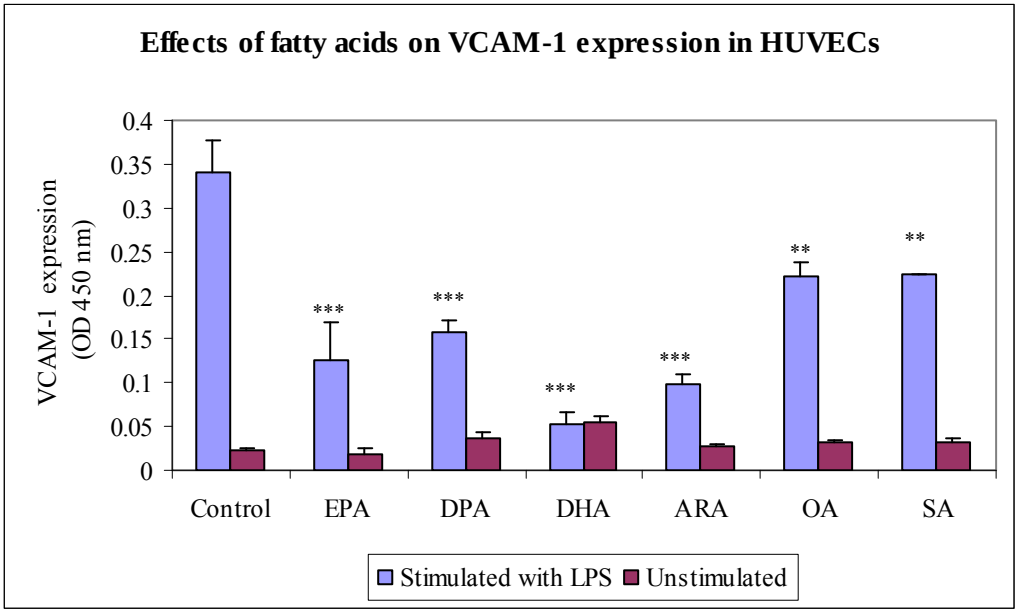


FIGURE 4.22: Comparative Effects of Fatty Acids on VCAM-1 Expression in HUVECs

Data are expressed as mean ± SEM (n = 3). Significantly different from control, **$P < 0.005$, ***$P < 0.001$.

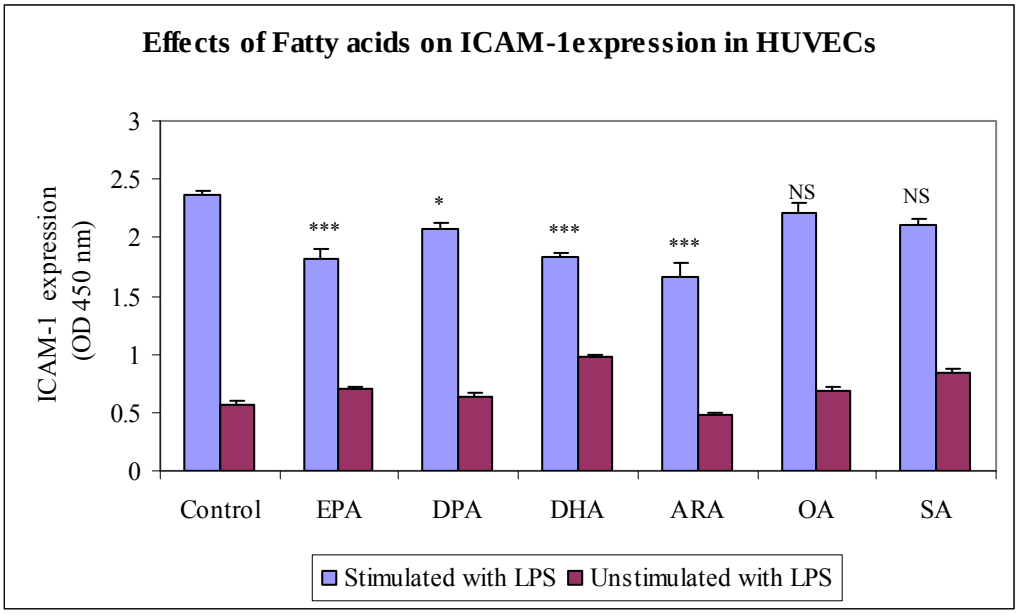


FIGURE 4.23: Comparative Effects of Fatty Acids on ICAM-1 Expression in HUVECs

Data are expressed as mean ± SEM (n = 3). Significantly different from control, * P < 0.05, ***P < 0.001, NS = non-significant.

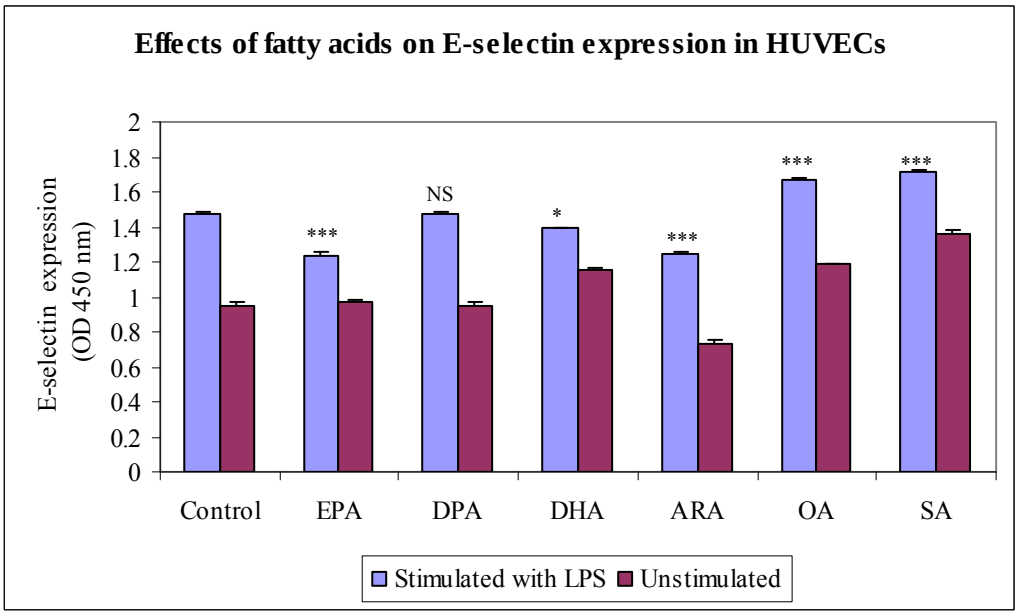


FIGURE 4.24: Comparative Effects of Fatty Acids on E-selectin Expression in HUVECs

Data are expressed as mean ± SEM (n = 3). Significantly different from control, * P < 0.05, ***P < 0.001, NS = non-significant.

Thus certain fatty acids were able to inhibit LPS-induced expression of VCAM-1, ICAM-1 and E-selectin in HUVECs, although the magnitude of VCAM-1 inhibition was the greatest, followed by ICAM-1 and E-selectin. Figure 4.25 attempts to summarise the pattern of inhibition seen across all fatty acids and all three adhesion molecules; this figure demonstrates that a very similar pattern of effects of fatty acids was seen, although the extent of the effect of a given fatty acid was different according to the adhesion molecule studied.

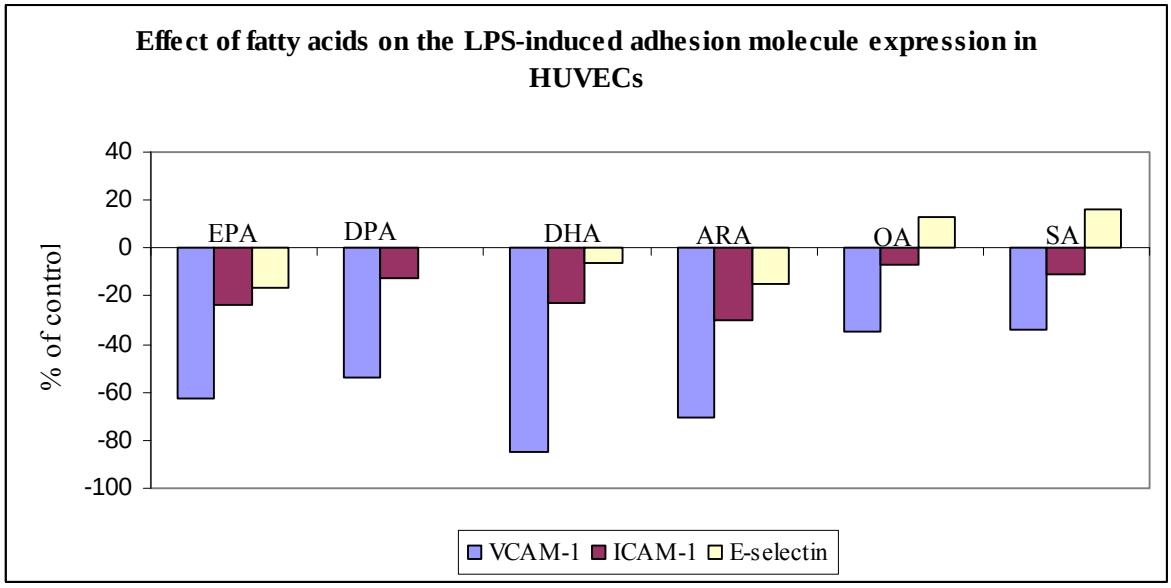


FIGURE 4.25: Comparative Effects of Fatty Acids on Adhesion Molecules Expression in HUVECs

4.4.3.2 Effects of Fatty Acids on Adhesion Molecule Expression in HUAECs

Figures 4.26-4.28 show the effects of six fatty acids on VCAM-1, ICAM-1 and E-selectin expression on unstimulated and LPS-stimulated HUAECs; this is the first time that the effects of fatty acids on these cells has been reported. It was observed that LPS significantly up-regulated adhesion molecule expression in HUAECs, particularly VCAM-1 and ICAM-1. None of the fatty acids demonstrated any significant effect on VCAM-1 expression in the absence of LPS stimulation ($P > 0.05$). However, ICAM-1 was induced by EPA ($P =$

0.001), OA (P = 0.001) and SA (P = 0.025) and E-selectin was induced by DHA and SA (both P < 0.001).

Under the optimized experimental conditions, all fatty acids studied significantly attenuated VCAM-1 expression in HUAECs (all P < 0.001) (Figure 4.26). DHA exerted the greatest inhibitory effect (average 95%), followed by ARA (85%), DPA (84%), SA (81%), EPA (77%) and OA (60%).

The effects of fatty acids on LPS-induced ICAM-1 and particularly E-selectin expression were less marked than the effects on VCAM-1 expression (Figures 4.27 and 4.28). DHA, DPA, EPA and SA inhibited ICAM-1 expression significantly (P < 0.05) by an average of 43%, 41%, 22% and 38%, respectively. Neither ARA nor OA showed any significant effects on ICAM-1 expression (P > 0.05). EPA and DPA significantly attenuated LPS-induced E-selectin expression by 21% and 19%, respectively. In contrast, DHA significantly enhanced LPS-induced E-selectin expression in HUAECs. ARA, OA and SA did not affect E-selectin expression.

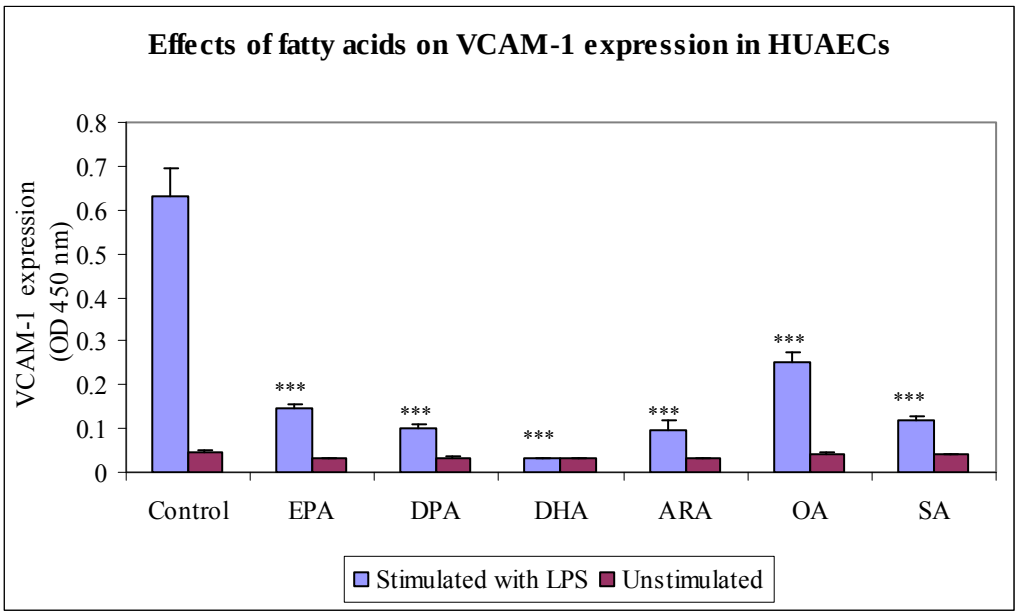


FIGURE 4.26: Comparative Effects of Fatty Acids on VCAM-1 Expression in HUAECs. Data are expressed as mean ± SEM (n = 3). Significantly different from control, ***P < 0.001.

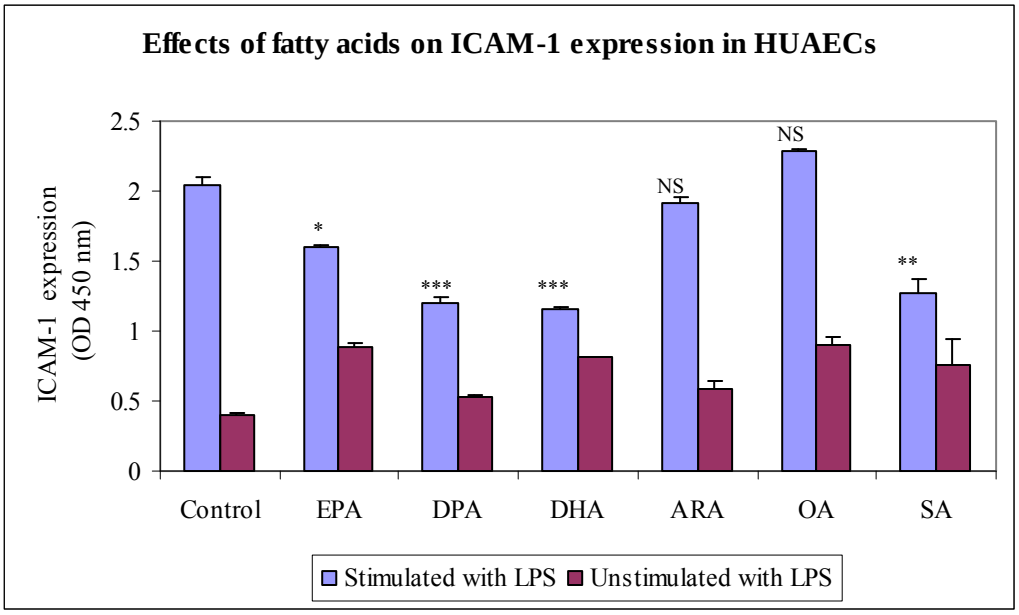


FIGURE 4.27: Comparative Effects of Fatty Acids on ICAM-1 Expression in HUAECs

Data are expressed as mean ± SEM (n = 3). Significantly different from control, * P < 0.05, **P < 0.005, ***P < 0.001, NS = non-significant.

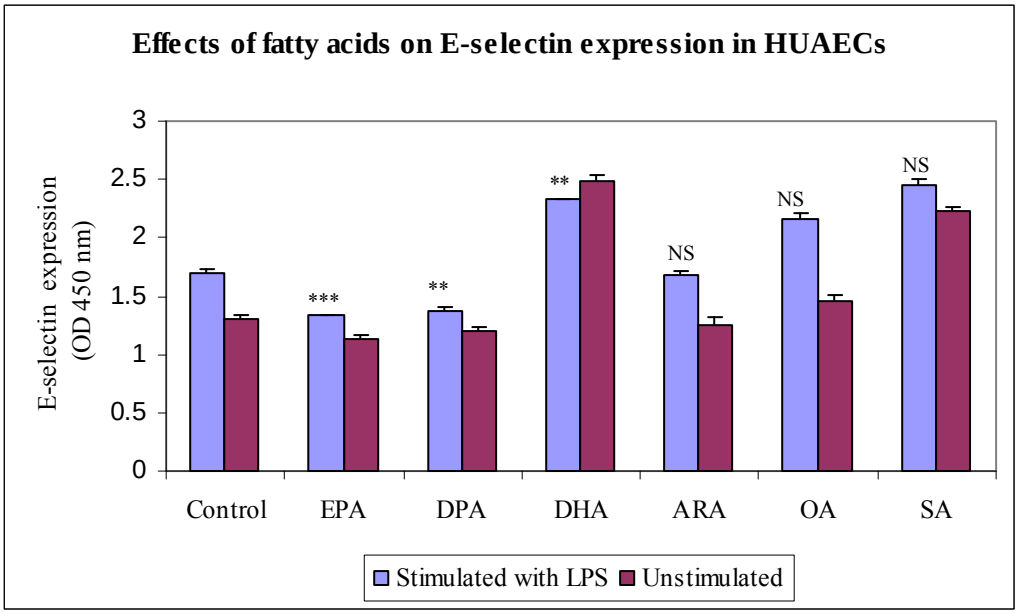


FIGURE 4.28: Comparative Effects of Fatty Acids on E-selectin Expression in HUAECs

Data are expressed as mean ± SEM (n = 3). Significantly different from control, **P < 0.005, ***P < 0.001, NS = non-significant.

Thus certain fatty acids were able to modulate LPS-induced expression of VCAM-1, ICAM-1 and E-selectin in HUAECs (Figure 4.29). Several fatty acids inhibited VCAM-1 and ICAM-1 expression with a greater effect on the former and DHA had the greatest effect. Effects of fatty acids on E-selectin included both inhibition and enhancement, although the magnitude of effects seen was smaller than those seen with VCAM-1 and ICAM-1 (Figure 4.29).

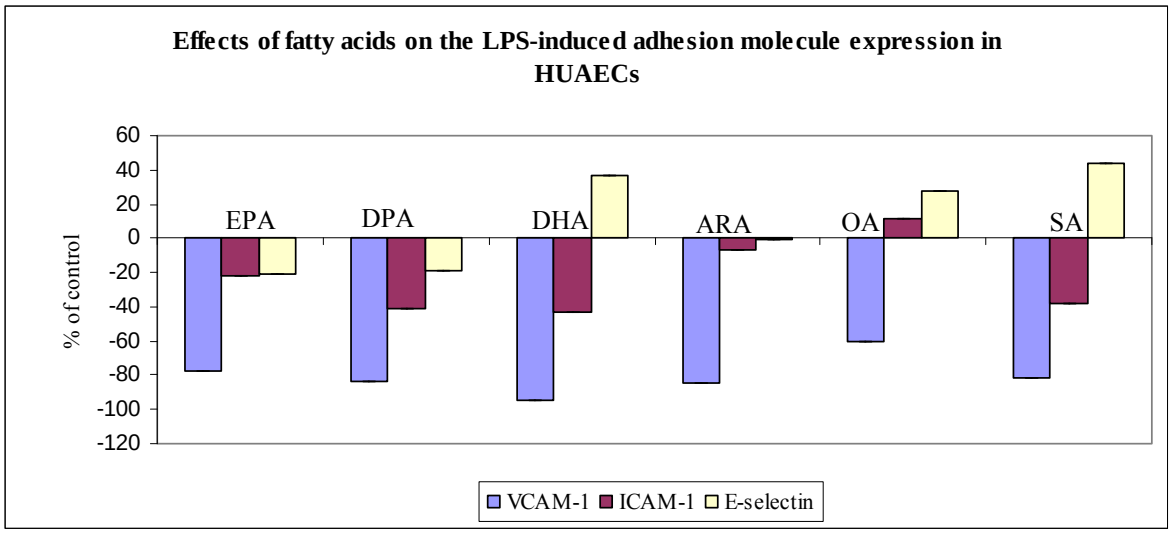


FIGURE 4.29: Comparative Effects of Fatty Acids on Adhesion Molecules Expression in HUAECs

4.4.3.3 Effects of Fatty Acids on Adhesion Molecule Expression in HCAECs

Figures 4.30-4.32 show the effects of six fatty acids on VCAM-1, ICAM-1 and E-selectin expression on unstimulated and LPS-stimulated HCAECs; this is the first time that the effects of fatty acids on these cells has been reported, apart from Reissig et al. (2003) in their study in respect to only linoleic acid. It was observed that LPS significantly up-regulated adhesion molecule expression in HCAECs, particularly VCAM-1. None of the fatty acids demonstrated any significant effect on VCAM-1 expression in the absence of LPS stimulation ($P > 0.05$). However, ICAM-1 was induced by DHA ($P = 0.005$), OA ($P = 0.035$) and SA ($P < 0.001$) and E-selectin was induced by ARA, OA and SA (all $P < 0.001$).

Under the optimized experimental conditions, ARA and DHA significantly attenuated VCAM-1 expression in HCAECs ($P = 0.014$ and $P < 0.001$, respectively) (Figure 4.31). ARA exerted the greatest inhibitory effect (average 40%), followed by DHA (13%). SA enhanced LPS-stimulated VCAM-1 expression by 40%.

None of the tested fatty acids affected LPS-induced ICAM-1 in HCAECs (Figure 4.31). Likewise no fatty acid inhibited LPS-induced E-selectin expression, but both OA and SA enhanced this by about 20% ($P < 0.05$).

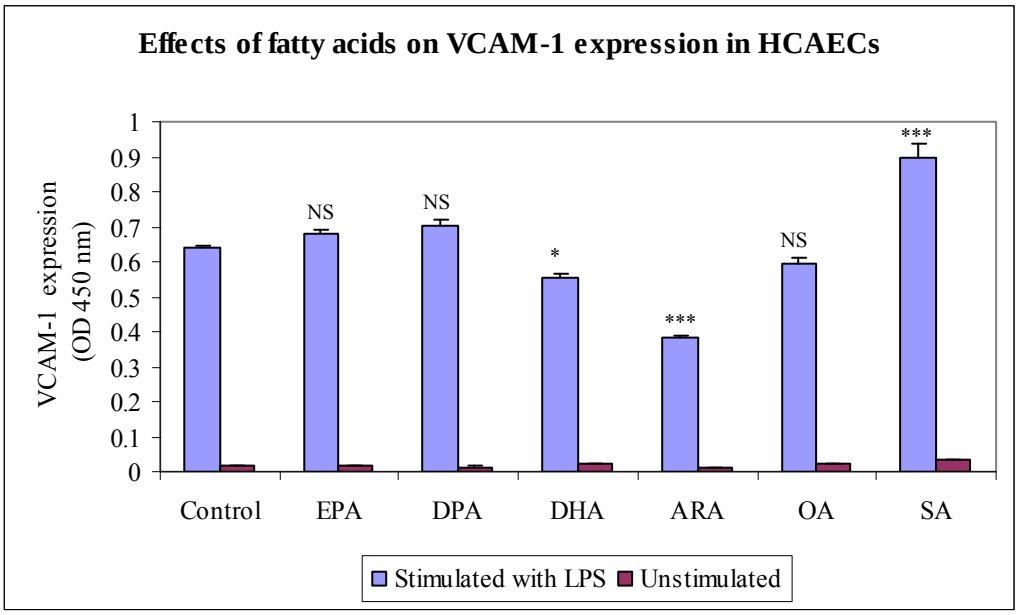


FIGURE 4.30: Comparative Effects of Fatty Acids on VCAM-1 Expression in HCAECs

Data are expressed as mean ± SEM (n = 3). Significantly different from control, * $P < 0.05$, ***$P < 0.001$, NS = non-significant.

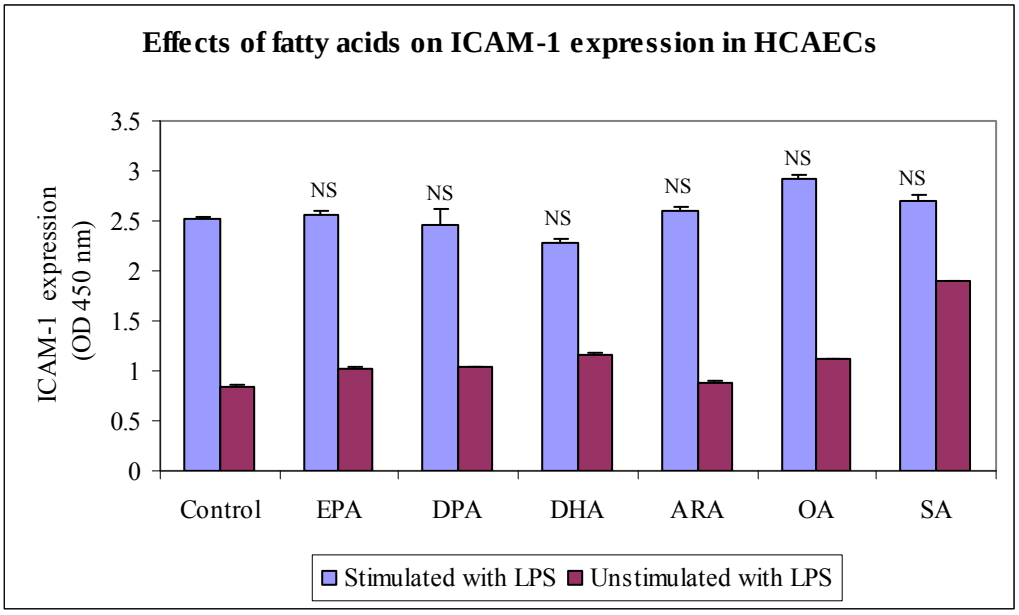


FIGURE 4.31: Comparative Effects of Fatty Acids on ICAM-1 Expression in HCAECs

Data are expressed as mean ± SEM (n = 3). NS = non-significant.

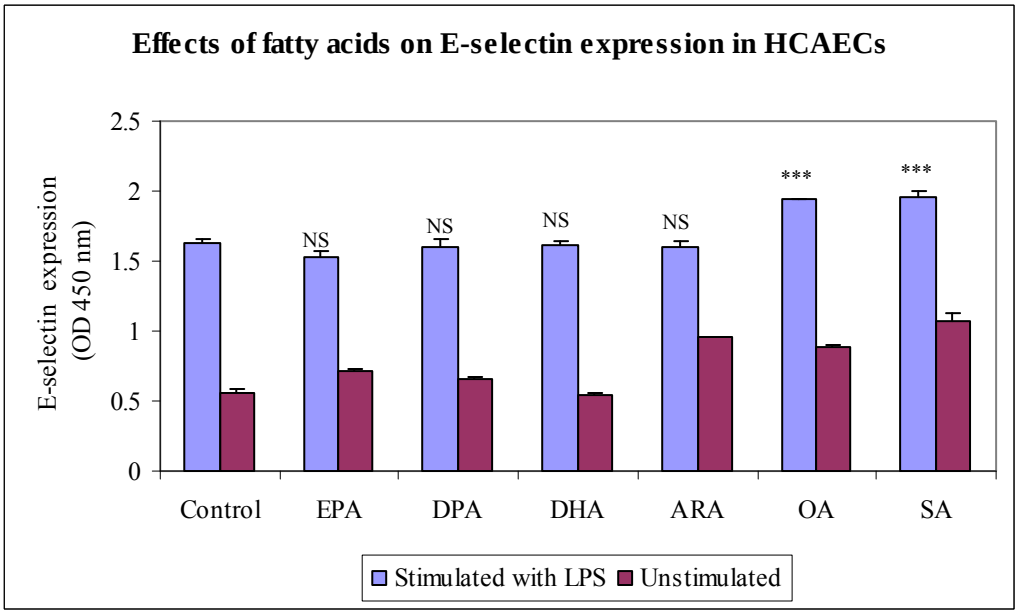


FIGURE 4.32: Comparative Effects of Fatty Acids on E-selectin Expression in HCAECs

Data are expressed as mean ± SEM (n = 3). Significantly different from control, ***P < 0.001, NS = non-significant.

Thus the extent of modulation of LPS-induced adhesion molecule expression in HCAECs by fatty acids was limited (Figure 4.33).

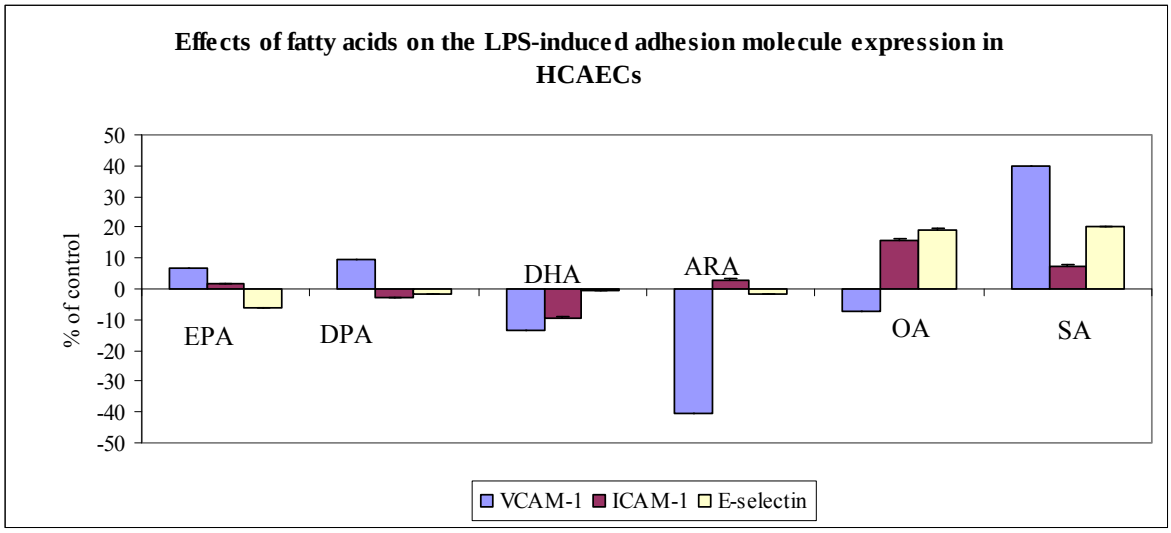


FIGURE 4.33: Comparative Effects of Fatty Acids on Adhesion Molecules Expression in HCAECs

4.4.3.4 Effects of Fatty Acids on Adhesion Molecule Expression in HSaVECs

Figures 4.34-4.36 show the effect of six fatty acids on VCAM-1, ICAM-1 and E-selectin expression on unstimulated and LPS-stimulated HSaVECs. It was observed that LPS significantly up-regulated adhesion molecule expression in HSaVECs, particularly VCAM-1. None of the fatty acids demonstrated any significant effect on VCAM-1 or E-selectin expression in the absence of LPS stimulation ($P > 0.05$). However, ICAM-1 was induced by DHA ($P = 0.001$), ARA ($P = 0.004$), OA ($P < 0.001$) and SA ($P = 0.001$).

Under the optimized experimental conditions, DHA, ARA, DPA and EPA significantly attenuated VCAM-1 expression in HSaVECs (all $P < 0.001$ except for EPA [$P = 0.006$]) (Figure 4.34). DHA exerted the greatest inhibitory effect (average 81%), with the other three fatty acids causing 23 to 29% inhibition. OA and SA both significantly enhanced LPS-stimulated VCAM-1 expression by 52% and 78% respectively (both $P < 0.001$).

None of the tested fatty acids affected LPS-induced ICAM-1 in HSaVECs (Figure 4.35). Only DHA affected LPS-induced E-selectin expression, an inhibition of 29% ($P < 0.05$) (Figure 4.36).

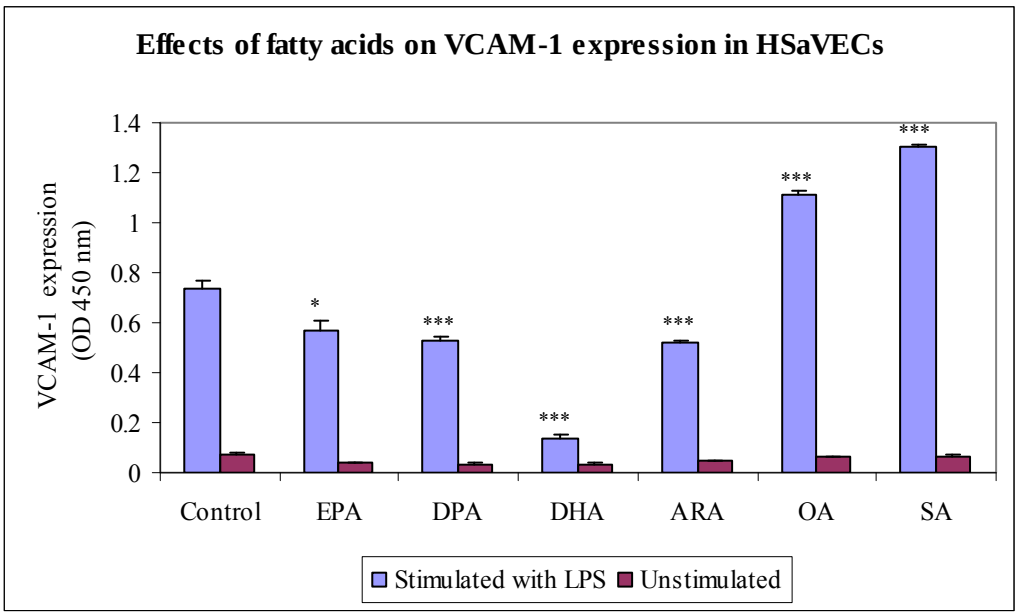


FIGURE 4.34: Comparative Effects of Fatty Acids on VCAM-1 Expression in HSaVECs

Data are expressed as mean ± SEM (n = 3). Significantly different from control * $P < 0.05$, ***$P < 0.001$.

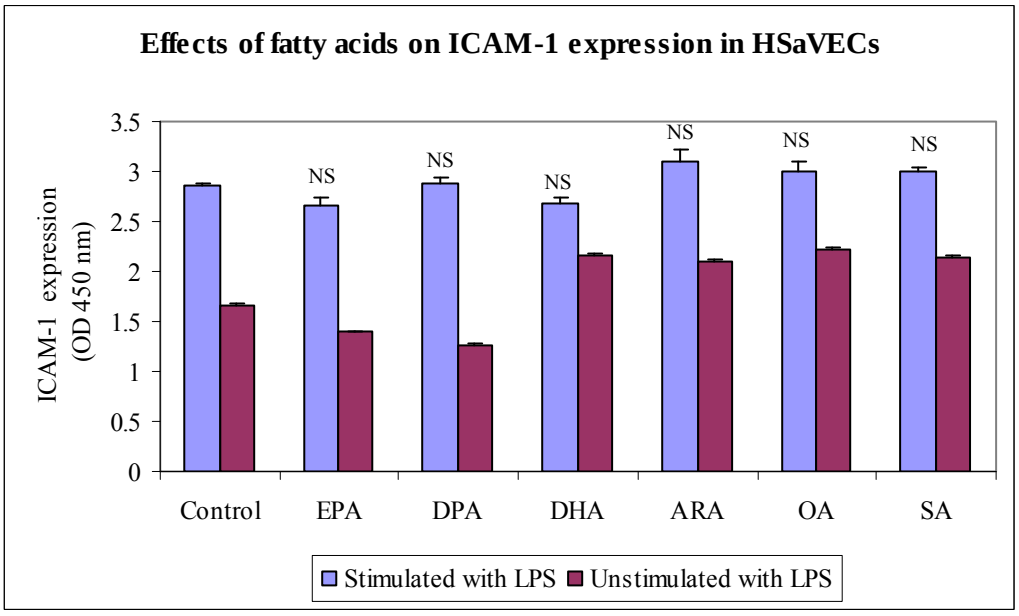


FIGURE 4.35: Comparative Effects of Fatty Acids on ICAM-1 Expression in HSaVECs

Data are expressed as mean ± SEM (n = 3). NS = non-significant.

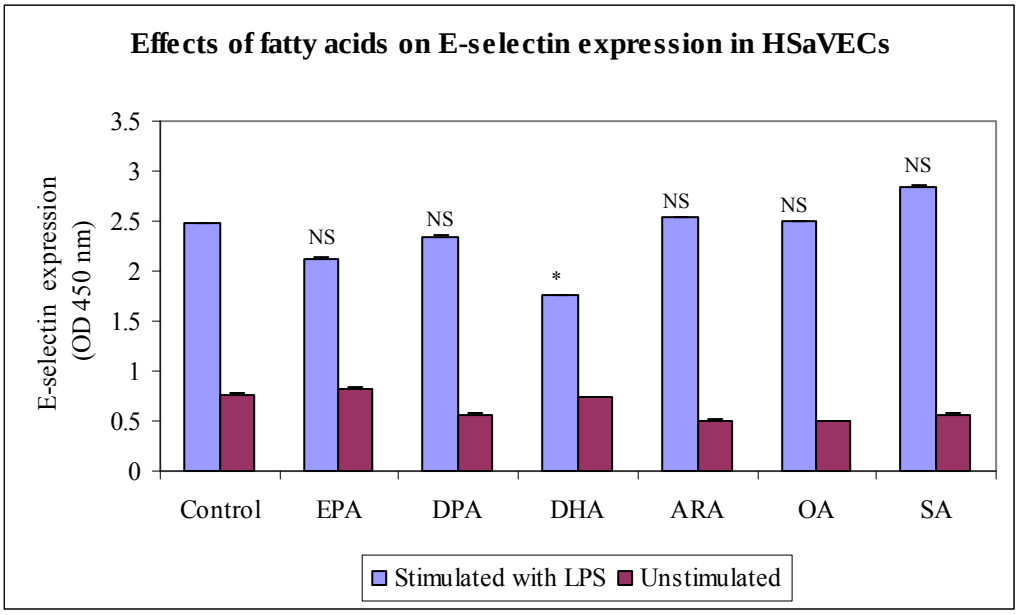


FIGURE 4.36: Comparative Effects of Fatty Acids on E-selectin Expression in HSaVECs

Data are expressed as mean ± SEM (n = 3). Significantly different from control, * $P < 0.05$, NS = non-significant.

Figure 4.37 summarises the effects of the different fatty acids on LPS-induced adhesion molecule expression in HSaVECs. Clearly the nature of the effects on VCAM-1 expression is different from those on the other two adhesion molecules.

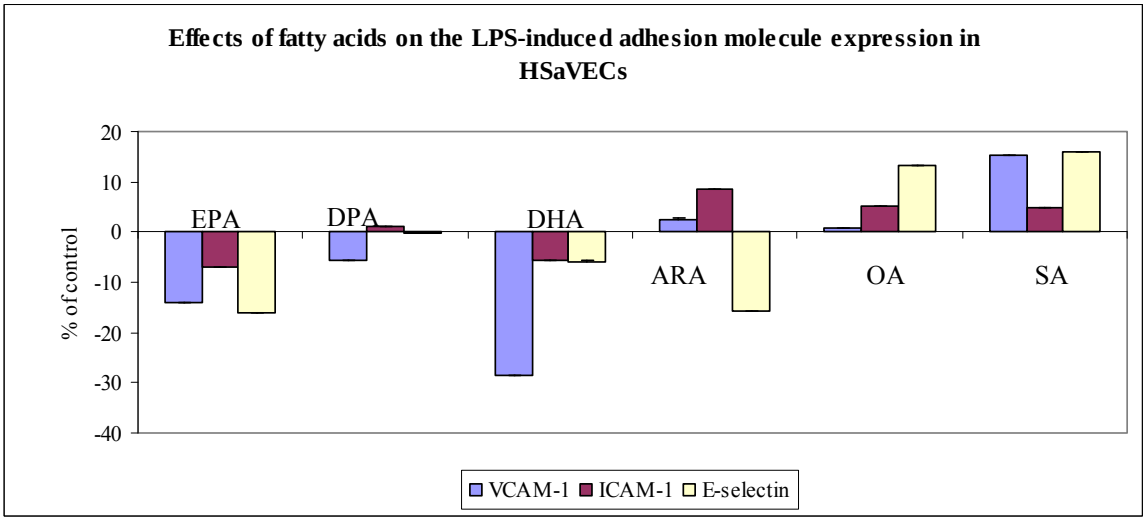


FIGURE 4.37: Comparative Effects of Fatty Acids on Adhesion Molecules Expression in HSaVECs

4.4.3.5 Effects of Fatty Acids on Adhesion Molecule Expression in HAOECs

Figures 4.38-4.40 show the effect of five fatty acids on VCAM-1, ICAM-1 and E-selectin expression on unstimulated and LPS-stimulated HAOECs; this is the first time that the effects of these selected fatty acids on these cells has been reported. Previous studies on the effects of fatty acids on adhesion molecule expression used α-linolenic acid, arachidonic acid analogs and linoleic acid (Stuhlmeier et al. 1997; Schleser et al. 2006). It was observed that LPS significantly up-regulated VCAM-1 and ICAM-1, but not E-selectin expression in HAOECs. None of the fatty acids demonstrated any significant effect on VCAM-1 or E-selectin expression in the absence of LPS stimulation ($P > 0.05$). However, ICAM-1 was inhibited by DPA ($P = 0.001$) and DHA ($P = 0.001$).

Under the optimized experimental conditions, DPA and DHA significantly attenuated VCAM-1 expression in HAOECs by almost 90% (both $P < 0.001$) (Figure 4.38). In addition SA significantly enhanced VCAM-1 expression in these cells (314%; $P < 0.001$).

DPA and DHA also significantly inhibited LPS-induced ICAM-1 in HAOECs, while EPA, ARA and SA significantly enhanced the effect of LPS on ICAM-1 in these cells (Figure 4.39). This stimulatory effect was approx. 20 to 25%.

EPA and SA significantly inhibited LPS-induced E-selectin expression by 31% ($P = 0.001$) and 36% ($P < 0.001$), respectively whilst DHA enhanced the effect of LPS on E-selectin by 38% ($P < 0.05$).

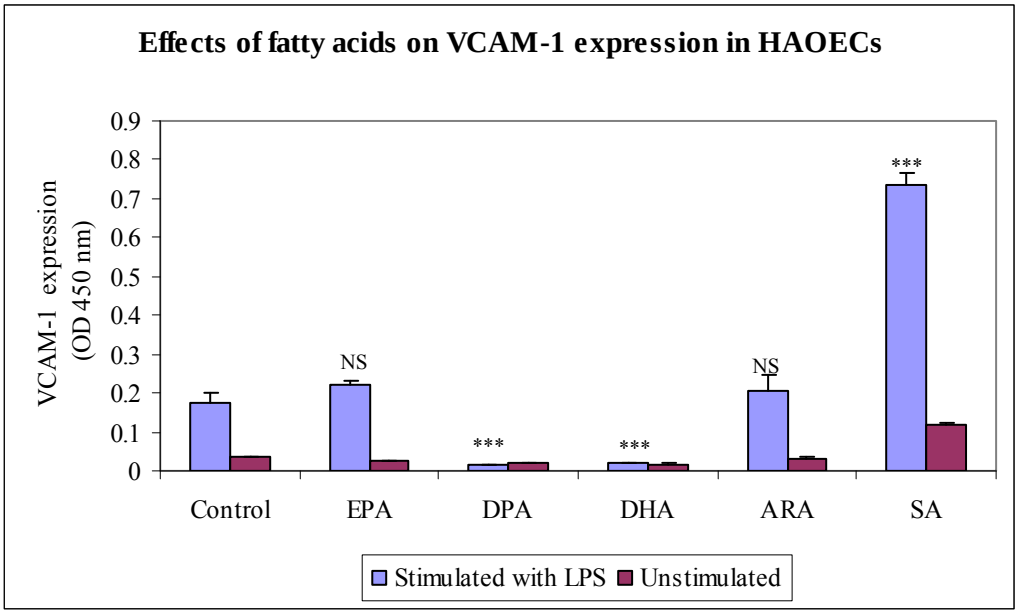


FIGURE 4.38: Comparative Effects of Fatty Acids on VCAM-1 Expression in HAOECs

Data are expressed as mean ± SEM (n = 3). Significantly different from control, ***P < 0.001, NS = non-significant.

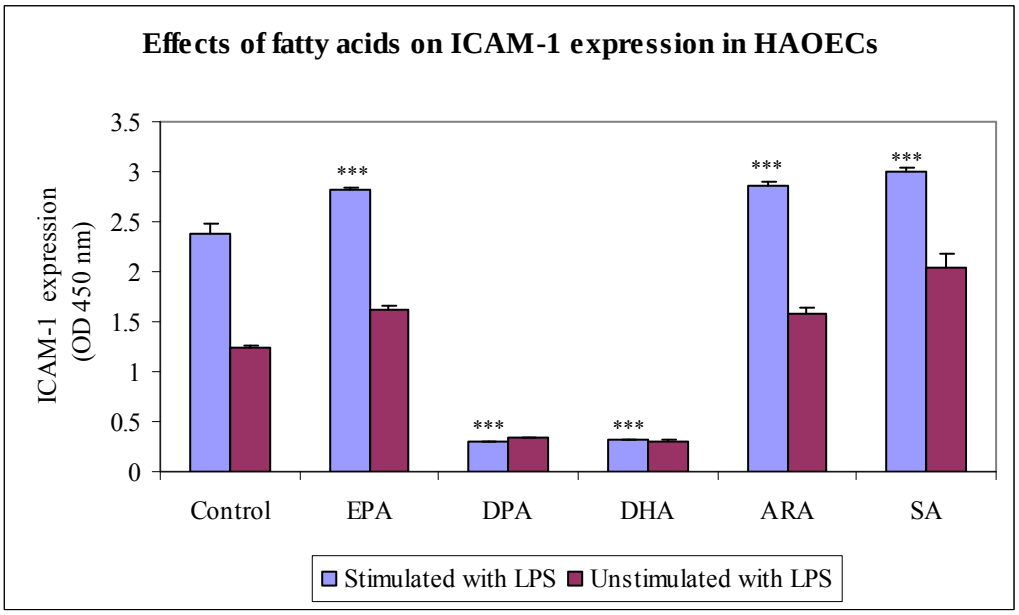


FIGURE 4.39: Comparative Effects of Fatty Acids on ICAM-1 Expression in HAOECs

Data are expressed as mean ± SEM (n = 3). Significantly different from control, ***P < 0.001.

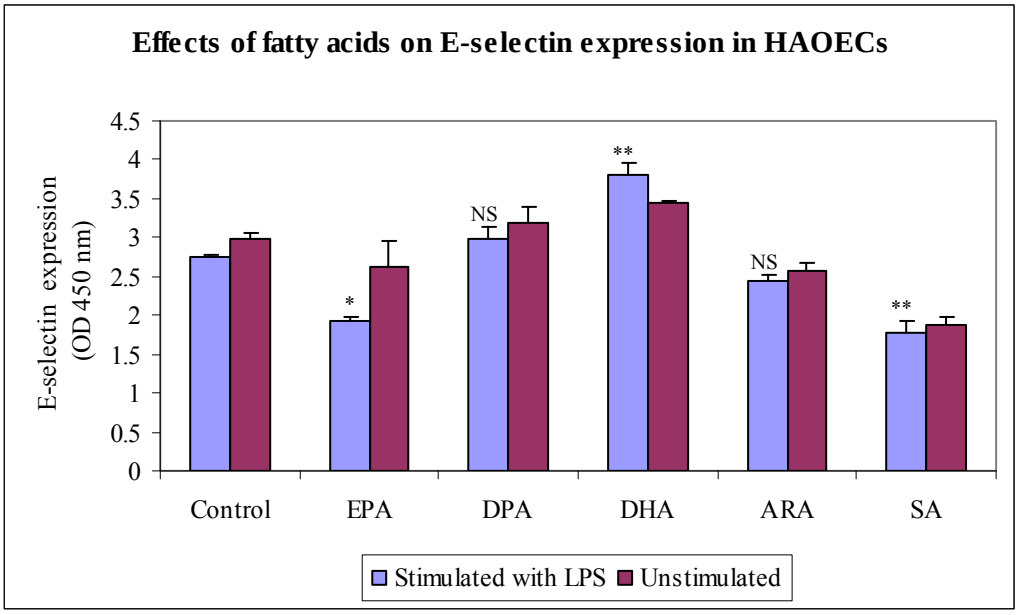


FIGURE 4.40: Comparative Effects of Fatty Acids on E-selectin Expression in HAOECs

Data are expressed as mean ± SEM (n = 3). Significantly different from control, * $P < 0.05$, **$P < 0.005$, NS = non-significant.

Figure 4.41 summarises the effects of the different fatty acids on LPS-induced adhesion molecule expression in HAOECs. Clearly there are fatty acid and adhesion molecule specific effects in these cells.

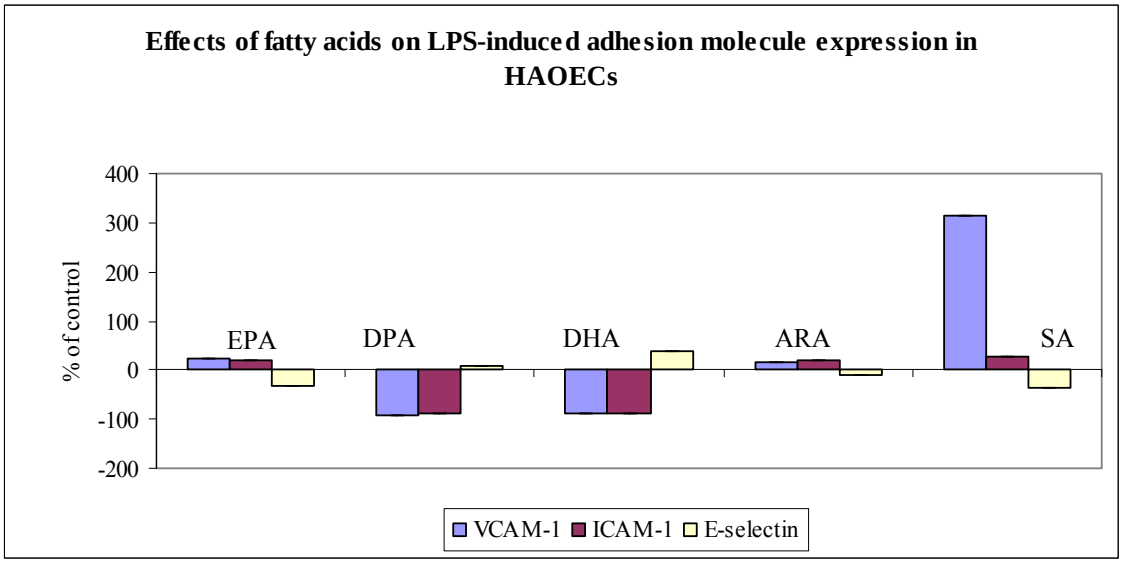


FIGURE 4.41: Comparative Effects of Fatty Acids on Adhesion Molecules Expression in HAOECs

4.5 Discussion

The protective role of LC n-3 PUFAs towards cardiovascular diseases has been known for many years (Iso et al. 2001; He et al. 2002; Hu et al. 2002; Albert et al. 1998). Part of this protective role may relate to an anti-inflammatory effect at the level of the vessel wall. However, support for such a role *in vivo* is not clear due to conflicting findings on the association between plasma inflammatory markers and LC n-3 PUFA exposure. Indeed in the studies described in Chapters 2 and 3 of this thesis I found that LC n-3 PUFAs could slightly decrease plasma sICAM-1 with no effect on sVCAM-1 or sE-selectin in healthy volunteers and in contrast, there were significant decreases in sVCAM-1 and sE-selectin in patients with advanced carotid atherosclerosis. *In vitro* experiments are useful in identifying potential effects and for undertaking mechanistic studies and a number of cell culture experiments using endothelial cells have investigated the potential anti-inflammatory effect of LC n-3 PUFAs on these cells, focusing upon adhesion molecule expression. The pioneering work of De Caterina et al. (1994) demonstrated that DHA could decrease cytokine-stimulated upregulation of the adhesion molecule VCAM-1 in cultured HSaVECs. Further work of this type has focused upon venous endothelial cells, including HSaVECs (De Caterina et al. 1996; De Caterina & Libby 1998; De Caterina et al. 1998) and HUVECs (Khoulfon et al. 1996; Collie-Duguid & Wahle 1996). Even in the highly controlled *in vitro* environment, conflicting effects of fatty acids have been reported (see Section 1.3.8). In addition no studies have examined the effect of fatty acids on adhesion molecule expression in arterial endothelial cells, which is surprising since cardiovascular diseases is mainly arterial in its location. Therefore, the aim of the study described in this chapter was to identify the effects of individual fatty acids on adhesion molecule expression in a range of human venous and arterial endothelial cells. In order to address this aim the conditions of endothelial cell culture were first optimized.

4.5.1 Optimized Condition for LPS Stimulation

Prior to the studying the effects of different fatty acids on adhesion molecule expression *in vitro*, endothelial cells were stimulated with LPS at different concentrations and for

different incubation times. Initially attempts were made to use flow cytometry to measure the expression of adhesion molecules. However this technique was unable to detect significant expression perhaps because flow cytometry requires that cells be detached from the culture plates and the use of proteases like trypsin to achieve this most likely cleaves surface adhesion molecules. Thus, ELISA was used instead to quantify adhesion molecules in endothelial cells that were still adherent to tissue culture plates. The work by De Caterina et al. used a similar assay (EIA) to measure endothelial adhesion molecules. In the present study, the optimum conditions for the expression of VCAM-1, ICAM-1 and E-selectin in five endothelial cell types (HUVECs, HUAECs, HCAECs, HSaVECs and HAOECs) were determined. For HUVECs, HUAECs, HCAECs and HSaVECs, the optimum conditions for LPS stimulation were 1 μg/mL for 12 hours; whereas for HAOECs, LPS concentration at 10 μg/mL for 12 hours stimulation time was optimal.

For HUVECs, the identified conditions partly agree with those used in several previous studies (Khalfoun et al. 1996; Massaro et al. 2002; Carluccio et al. 2003; Holthe et al. 2005) in which LPS was used at 1 μg/mL but cells were incubated with LPS for 4 hrs (Massaro et al. 2002; Carluccio et al. 2003), 6 hrs (Holthe et al. 2005) or 8 hrs (Khalfoun et al. 1996) after pre-incubation with fatty acids. On the other hand, Kalogeris et al. (1999) reported that no effect of 1 μg/mL LPS on VCAM-1 expression in HUAECs for 4 hours. However, the present study clearly shows that LPS does stimulate VCAM-1 expression and to a lesser extent of ICAM-1 and E-selectin on HUAECs after 12 hrs. For HCAECs, my results partly agree with Zouki et al. (2000) which used LPS at 1 μg/mL to stimulate ICAM-1 and E-selectin expression, however these cells were stimulated for 4 hours. Studies with HAOECs used much lower LPS concentrations than used here including 0.1 μg/mL (Tummala et al. 2000) and 0.01 μg/mL (Tsao et al. 1996).

4.5.2 Optimized Conditions for Fatty Acid Studies

In the present study, the optimal conditions for studying the effects of fatty acids were identified by preliminary studies using DHA in HUVECs and HAOECs. Inhibitory effects were observed at 48 hours pre-incubation with 50 μM DHA followed by stimulation with LPS for another 12 hours. These conditions are in general accordance with De Caterina et

al. (1994; 1995) who reported that in order to achieve an inhibitory effect of fatty acids, a relatively prolonged fatty acid incubation time ranging from 48 to 72 hours before the addition of the stimulating cytokines was required. The prolonged incubation time with fatty acids is presumably to allow their incorporation into the endothelial cell membranes. Indeed De Caterina et al. (1994) found that the incorporation of DHA and ARA reached plateau at about 72 hours of incubation at 25 μM concentration. Under the conditions of a prolonged fatty acid pre-exposure followed by six hrs stimulation with an inflammatory cytokine, DHA in particular exhibited inhibition of VCAM-1 expression. These results suggested that fatty acids at a concentration of 50 μM should be used in further experiments.

4.5.3 Effects of Stearic Acid on Adhesion Molecule Expression

De Caterina et al. (1998) are the only researchers to have reported the influence of saturated fatty acids on adhesion molecule protein expression in cultured endothelial cells; they found no effects of palmitic acid or stearic acid on VCAM-1 expression in HSaVECs. On this basis they concluded that only unsaturated fatty acids affected adhesion molecule expression. In contrast, the current study found that stearic acid did affect adhesion molecule expression, and that it had different effects on different cell types and on different adhesion molecules. Table 4.1 summarises the effects of all fatty acids tested on all three adhesion molecules in all five endothelial cell types. Stearic acid affected VCAM-1 expression in all five cell types. It attenuated VCAM-1 expression in HUVECs and HUAECs, but enhanced VCAM-1 expression in HCAECs, HSaVECs and HAOECs. SA affected ICAM-1 expression in only two endothelial cell types: it decreased expression in HUAECs and increased expression in HAOECs. Stearic acid increased E-selectin expression in HUVECs, HUAECs and HCAECs; and decreased expression in HAOECs. In the current study, cells were pre-incubated with stearic acid at 50 μM for 48 hours before stimulation with LPS for 12 hours, while De Caterina et al. (1998) used 25 μM stearic acid for 72 hours and then stimulated with IL-1α for 12 hours. It is possible that the difference between the current findings and those of De Caterina et al. (1998) relate to the higher stearic acid concentration used here.

The present study is the first to compare fatty acid effects among different types of endothelial cells. Table 4.1 indicates that fatty acids have different effects on different cell types, despite the generally similar functional characteristics of those cell types.

TABLE 4.1: Summary of the Significant Effects of Fatty Acids on LPS-stimulated Adhesion Molecule Expression in Endothelial Cells

	Cell	EPA	DPA	DHA	ARA	OA	SA
VCAM-1	1	↓↓	↓↓	↓↓↓	↓↓	↓	↓
	2	↓	↓	↓↓↓	↓	↑	↑
	3	↓↓	↓↓	↓↓↓	↓↓	↓↓	↓↓
	4	-	-	↓	↓↓	-	↑
	5	-	↓↓↓	↓↓↓	-	NA	↑↑↑
ICAM-1	1	↓	↓	↓	↓	-	-
	2	-	-	-	-	-	-
	3	↓	↓↓	↓↓	-	-	↓
	4	-	-	-	-	-	-
	5	↑	↓↓↓	↓↓↓	↑	NA	↑
E-selectin	1	↓	-	↓	↓	↑	↑
	2	-	-	↓	-	-	-
	3	↓	↓	↑↑	-	↑	↑↑
	4	-	-	-	-	↑	↑
	5	↓	-	↑	-	NA	↓

1 = HUVECs; 2 = HSaVECs; 3 = HUAECs; 4 = HCAECs; 5 = HAOECs
NA = not available; ↓↓↓/↑↑↑ = extremely significant; ↓↓/↑↑ = highly significant; ↓/↑ = significant ($P < 0.05$)

4.5.4 Effects of Oleic Acid on Adhesion Molecule Expression

In the present study, OA did not affect ICAM-1 expression on any of the four endothelial cell types studied (Table 4.1). OA increased E-selectin expression in HUVECs, HUAECs and HCAECs, increased VCAM-1 expression in HSaVECs and decreased VCAM-1 expression ion HUVECs and HUAECs. De Caterina et al. (1998) in their HSaVECs study reported that pre-incubation with 50 μM OA for 72 hours and then stimulation with IL-1α (1 ng/mL) resulted in modest VCAM-1 inhibition. Carluccio et al. (1999) also demonstrated that OA at a higher concentration (100 μM) pre-incubated for 72 hrs followed by stimulation with LPS at 1 μg/mL inhibited VCAM-1 and E-selectin expression to a similar extent and ICAM-1 to a lesser extent in HSaVECs. Clearly the current study reporting that OA increases VCAM-1 and E-selectin in HSaVECs contradicts these earlier findings. Holthe et al. (2005) reported no significant effects on the expression of VCAM-1, ICAM-1

and E-selectin following 100 μM OA incubated together with LPS (1 μg/mL), either at 6 or 24 hours in HUVECs. This may due to shorter pre-incubation time as compared to previous studies and to the lack of a fatty acid pre-incubation prior to stimulating the cells.

4.5.5 Effects of Arachidonic Acid on Adhesion Molecule Expression

In the present study, ARA attenuated VCAM-1 expression in four endothelial cell types, with no effect seen in HAOECs (Table 4.1). However, few effects of ARA were seen for ICAM-1 and E-selectin, apart from a reduction in ICAM-1 and E-selectin expression in HUVECs (Table 4.1). Using HSaVECs, De Caterina et al. (1998) reported that ARA produced significant inhibition of VCAM-1 expression. The current study is in accordance with this. Mayer et al. (2002) reported no significant effects of ARA on VCAM-1, ICAM-1 and E-selectin expression in TNF-α-stimulated HUVECs. In their study, the concentration of ARA used was much lower than used here (10 μM) and there was a shorter pre-incubation time (6 hrs) but a longer exposure to TNF-α (20 hrs). The lower concentration and shorter pre-incubation time may account for the differences in results seen.

4.5.6 Effects of n-3 PUFAs on Adhesion Molecule Expression

Three n-3 PUFAs were used in this study: EPA, DPA and DHA. They were found to have a different range of effects (Table 4.1) and different potencies. DHA consistently resulted in a highly significant reduction in VCAM-1 expression in all endothelial cell types studied, and in ICAM-1 in three endothelial cell types and in E-selectin in two endothelial cell types (Table 4.1). DPA and EPA also inhibited VCAM-1, ICAM-1 and E-selectin in some of the endothelial cell types, but the results clearly showed that among the three n-3 PUFAs compared, DHA is the most potent inhibitor of endothelial activation. The effects of EPA and DPA were very similar. EPA and DPA contain the same number of double bonds (five) but are different chain lengths (20 vs. 22), while DPA and DHA are the same chain length (22 carbons) but have different numbers of double bonds (5 vs. 6). Thus, the findings of the current study support the contention of De Caterina et al. (1998) that the degree of

unsaturation, but not the chain length alone, is important in influencing the inhibition of endothelial activation.

Previously, it has been reported that DHA, but not EPA, reduced cytokine-induced adhesion molecule expression in HSaVECs (De Caterina et al. 1995). In the current study both EPA and DHA inhibited VCAM-1 expression in HSaVECs, but DHA was much more potent. This discrepancy in the effect of EPA seen may due to the fatty acid concentration used: 50 μM in the current study and 10-25 μM in De Caterina et al. (1995). In support of this, other studies have demonstrated a reduction in adhesion molecules in HUVECs by EPA at 65 μM (Collie-Duguid et al., 1996) or 30 μM (Khalfoun et al. 1996). Kim et al. (1995) reported that EPA at 50-100 μM reduced the expression of VCAM-1, ICAM-1 and E-selectin in HUVECs.

Unexpectedly DHA increased E-selectin expression in HUAECs and HAOECs. This may suggest a different effect on signaling processes leading to E-selectin expression in these particular cells.

In general n-3 PUFAs inhibited LPS-stimulated adhesion molecule expression. This is indicative of an anti-inflammatory effect that might come about as a result of inhibiting cell signaling processes that result in enhanced gene expression. It is likely that inhibition of signaling leading to NF-κB activation is involved (Wahle & Rotondo 1999; Hennig & Toborek 2000; De Caterina & Massaro 2005).

4.6 Conclusion

The current study has identified that fatty acids can influence expression of adhesion molecules in endothelial cells of venous, arterial and aortic origin. By studying the effects of six different fatty acids on expression of three adhesion molecules on five endothelial cell types, it has been possible to identify that effects are fatty acid, adhesion molecule and endothelial cell specific. VCAM-1 is affected by fatty acids to a greater extent than ICAM-1 or E-selectin. Cells of venous, arterial and aortic origin can all be affected by fatty acids.

Amongst the fatty acids, DHA has the greatest and the most consistent effects, at least under the conditions of concentration and timing used here. Differences in findings reported in the literature most likely relate to fatty acid concentration and exposure time used, rather than cell type. If n-3 fatty acids, particularly DHA behave *in vivo* in a similar manner to the effects seen here *in vitro*, then a significant anti-inflammatory effect may occur at the vascular wall and this might contribute to the protective effect of these fatty acids on CVD. The greater potency of DHA than EPA or DPA seen in this study suggests that DHA is a very important functional component of fish and of fish oils. Thus functional effects of fish oil may not be seen if the amount of DHA provided is insufficient. The identification of an important role for DHA is consistent with the conclusion of Chapter 2 in relation to the limited impact of the fish oil used in the study described therein.

Chapter 5

The Effects of Fatty Acids on the Expression of Adhesion Molecule mRNA Levels in Cultured Endothelial Cells

5.1 Introduction

5.1.1 Background of the study

In Chapter 4, I described the effects of fatty acids on the expression of adhesion molecule proteins in cultured human endothelial cells. It was found that fatty acids can affect expression of adhesion molecules in endothelial cells and that effects are fatty acid, adhesion molecule and endothelial cell specific. My results demonstrated that VCAM-1 is

affected by fatty acids to a greater extent than ICAM-1 or E-selectin. Amongst the fatty acids studied, DHA had the greatest and the most consistent effects, under the conditions of concentration and timing used. The greater potency of DHA rather than EPA or DPA seen in this study suggests that DHA is an important functional component of fish and of fish oil in the diet. Therefore, functional effects of fish oil *in vivo* may not be seen if the amount of DHA provided is insufficient. The identification of an important role for DHA is consistent with the conclusion of Chapter 2 in relation to the limited impact of the fish oil used in the study described therein.

Evidence illustrates that selected fatty acids can stimulate inflammatory responses through the transcriptional regulation of inflammatory genes, for example, genes encoding for adhesion molecules and inflammatory cytokines (Toborek et al. 1998). Since fatty acids may be released by triacylglycerol hydrolysis at the vascular endothelium by the action of

lipoprotein lipase, endothelial cells can be directly exposed to high concentrations of free fatty acids (Zilversmit et al. 1973). It has been reported that n-3 PUFAs, particularly EPA and DHA inhibit migration of inflammatory cells into local tissue by down regulating adhesion molecule expression (VCAM-1, ICAM-1, E-selectin) and monocyte adherence to the vascular endothelium (Hughes et al. 1996). The effects of these fatty acids involve several mechanisms including actions on signal transduction and on gene expression (Grimm et al. 2002; De Caterina et al. 1994; Collie-Duguid et al. 1996; Wahle et al. 1999).

The present study is an extension of the previous work on the effects of fatty acids on endothelial inflammatory protein expression as described in Chapter 4. Previously, *in vitro* work of this sort has mostly focused on protein expression (De Caterina et al. 1995; Holthe et al. 2005; ReiBig et al. 2003; Khalfoun et al. 1996), although there have been a limited number of studies which have investigated the effect of fatty acids on pro-inflammatory gene expression (De Caterina et al. 1996; Huang et al. 1997; Holthe et al. 2005; Shaw et al. 2007). The outcomes however, were reported to be inconsistent. This may due to many experimental variables, such as the type and concentration of fatty acid used and incubation time with fatty acid, type of stimulus used and its concentration, duration of exposure to stimulant, and type of endothelial cell studied. These factors differ considerably between studies, making comparisons between them difficult. Moreover, the methods used were also varied, from flow cytometry for determination at the protein level to polymerase chain reaction (PCR), for assessing the expression at the genomic level. Based on the pioneering *in vitro* studies using HSaVECs, it has been concluded that a minimum of single double bond is required for fatty acids to inhibit adhesion molecule expression (therefore saturated fatty acids are inactive); it was also concluded that the effectiveness does not depend on the chain length and the degree of effectiveness increases correspondingly with unsaturation (double bond insertion) (De Caterina et al. 1998). However, some of my findings in Chapter 4 dispute this conclusion. Therefore, in order to further explore the inhibition of adhesion molecules production by selected fatty acids at the transcriptional level, I studied mRNA expression using real-time reverse transcriptase PCR. The main aim of the present study was to provide a systematic investigation of the comparative effects of fatty acids on adhesion molecule expression at the mRNA level using different types of endothelial cells.

5.2 Aim of this Study

The aim of this study was to identify the effects of individual fatty acids on endothelial cell adhesion molecule mRNA levels and to compare this with results on protein expression (as described in Chapter 4). To investigate this aim, different endothelial cell types were used under similar experimental conditions and the expression of genes encoding a number of inflammatory proteins was assessed by reverse transcription and quantitative real time polymerase chain reaction (real-time-PCR). The objectives of the experiments were:

- To compare the effect of individual fatty acids (EPA, DPA, DHA, arachidonic acid (ARA), oleic acid (OA) and stearic acid (SA)) on VCAM-1 mRNA levels in five different endothelial cell types (HUVECs, HUAECs, HCAECs, HSaVECs and HAOECs);

- To compare the effect of individual fatty acids on ICAM-1 mRNA levels in five different endothelial cell types;

- To compare the effect of individual fatty acids on E-selectin mRNA levels in five different endothelial cell types.

5.3 Methods

5.3.1 Reagents

Sodium salts of EPA, DPA, DHA, ARA, OA and SA were from Sigma-Aldrich, Poole and each was dissolved in ethanol to prepare 100 mM fatty acid stock solutions at room temperature or at 37^{o} C, to overcome fatty acid insolubility in aqueous solution. Endothelial cell growth media were from PromoCell, Heidelberg and ECACC, Salisbury (only for HAOECs). Cell detaching solution (HepesBSS: 30 mM Hepes, D-glucose, NaCl, KCl, NaPhosphate, Phenol red), trypsin/EDTA solution and soybean trypsin neutralizing solution from soybean (0.05%) were from PromoCell, Heidelberg. The antibiotics penicillin and streptomycin were from PAA Laboratories, Somerset, UK. Trizol was from Invitrogen, Paisley. Chloroform and ethanol were from BDH Laboratory, Poole and isopropanol was

from Sigma, Dorset. RT-PCR reagents were from Promega, Madison and PCR reagents were from PrimerDesign, Southampton.

5.3.2 Experimental Design

In order to determine a possible stimulatory effect of fatty acids at the mRNA level, endothelial cells were pre-incubated with fatty acids at 50 μM for 48 hours. Then the cells were stimulated with LPS at 1 μg/mL for 12 hours. This optimum experimental condition was chosen based on my previous preliminary results (See Section 4.3.3). Endothelial cells of HUVECs, HUAECs, HSaVECs and HCAECs were purchased from PromoCell, Heidelberg, whereas HAOECs were from European Collection of Cell Culture (ECACC), Salisbury. The cells were thawed and subcultured following the supplier's instructions. Prior to use in experiments, cells at passage four were grown in 6-well plates until confluence.

5.3.3 Isolation of RNA

Following manufacturer's instructions, endothelial cell cultures were lysed by the direct addition of 1 mL trizol reagent and the cell lysate was passed several times with a pipette to form a homogenous lysate. Then the phases were separated by adding 0.2 mL chloroform per 1 mL trizol and RNA was precipitated from the top aqueous layer by adding to 0.5 mL isopropanol before washing with 70% ethanol. At the end of the procedure, the RNA was redissolved in RNase-free water. The concentration and purity of total RNA were determined by measuring the absorbance at 260 nm (one absorbance unit at 260 nm equals 40 μg RNA/mL) and 280 nm using a Nano Drop 1000 Spectrophotometer (Thermo Fisher Scientific, USA). The ratio between the absorbance at 260 and 280 nm reflects RNA purity and value should be more than 1.7.

5.3.4 Synthesis of cDNA

cDNA was generated from isolated RNA by heating to 70 ^{o}C for 5 min (reaction volume 15 μL) in the presence of random primers (400 ng) and subsequently was reverse transcribed using mixture reagents of 5 μL RT Buffer (5 x concentration), 1.25 μL dNTPs (PCR MIX 10 mM, Promega), 0.625 μL RNAsin (40 u/μL) and 1 μL M-MLV Transcriptase (200 u/μL) (reaction volume 10 μL). The samples were loaded into a Hybaid Thermal Reactor and the program used was 1 hour at 37 ^{o}C, followed by 10 min at 42 ^{o}C and 10 min at 75 ^{o}C.

5.3.5 Real Time RT-PCR

The sequences of primers are as follows:
VCAM-1 forward primer: 5'-CAGGCTAAGTTACATATTGATGACAT-3'
VCAM-1 reverse primer: 5'-GAGGAAGGGCTGACCAAGAC-3'
ICAM-1 forward primer: 5'-CCTATGGCAACGACTCCTTC-3'
ICAM-1 reverse primer: 5'-TCTCCTGGCTCTGGTTCC-3'
E-selectin forward primer: 5'-TTCTTGCCTACTATGCCAGATG-3'
E-selectin reverse primer: 5'-AGGAAAGGGAACACTGAGTCT-3'
with the accession numbers NM_001078, NM_000201 and NM_000450, respectively. The following primers were then obtained from PrimerDesign Ltd (Southampton, UK): VCAM-1 PerfectProbe CGAACCCAAACAAAGGCAGAGTACGCAAACACggttcg
ICAM-1 PerfectProbe CTGGGTGCCCTCGTCCTCTCGGGccag
E-selectin PerfectProbe AACCGCAACACCCATCACCACTTCAATAAgcggtt.

Real time RT-PCR was performed using Absolute QPCR SYBR Green PCR Master Mix (ABgene, Epsom, UK) for β-actin and PerfectProbe for VCAM-1, ICAM-1 and E-selectin using a 7500 Fast Real time PCR system (Applied Biosystems, Foster City, CA). The PCR was run for 10 min at 95 ^{o}C followed by 50 cycles of 15 sec at 95 ^{o}C, 30 sec at 50 ^{o}C and 15 sec at 72 ^{o}C (for VCAM-1, ICAM-1 and E-selectin), whereas the program for β-actin: 2 min at 50 ^{o}C followed by 40 cycles of 10 min at 95 ^{o}C, 15 sec at 95 ^{o}C and 1 min at 60 ^{o}C. After amplification, melting curve analysis was performed to verify specificity of the reactions with SYBR Green. The end point used in the real time RT-PCR quantification, Ct, was defined as the PCR cycle number at which threshold fluorescence was reached. A

standard curve was constructed using stock cDNA from HCAECs in a series of dilutions. The expression of β-actin and of each target gene of cDNA in each sample was quantified against a standard curve by allowing the software to determine the sample units accurately. Then the expression of each target gene was normalized to β-actin expression, whereby relative adhesion molecule production units were expressed as arbitrary units, calculating according to the following formula:

relative adhesion molecule production units = sample adhesion units/sample β-actin units.

5.3.6 Statistical Analysis

Data were analyzed using SPSS Version 16.0 (SPSS Inc., Chicago, IL) and results were presented as means ± SEM. The statistical significance of differences ($P < 0.05$) was determined using unpaired two-tailed Student t-test with GraphPad Software.

5.4 Results

5.4.1 Effects of Fatty Acids on Adhesion Molecule mRNA Levels in HUVECs

Figures 5.1-5.3 show the effects of five fatty acids (50 μM) on VCAM-1, ICAM-1 and E-selectin expression on unstimulated and LPS-stimulated HUVECs; no data are shown for DHA because it was not possible to recover mRNA from HUVECs after exposure to DHA. HUVECs were cultured with the fatty acids for 48 hrs and then with LPS for a further 12 hrs. Adhesion molecule mRNA was hardly detectable in unstimulated endothelial cells. However, LPS significantly up-regulated adhesion molecule mRNA levels in HUVECs ($P < 0.05$ vs. unstimulated cells).

Under the experimental conditions used EPA, OA (both at $P < 0.01$) and SA ($P < 0.001$) significantly attenuated VCAM-1 mRNA levels in HUVECs, indicating possible regulation by these fatty acids at the transcriptional level. Interestingly, SA was found to exert the greatest inhibitory effect (average 62%), followed by EPA (40%) and OA (31%). DPA and ARA had no significant effect on VCAM-1 mRNA levels in HUVECs.

EPA ($P < 0.01$), OA and SA (both at $P < 0.05$) significantly inhibited LPS-induced ICAM-1 mRNA levels by 56%, 55% and 46%, respectively (Figure 5.2). As seen for VCAM-1 mRNA, DPA and ARA showed no significant effects on ICAM-1 mRNA levels ($P > 0.05$).

EPA, DPA, ARA and SA all significantly inhibited LPS-stimulated E-selectin mRNA levels in HUVECs (all $P < 0.001$); inhibition was by 62%, 20%, 62% and 53%, respectively (Figure 5.3). Only OA was without effect.

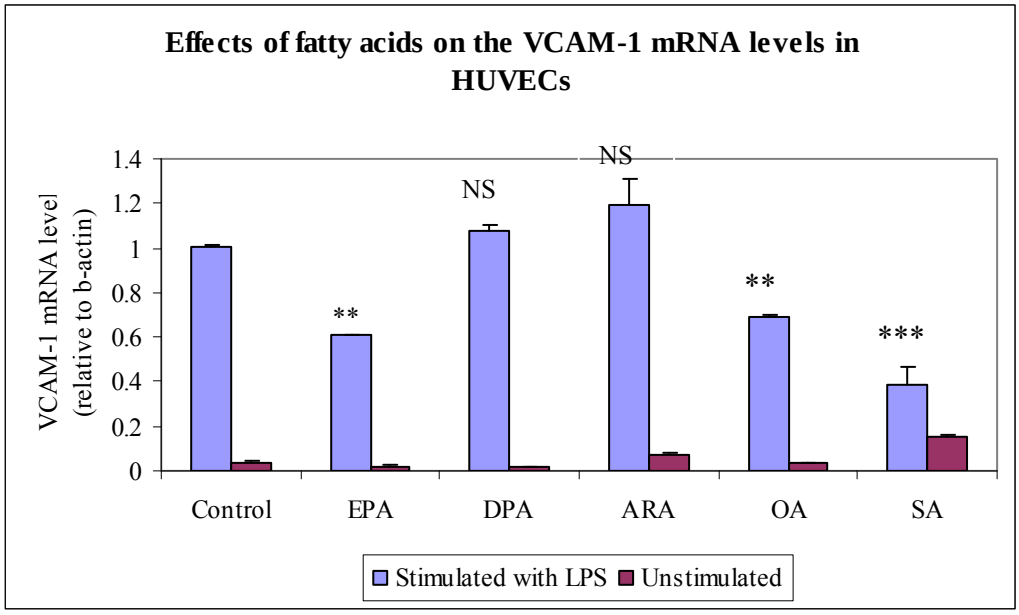


FIGURE 5.1: Effects of Fatty Acids on VCAM-1 mRNA Levels in HUVECs

Data are expressed as mean ± SEM (n = 2). Significantly different from control, **P < 0.005, ***P < 0.001, NS = non-significant.

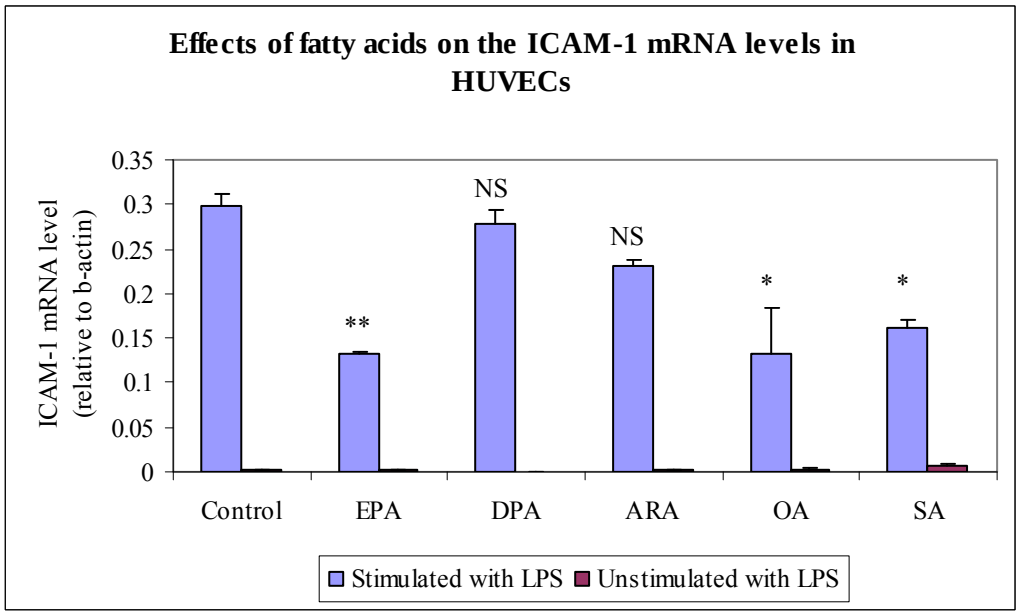


FIGURE 5.2: Effects of Fatty Acids on ICAM-1 mRNA Levels in HUVECs

Data are expressed as mean ± SEM (n = 2). Significantly different from control, * P < 0.05, **P < 0.005, NS = non-significant.

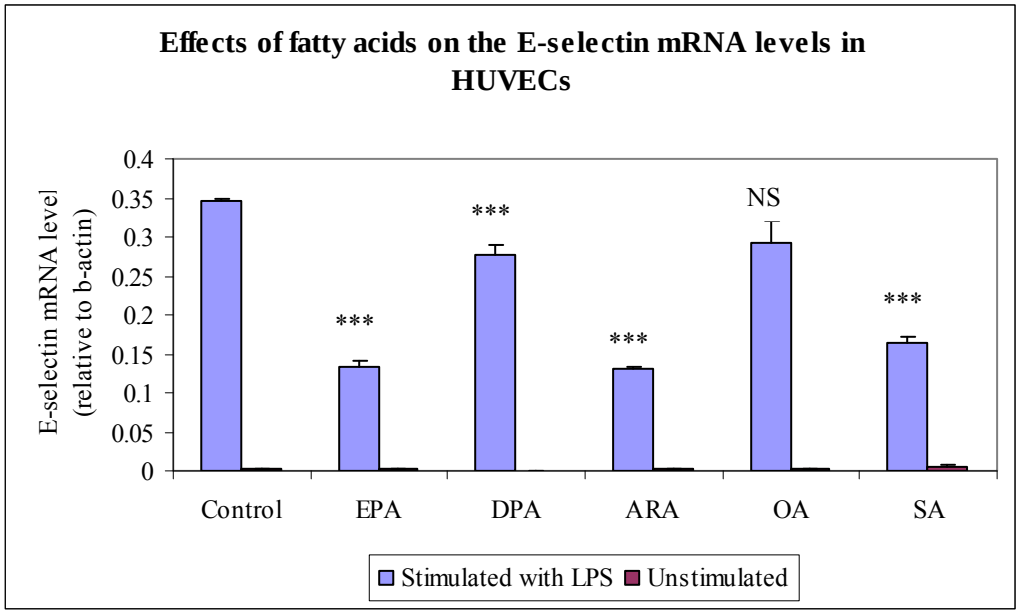


FIGURE 5.3: Effects of Fatty Acids on E-selectin mRNA Levels in HUVECs

Data are expressed as mean ± SEM (n = 2). Significantly different from control, ***P < 0.001, NS = non-significant.

Thus certain fatty acids were able to inhibit LPS-induced expression of VCAM-1, ICAM-1 and E-selectin at the mRNA levels in HUVECs. Figure 5.4 attempts to summarise the pattern of inhibition seen across all fatty acids and all three adhesion molecules; this figure demonstrates that a similar pattern of effects of fatty acids was seen, although the extent of the effect of a given fatty acid was different according to the adhesion molecule mRNA studied. EPA and SA were consistently shown to be the most potent fatty acid inhibitors for the three adhesion molecules studied.

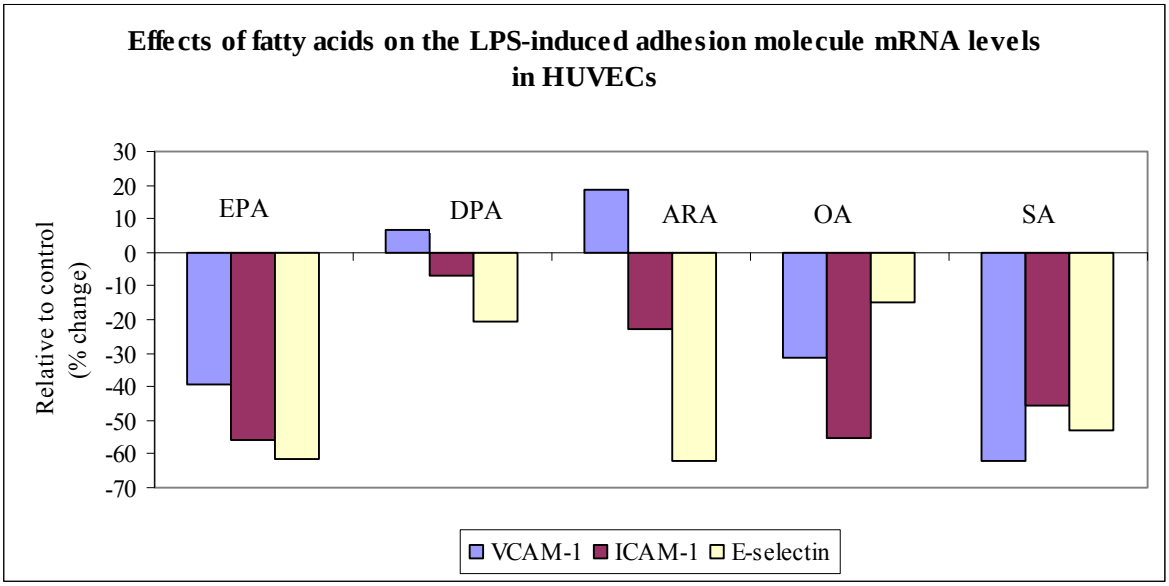


FIGURE 5.4: Comparative Effects of Fatty Acids on Adhesion Molecules mRNA Levels in HUVECs (Relative to Control)

5.4.2 Effects of Fatty Acids on Adhesion Molecule mRNA Levels in HUAECs

Figures 5.5-5.7 show the effects of five fatty acids (50 μM) on VCAM-1, ICAM-1 and E-selectin mRNA levels on unstimulated and LPS-stimulated HUAECs; no data are shown for DHA because it was not possible to recover mRNA from HUAECs after exposure to DHA. HUAECs were cultured with the fatty acids for 48 hrs and then with LPS for a further 12 hrs. Adhesion molecule mRNA was hardly detectable in unstimulated endothelial cells. However, LPS significantly up-regulated adhesion molecule mRNA levels in HUAECs ($P < 0.05$ vs. unstimulated cells).

Under the experimental conditions used EPA, DPA (both at $P < 0.01$) and SA ($P = 0.05$) significantly attenuated VCAM-1 mRNA levels in HUAECs, indicating regulation by these fatty acids at the transcriptional level (Figure 5.5). EPA was found to exert the greatest inhibitory effect (average 80%), followed by DPA (72%) and SA (47%). ARA and OA had no significant effect on VCAM-1 mRNA evels in HUAECs.

EPA, DPA (both at $P < 0.01$) and OA ($P < 0.001$) significantly inhibited LPS-induced ICAM-1 mRNA levels in HUAECs by 52%, 66% and 53%, respectively (Figure 5.6). As seen for VCAM-1 mRNA, ARA showed no significant effect on ICAM-1 mRNA levels ($P > 0.05$). SA tended to increase LPS-induced ICAM-1 mRNA levels (by 78%) but the effect did not reach statistical significance.

Only EPA and ARA significantly inhibited LPS-stimulated E-selectin mRNA levels in HUAECs (both $P < 0.001$); inhibition was by 60% and 46%, respectively (Figure 5.7).

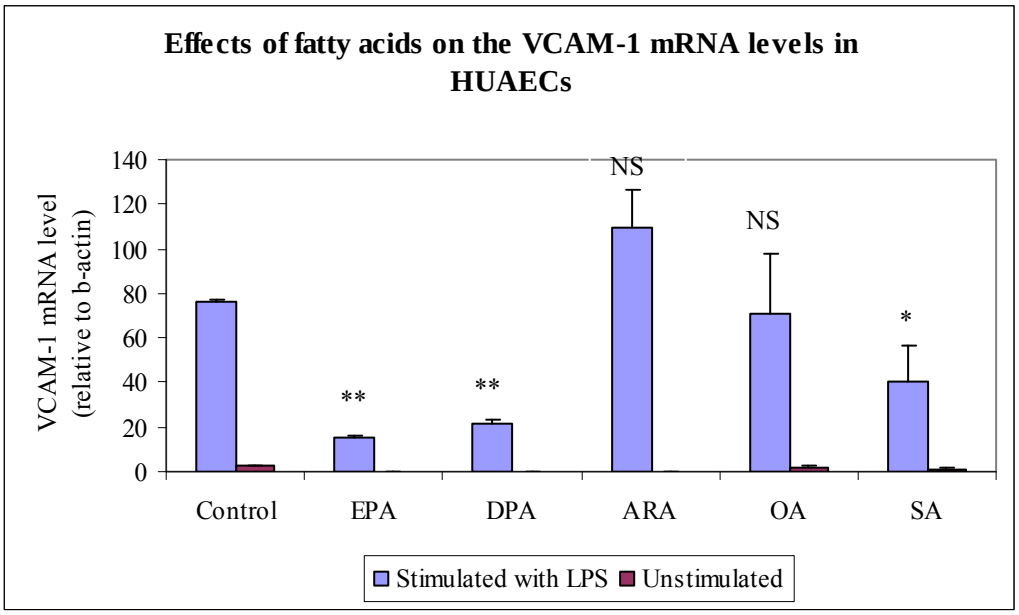


FIGURE 5.5: Effects of Fatty Acids on VCAM-1 mRNA Levels in HUAECs.

Data are expressed as mean ± SEM (n = 2). Significantly different from control, * $P < 0.05$, **$P < 0.005$, NS = non-significant.

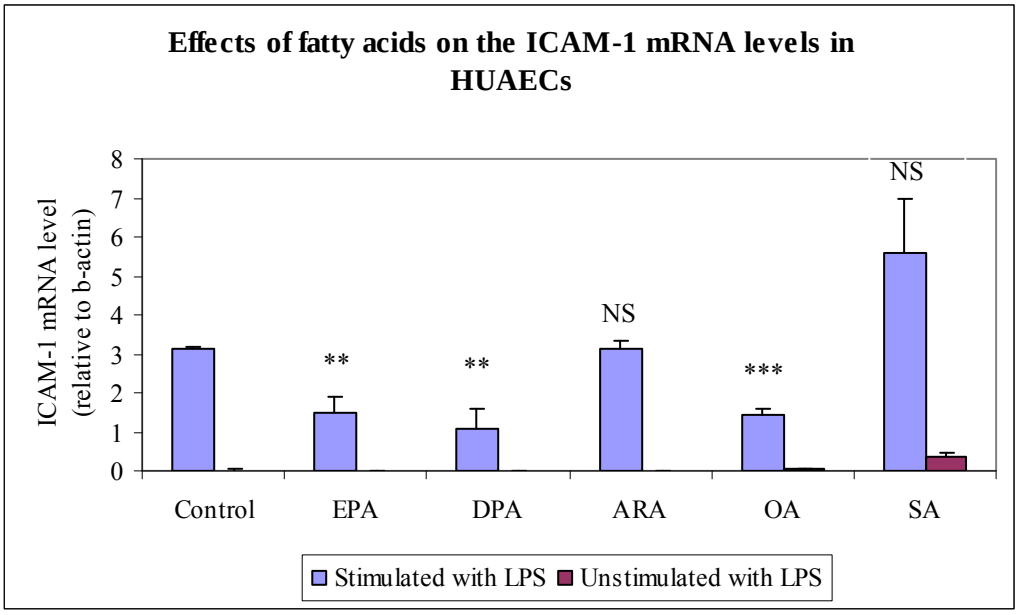


FIGURE 5.6: Effects of Fatty Acids on ICAM-1 mRNA Levels in HUAECs

Data are expressed as mean ± SEM (n = 2). Significantly different from control, **P < 0.005, ***P < 0.001, NS = non-significant.

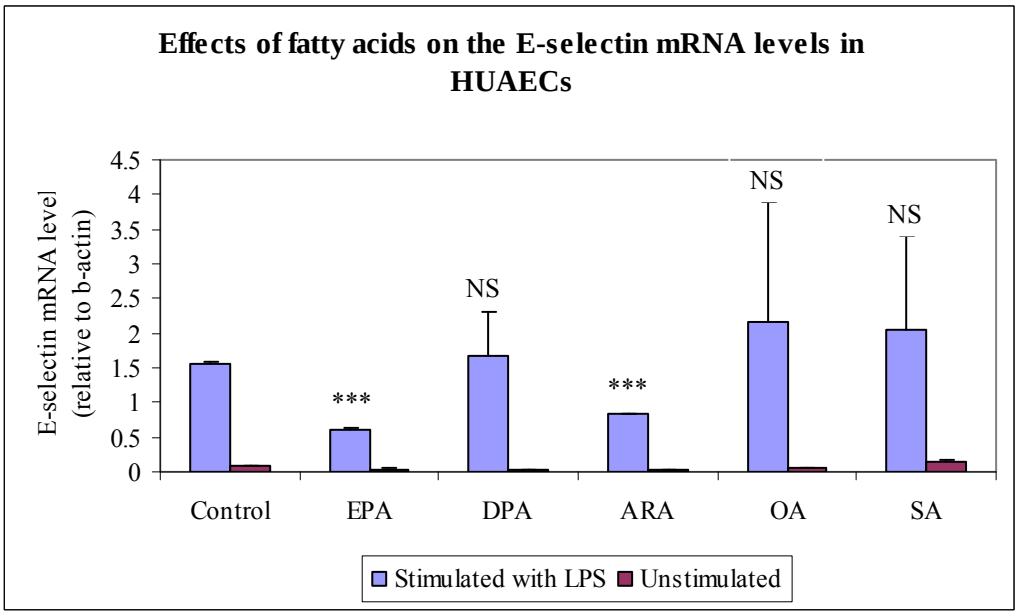


FIGURE 5.7: Effects of Fatty Acids on E-selectin mRNA Levels in HUAECs

Data are expressed as mean ± SEM (n = 2). Significantly different from control, ***P < 0.001, NS = non-significant.

Thus only EPA inhibited mRNA levels of all three adhesion molecules in HUAECs (Figure 5.8).

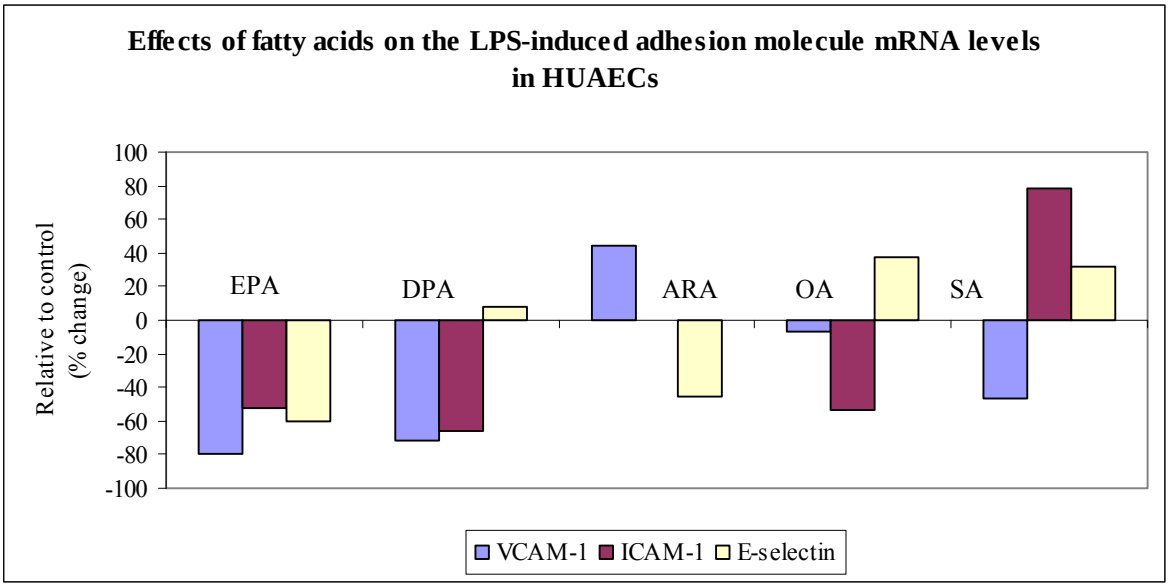


FIGURE 5.8: Comparative Effects of Fatty Acids on Adhesion Molecules mRNA Levels in HUAECs (Relative to Control)

5.4.3 Effects of Fatty Acids on Adhesion Molecule mRNA Levels in HCAECs

Figures 5.9-5.11 show the effects of six fatty acids (50 μM) on VCAM-1, ICAM-1 and E-selectin mRNA levels on unstimulated and LPS-stimulated HCAECs. HCAECs were cultured with the fatty acids for 48 hrs and then with LPS for a further 12 hrs. Low levels of adhesion molecule mRNA were detectable in unstimulated HCAECs but these were not affected by any of the fatty acids. LPS significantly up-regulated adhesion molecule mRNA levels in HCAECs ($P < 0.05$ vs. unstimulated cells).

Under the experimental conditions used none of the fatty acids significantly affected VCAM-1 mRNA levels in HCAECs, although EPA and DHA tended to decrease (by 83% and 68%, respectively) and ARA tended to increase it. A similar set of findings was made for ICAM-1 mRNA in HCAECs as seen for VCAM-1 mRNA (Figure 5.10). Once again, EPA and DHA, and also SA, tended to decrease mRNA levels (by 93%, 89% and 67%,

respectively) and ARA tended to increase it, but these effects were not significant. However, the inhibition of E-selectin mRNA levels seen for EPA, DPA and OA in HCAECs was significant (Figure 5.11). Reductions were by 94% (P = 0.0018), 78% (P = 0.0033) and 73% (P = 0.0488), respectively.

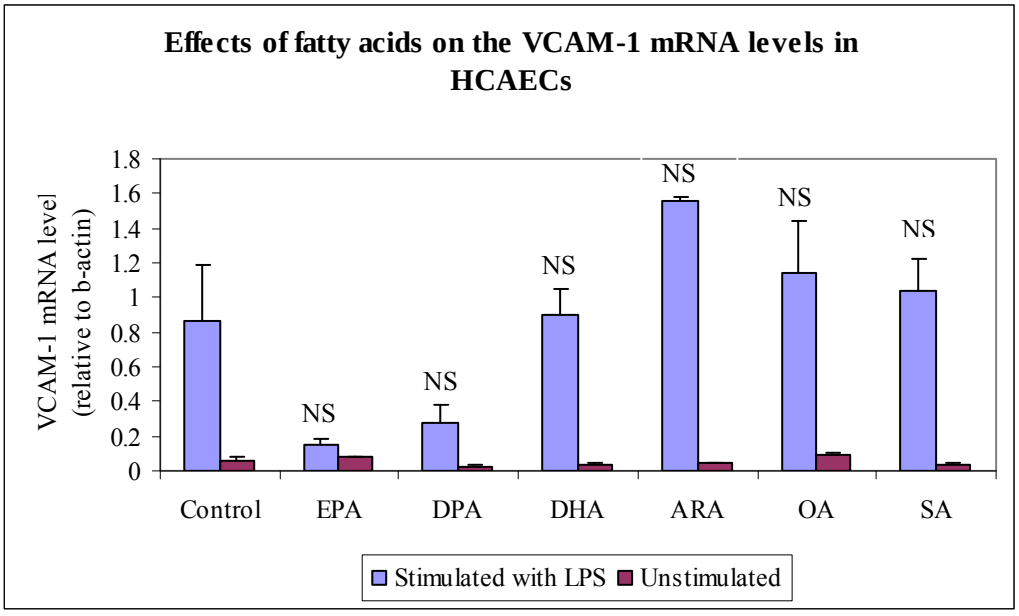


FIGURE 5.9: Effects of Fatty Acids on VCAM-1 mRNA Levels in HCAECs

Data are expressed as mean ± SEM (n = 2). NS = non-significant.

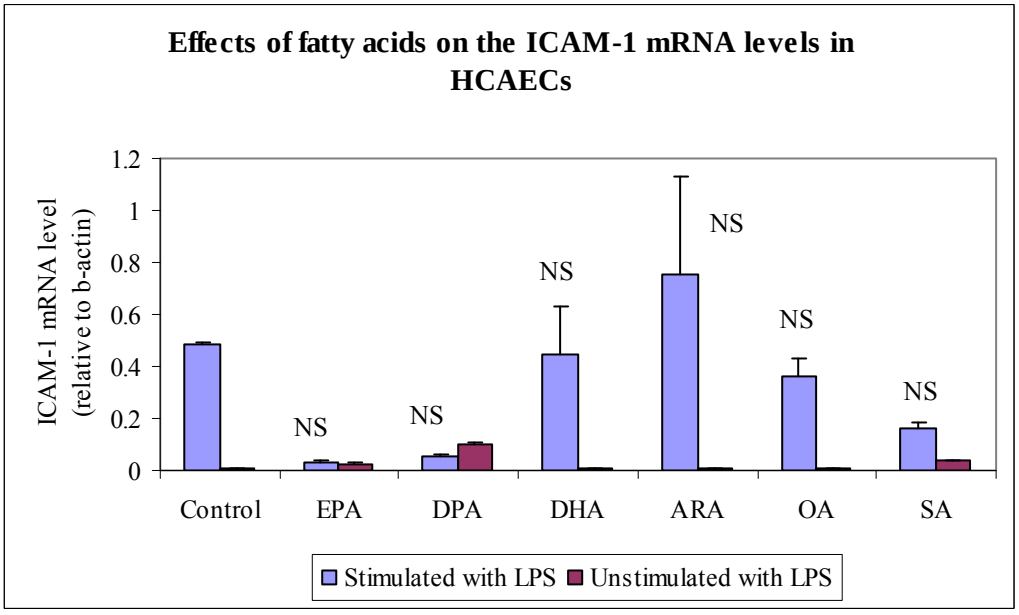


FIGURE 5.10: Effects of Fatty Acids on ICAM-1 mRNA Levels in HCAECs

Data are expressed as mean ± SEM (n = 2). NS = non-significant.

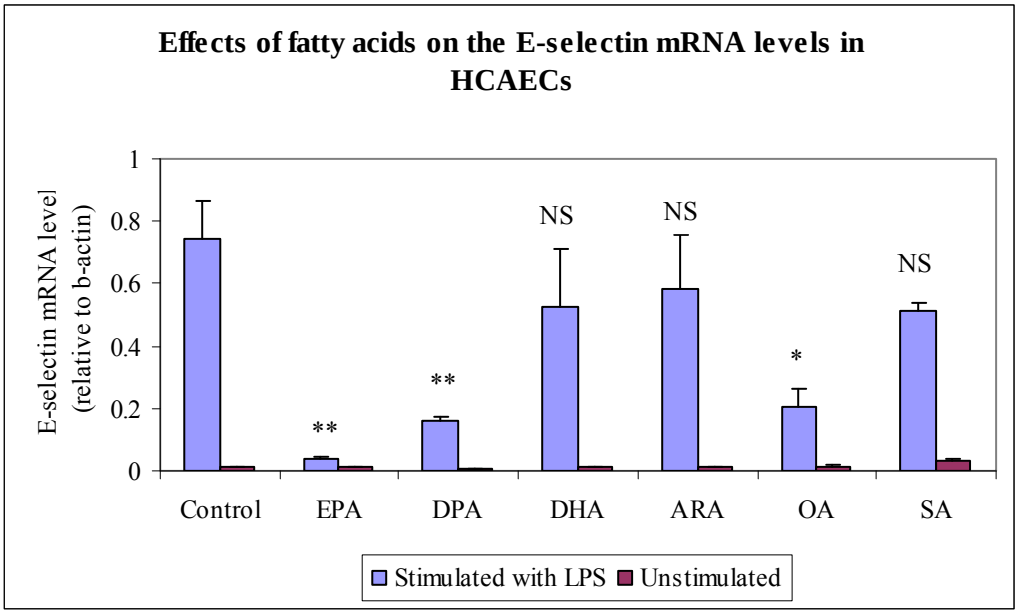


FIGURE 5.11: Effects of Fatty Acids on E-selectin mRNA Levels in HCAECs

Data are expressed as mean ± SEM (n = 2). Significantly different from control, * $P < 0.05$, **$P < 0.005$, NS = non-significant.

Figure 5.12 indicates that EPA and DPA caused a similar degree of inhibition of LPS-induced mRNA for all three adhesion molecules and that the effects of these two fatty acids were near identical. The figure also shows the limited impact of DHA and of the other fatty acids studied.

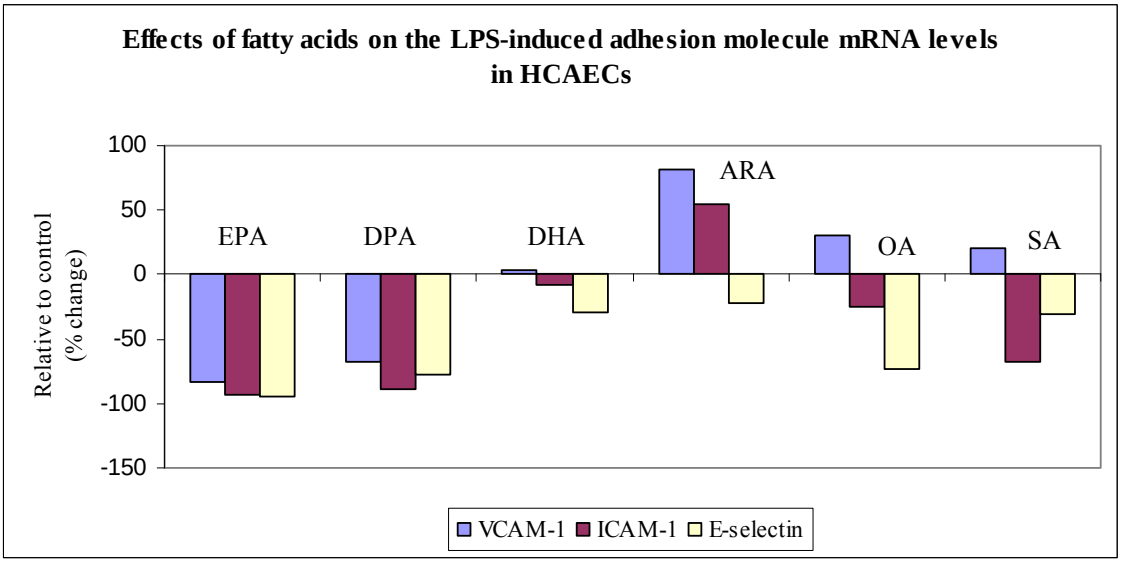


FIGURE 5.12: Comparative Effects of Fatty Acids on Adhesion Molecules mRNA Levels in HCAECs (Relative to Control)

5.4.4 Effects of Fatty Acids on Adhesion Molecule mRNA Levels in HSaVECs

Figures 5.13-5.15 show the effects of six fatty acids (50 μM) on VCAM-1, ICAM-1 and E-selectin mRNA levels on unstimulated and LPS-stimulated HSaVECs. HSaVECs were cultured with the fatty acids for 48 hrs and then with LPS for a further 12 hrs. Low levels of adhesion molecule mRNA were detectable in unstimulated HSaVECs but these were not affected by any of the fatty acids. LPS significantly up-regulated adhesion molecule mRNA levels in HUAECs ($P < 0.05$ vs. unstimulated cells).

Under the experimental conditions used EPA, DPA, DHA and ARA all significantly inhibited LPS-induced VCAM-1 mRNA levels in HSaVECs (Figure 5.13). DHA was found to exert the greatest inhibitory effect (average 86%; $P = 0.0011$), followed by ARA (72%; $P = 0.0019$), EPA (68%; $P = 0.0013$) and DPA (52%; $P = 0.0131$). OA and SA had no significant effect on VCAM-1 mRNA levels in HSaVECs.

Only EPA and DHA inhibited ICAM-1 mRNA levels in HSaVECs (Figure 5.14). Inhibition was by 54% ($P = 0.0042$) and 61% ($P = 0.0064$), respectively. SA significantly enhanced the effect of LPS on ICAM-1 in HSaVECs, increasing mRNA levels by 262% ($P = 0.0002$).

EPA, DPA, DHA and ARA all inhibited LPS-induced E-selectin mRNA levels with decreases of 43% ($P = 0.0123$), 23% ($P = 0.0313$), 28% ($P = 0.0678$) and 48% ($P = 0.0012$), respectively. Once again, SA tended to enhance mRNA levels in this case by 37%, though this was not significant.

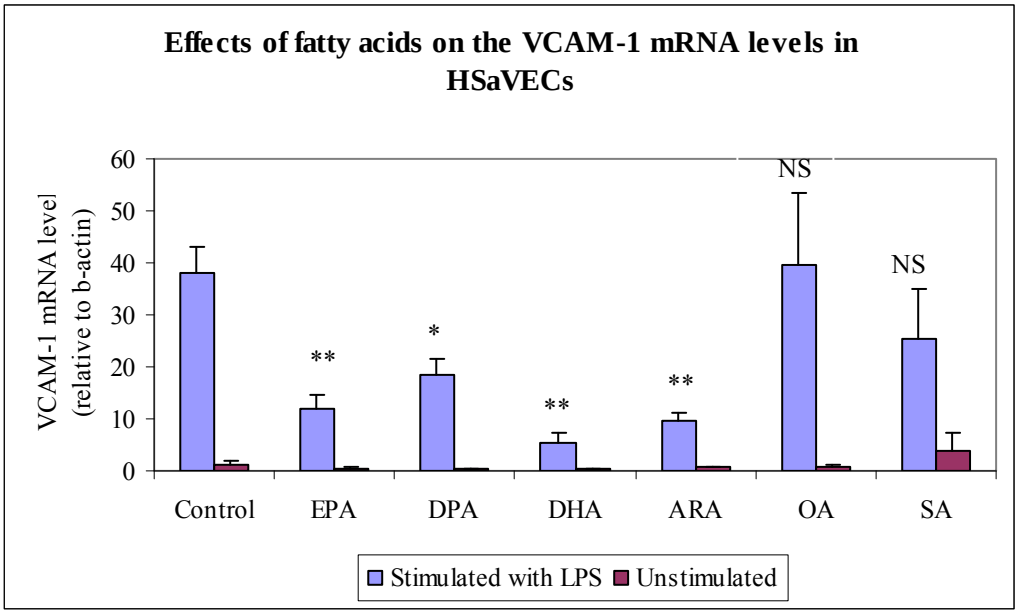


FIGURE 5.13: Effects of Fatty Acids on VCAM-1 mRNA Levels in HSaVECs

Data are expressed as mean ± SEM (n = 2). Significantly different from control, * P < 0.05, **P < 0.005, NS = non-significant.

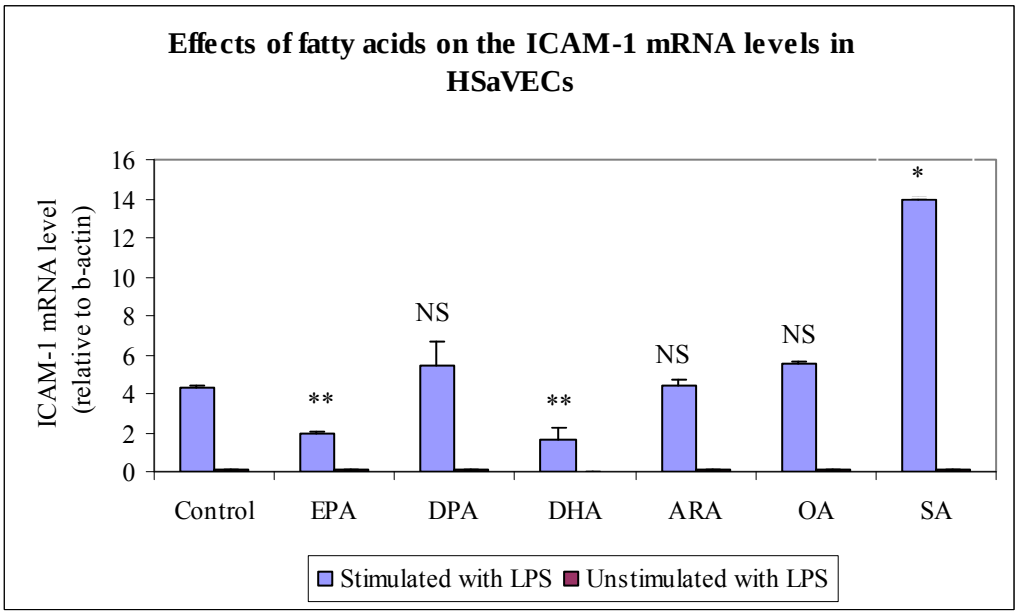


FIGURE 5.14: Effects of Fatty Acids on ICAM-1 mRNA Levels in HSaVECs

Data are expressed as mean ± SEM (n = 2). Significantly different from control, **P < 0.005, NS = non-significant.

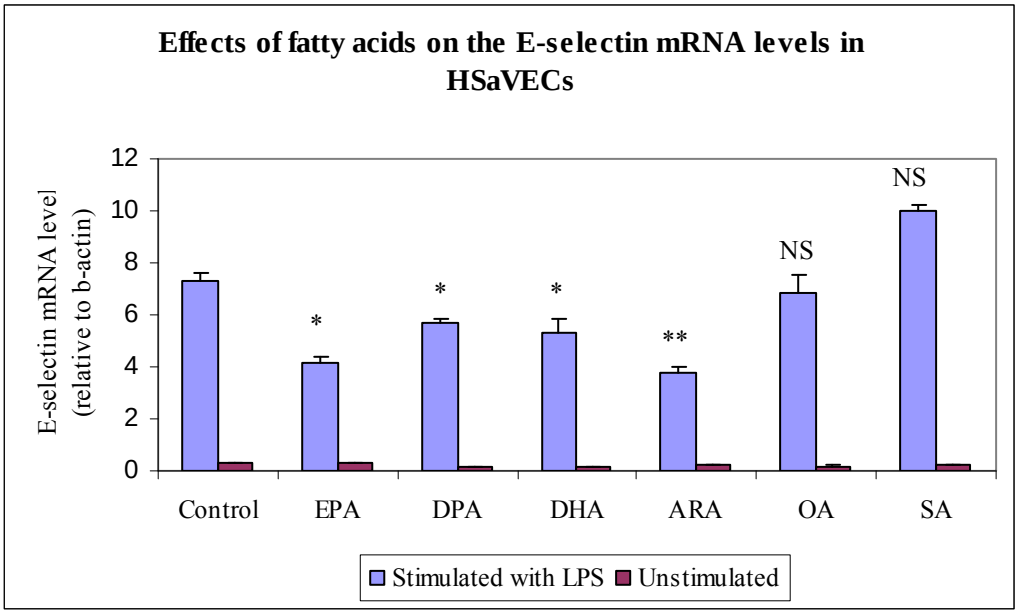


FIGURE 5.15: Effects of Fatty Acids on E-selectin mRNA Levels in HSaVECs

Data are expressed as mean ± SEM (n = 2). Significantly different from control * $P < 0.05$, **$P < 0.005$, NS = non-significant.

Figure 5.16 indicates that DHA, EPA and DPA all caused a fairly similar degree of inhibition of LPS-induced mRNA for all three adhesion molecules, with DHA being slightly more potent. The figure also shows the adhesion molecule specific effects of the other fatty acids.

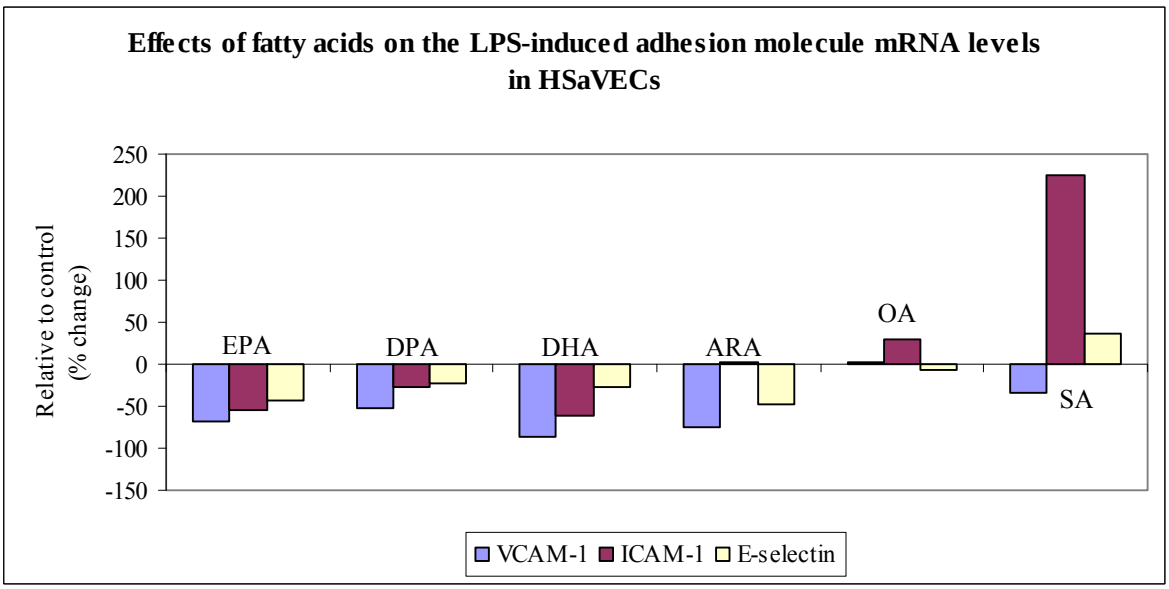


FIGURE 5.16: Comparative Effects of Fatty Acids on Adhesion Molecules mRNA Levels in HSaVECs (Relative to Control)

5.4.5 Effects of Fatty Acids on Adhesion Molecule mRNA Levels in HAOECs

Figures 5.17-5.19 show the effects of four fatty acids (50 μM) on VCAM-1, ICAM-1 and E-selectin mRNA levels on unstimulated and LPS-stimulated HAOECs; no data are shown for DPA and DHA because no mRNA could be isolated from these cells after culture with these two fatty acids. HAOECs were cultured with the fatty acids for 48 hrs and then with LPS for a further 12 hrs. Low levels of adhesion molecule mRNA were detectable in unstimulated HAOECs. These were not affected by EPA, ARA or SA, but OA induced expression of mRNA for all three adhesion molecules. LPS significantly up-regulated adhesion molecule mRNA levels in HAOECs ($P < 0.05$ vs. unstimulated cells).

Under the experimental conditions used none of the four fatty acids for which data are available inhibited levels of mRNA for any of the three adhesion molecules, although EPA and ARA showed a strong tendency to decrease VCAM-1, ICAM-1 and E-selectin. In contrast SA significantly increased LPS-induced ICAM-1 mRNA and had a tendency to increase E-selectin

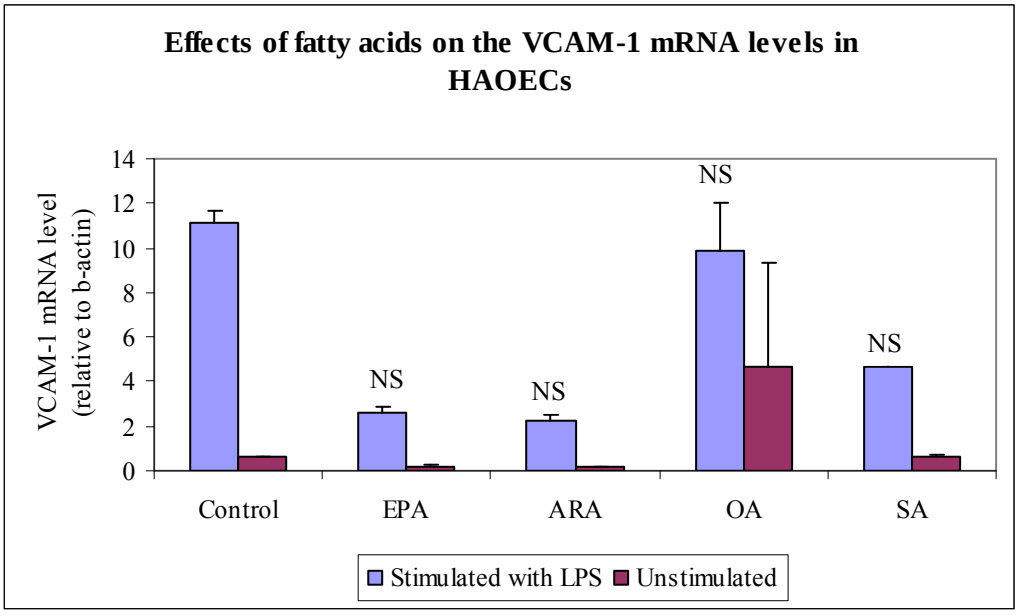


FIGURE 5.17: Effects of Fatty Acids on VCAM-1 mRNA Levels in HAOECs
Data are expressed as mean ± SEM (n = 2). NS = non-significant.

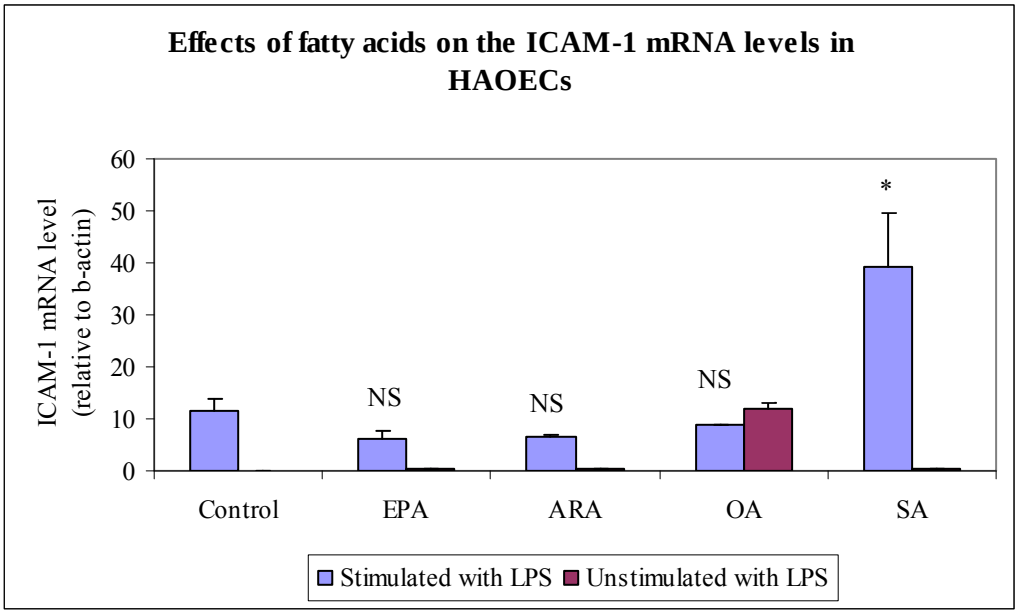


FIGURE 5.18: Effects of Fatty Acids on ICAM-1 mRNA Levels in HAOECs

Data are expressed as mean ± SEM (n = 2). Significantly different from control, * $P < 0.05$, NS = non-significant.

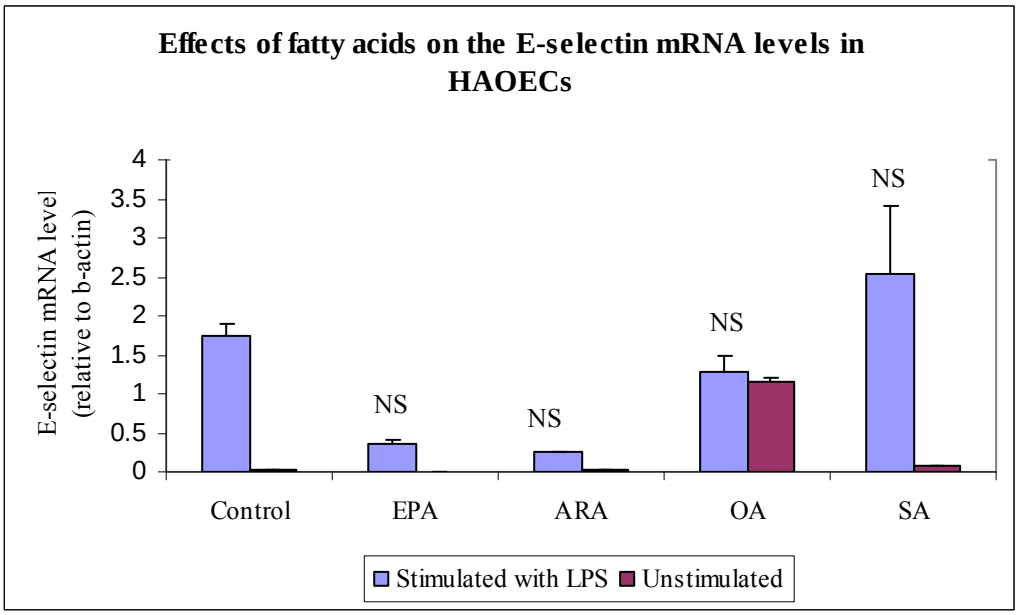


FIGURE 5.19: Effects of Fatty Acids on E-selectin mRNA Levels in HAOECs

Data are expressed as mean ± SEM (n = 2). NS = non-significant.

Figure 5.20 indicates a general lack of effect of the fatty acids tested on adhesion molecule mRNA levels in HAOECs.

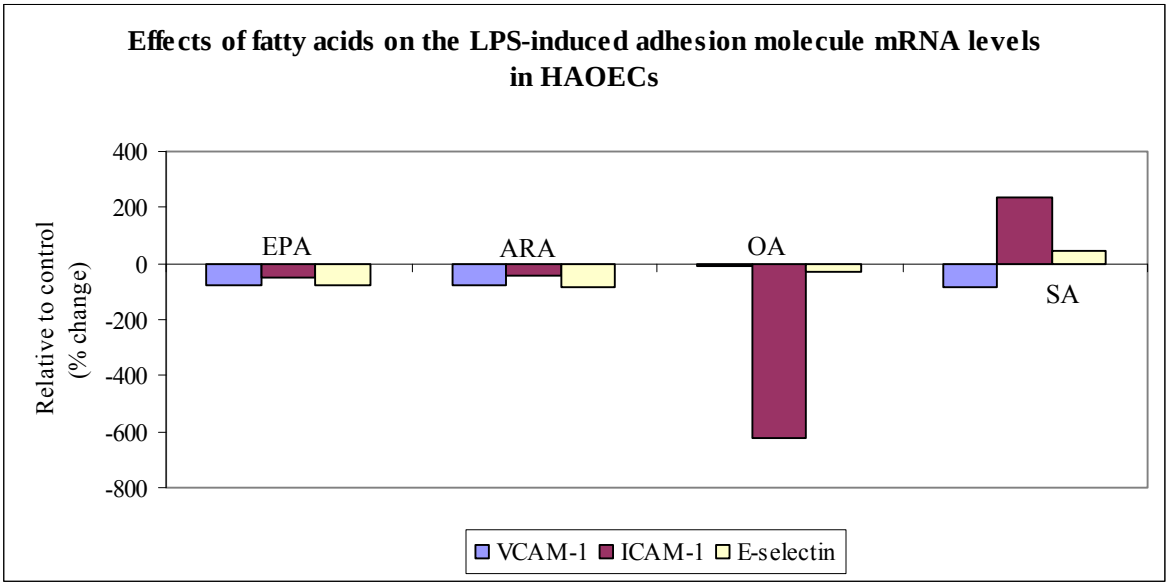


FIGURE 5.20: Comparative Effects of Fatty Acids on Adhesion Molecules mRNA Levels in HAOECs (Relative to Control)

5.5 Discussion

Long chain fatty acids, particularly mono- and polyunsaturated 18-carbon fatty acids, provide a unique model for studying the cellular effects of fatty acids that differ in unsaturation whilst retaining the same carbon length (Toborek & Hennig 1994). Almost all of the currently available data on the effects of fatty acids on adhesion molecule expression has been obtained with venous endothelial cells. Apparent differences in leukocyte adhesiveness to the arterial and the venous endothelium under inflammatory conditions *in vivo,* suggest that the venous enodothelium is more adhesive for leukocytes (particularly neutrophils) than arterial endothelium (Granger & Kubes 1994). Thus there may be different components to the leukocyte-endothelium interaction within veins and arteries and these may be regulated differently. Therefore it is important to study potential regulators at both sites and to do this under similar experimental conditions.

In the present study, similar conditions were used for studying the effects of fatty acids as those that had been identified as optimal for the previous study of protein expression (as described in Chapter 4). Endothelial cells were pre-incubated with fatty acids at 50 μM for 48 hours and then stimulated for 12 hours with LPS at a concentration of 1 μg/mL. Even though the expression of E-selectin occurred much earlier and to a greater extent than VCAM-1 and ICAM-1, Sawa and Tsuruga (2008) showed that increased mRNA expression in LPS stimulated endothelial cells only reached a plateau after 48 hours of stimulation. My study clearly shows markedly enhanced expression of all three adhesion molecule mRNAs in all five endothelial cells by LPS under the conditions used here.

5.5.1 Effects of Saturated Stearic Acid on Adhesion Molecule mRNA Levels

Until recently, De Caterina et al. (1998) were the only researchers to have reported the influence of saturated fatty acids on adhesion molecule expression in cultured endothelial cells; they found no effects of palmitic acid or SA on VCAM-1 mRNA expression in HSaVECs stimulated with IL-1α. In contrast, in the present study, I found that SA can affect adhesion molecule mRNA expression, but that it had different effects on different cell

types and on different adhesion molecules. Table 5.1 shows that the effects of SA on endothelial cells adhesion molecule mRNA expression were varied. I found that SA has an inhibitory effect on LPS-induced VCAM-1, ICAM-1 and E-selectin expression at the mRNA level in HUVECs. However, SA attenuated only VCAM-1 mRNA expression in HUAECs by 47% whereas in contrast, SA increased ICAM-1 (by 262%) and E-selectin (by 37%) mRNA levels in HSaVECs. No effects of SA on adhesion molecule mRNA expression in HCAECs and HAOECs were observed.

Apart from De Caterina et al. (1998) who studied SA and VCAM-1 mRNA in HSaVECs, this is the first study to investigate the effects of SA on adhesion molecule mRNA levels in endothelial cells. Shaw et al. (2007) in their study of comparative effects of fatty acids reported that the saturated fatty acid palmitate enhanced E-selectin mRNA levels in HUVECs. Their findings were consistent with those of Stentz et al. (2006) who found that palmitic acid increased E-selectin cell surface expression in HAOECs even though other studies demonstrated no effect (Holthe et al. 2005; ReiBig et al. 2003). None of these more recent studies examined the effect of SA.

My findings do not support the theory that the presence of a double bond within the structure of a fatty acid is a necessary requirement for alteration in endothelial function as suggested by De Caterina et al. (1998) which was further supported by Carluccio et al. (1999). Indeed, I observed that SA exerted anti-inflammatory effects in HUVECs and, to a lesser extent in HUAECs, but was associated with pro-inflammatory effects in HSaVECs. Dichtl et al. (2002) reported that palmitic acid induced VCAM-1 and MCP-1 gene expression in HUVECs.

Importantly, since the present study is the first one using different types of endothelial cells, I found that fatty acids do not necessarily influence endothelial inflammatory gene expression in a similar direction. Furthermore, although previous work has suggested the degree of fatty acid unsaturation was positively associated with inhibition of cytokine-induced endothelial activation as assessed by cell surface protein expression, my findings on the effects on endothelial mRNA levels do not accord with this paradigm. In terms of the effect of saturated fatty acids on the expression of adhesion molecule mRNA, the present work shows that SA can have down-regulatory effects on the expression of some

inflammatory genes in some cells, while having neutral (no significant effect) or up-regulatory effects on genes associated with inflammation in other cells.

TABLE 5.1**:** Summary of the Significant Effects of Fatty Acids on LPS-stimulated mRNA Adhesion Molecule Levels in Endothelial Cells

	Cell	**EPA**	**DPA**	**DHA**	**ARA**	**OA**	**SA**
VCAM-1	1	↓	↔	NA	↔	↓	↓↓
	2	↓	↔	↓	↔	↔	↔
	3	↓↓	↓	NA	↓	↔	↓
	4	↔	↓↓	↔	↔	↔	↔
	5	↔	NA	NA	↔	↔	↔
ICAM-1	1	↓	↔	-	↔	↓	↓
	2	↓	↔	↓	↔	↔	↑
	3	↓	↓	-	↔	↓	↔
	4	↔	↓↓	↔	↔	↔	↔
	5	↔	NA	NA	↔	↔	↑
E-selectin	1	↓	↓	-	↓	↔	↓
	2	↓	↓	↓	↓	↔	↑
	3	↓	↔	-	↓	↔	↔
	4	↓↓	↓↓	↔	↔	↓	↔
	5	↔	NA	NA	↔	↔	↔

1 = HUVECs; 2 = HSaVECs; 3 = HUAECs; 4 = HCAECs; 5 = HAOECs
NA = not available; ↓↓↓/ ↑↑↑ = extremely significant; ↓↓/ ↑↑ = highly significant; ↓/ ↑ = significant ($P < 0.05$). Student's T-test between control and fatty acid treatment was used to determine any significant level ($P < 0.05$). NA = data not available.

5.5.2 Effects of Monounsaturated Oleic Acid on Adhesion Molecule mRNA Levels

In the present study, I found that OA can affect adhesion molecule expression, but that it has different effects on different cell types and on different adhesion molecules as shown in Table 5.1. I found that VCAM-1 and ICAM-1 mRNA levels were inhibited significantly by OA in HUVECs. In HUAECs, attenuation was only significant on ICAM-1 mRNA expression. OA did not have significant effects on other endothelial cells. De Caterina et al. (1998) reported that treatment of HSaVECs with OA resulted in pronounced reduction (> 50%, as assessed by Northern analysis) in VCAM-1 mRNA, suggesting that the effect of

OA on VCAM-1 is not only seen at the protein level, but also involves a pre-translational process. Carluccio et al. (1999) also reported reduced VCAM-1 mRNA expression with OA (10, 50, 100 μM) in HUVECs, whereas decreased ICAM-1 mRNA level was demonstrated in HUVECs (incubated with 60 and 90 μM OA for 24 hours) (Toborek et al. 2002). However, Shaw et al. (2007) indicated that OA (at 10, 25, 100 μM and incubated for 6 and 24 hours) caused increased expression of VCAM-1 mRNA in TNF-α-stimulated HUVECs. Their findings were further supported by the observed up-regulation of NF-κB expression after incubation with this fatty acid. This is in agreement with Park et al. (2003) who used much higher concentrations of 150, 300 and 450 μM OA (incubated for 24 hours) in HAOECs. In my study using HAOECs, VCAM-1, ICAM-1 and E-selectin were induced significantly by OA in unstimulated cells. The present work is the first to report the inhibitory effect of OA on E-selectin mRNA expression which was only shown in HCAECs.

Upregulation of adhesion molecules on endothelial cells is considered to be a key part of inflammatory conditions including atherosclerosis (Ross 1999). The Mediterranean diet is believed to protect against atherosclerosis and other inflammatory conditions (de Lorgeril & Salen 2008; Carluccio et al. 2007; Perona et al. 2006; Massaro & De Caterina 2002). Olive oil, which contains OA, is the major culinary fat used in the Mediterranean diet and is believed to contribute to the protective effect of that diet. It is possible part of the benefits of the Mediterranean diet are mediated by the anti-inflammatory effects of OA seen here with respect to down-regulating inflammation-triggered adhesion molecule gene expression.

5.5.3 Effects of n-6 Arachidonic Acid on Adhesion Molecule mRNA Levels

Table 5.1 shows that exposure of endothelial cells to ARA attenuated E-selectin mRNA expression in HUVECs, HUAECS and HSaVECs. No significant effect of ARA on ICAM-1 mRNA expression was found, whereas VCAM-1 mRNA expression was inhibited in HUAECs. These findings are in accordance with Stuhlmeier et al. (1996) which reported that ARA attenuated ICAM-1 and E-selectin mRNA expression, but not VCAM-1 mRNA, in porcine aortic endothelial cells. Khalfoun et al. (1996) reported no effect on adhesion

molecule (VCAM-1, ICAM-1 and E-selectin) expression in HUVECs with 150-300 μM ARA. De Caterina et al. (1998) demonstrated that ARA decreased VCAM-1 protein expression (mRNA data was not available) in HSaVECs and that the effect was much greater compared with OA. I also demonstrated that ARA consistently attenuated VCAM-1 protein expression (as assessed by ELISA) (see Section 4.5.5), but it appears that the effect does not involve a change in transcription.

5.5.4 Effects of n-3 PUFAs on Adhesion Molecule mRNA Levels

n-3 Fatty acids, particularly EPA and DHA, are currently receiving remarkable attention as potential anti-atherogenic and anti-inflammatory substances. In Chapter 4, I reported that n-3 fatty acids exhibited pronounced inhibitory effects on the surface adhesion molecule protein expression as determine by ELISA. In the present study, treatment of endothelial cells with EPA consistently resulted in a pronounced inhibition of VCAM-1, ICAM-1 and E-selectin mRNA in HUVECs, HUAECs and HSaVECs (Table 5.1). Furthermore inhibitory effects of EPA were seen in HCAECs and HAOECs, but these results were not significant, except for E-selectin mRNA expression in HCAECs. This lack of significance is most likely due to having too few samples; thus this experiment should be repeated with more samples to confirm the precise effect of EPA. Figure 5.21 shows the percent of inhibition seen with EPA on LPS-stimulated endothelial cells (only significant results). It is clear that EPA has a fairly similar inhibitory effect (approx. 50%) on all three adhesion molecules (at the mRNA level) in all three cell types shown. This effect is consistent with what was seen in Chapter 4 for the effect of EPA on adhesion molecule proteins.

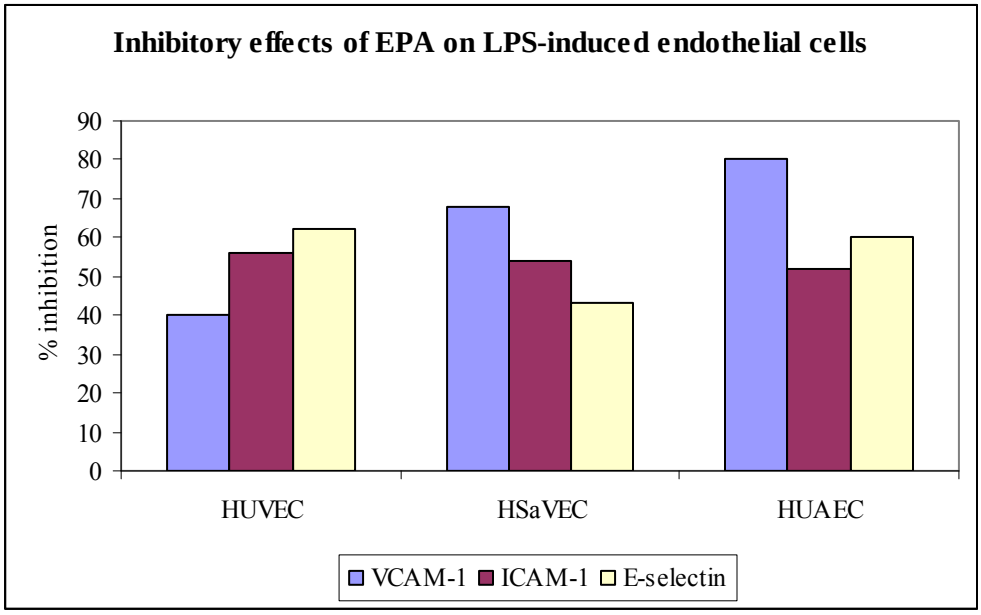


FIGURE 5.21: Comparison of % Inhibitory Effects of EPA on Adhesion Molecule mRNA Levels

In HSaVECs, VCAM-1, ICAM-1 and E-selectin mRNA expression were attenuated by DHA but no significant effect of this fatty acid was observed on HCAECs. DPA, decreased VCAM-1 and ICAM-1 mRNA expression in HUAECs, whereas E-selectin mRNA expression was inhibited in HUVECs and HSaVECs. Unfortunately, I was not able to obtain complete data for the effects of DHA on all five different types of endothelial cells. It is not clear why sufficient mRNA was not obtained from HUVECs, HUAECs and HAOECs following DHA treatment. In the experiments described in Chapter 4, I was able to gather protein data (as assessed by ELISA) on the effect of fatty acids, including DHA on adhesion molecule expression in all cell types. Thus the cells were viable. The fatty acid concentration used in the present study was 50 μM, compared with 20, 25 and 65 μM used by others (Weber et al. 1995; De Caterina et al. 1998; Collie-Duguid & Wahle 1996). Thus the DHA concentration I used is not likely to have been responsible. Future work should try to understand what is happening when these cells are exposed to DHA.

Among the three n-3 PUFAs studied, EPA appears to be the most potent inhibitor of endothelial activation. De Caterina's group first reported the effects of n-3 fatty acids on adhesion molecule expression, both at the protein (flow cytometry) and gene expression levels (Northern analysis). Using HUVECs, they found that inhibition of adhesion molecule

expression occurred in a range of DHA concentrations that they said were achievable by normal dietary intake. Furthermore, the extent of inhibitory effects was paralleled with DHA incorporation into the cells and was inversely associated with the amount of n-6 fatty acids. In the present work, compared with other fatty acids studied, n-3 fatty acids demonstrated remarkable inhibition on adhesion molecule mRNA expression and my findings are in agreement with previous studies (De Caterina et al. 1999; Collie-Duguid & Wahle 1996; Weber et al. 1995), although they extend these studies by comparing effects on different endothelial cell types.

All three n-3 fatty acids I studied (EPA, DPA, DHA reduced mRNA levels induced by LPS. High intakes of these fatty acids are associated with protection against cardiovascular disease (Nordoy et al. 2001; von Schacky 2003; Harris 2005) and can be used to treat some inflammatory conditions (Calder 2006; Sijben & Calder 2007; Calder 2008). Thus, the observed inhibitory actions of these fatty acids as observed here, may provide part of the explanation for their benefits *in vivo*.

The present study demonstrates that the effects of fatty acids on adhesion molecule expression at the protein level is not always consistent with the effects seen at the transcriptional level. These observed differences in the inhibitory effects of fatty acids on specific adhesion molecule expression might vary according to endothelial types and also could relate to the method used.

Most of the data previously available for the effects of n-3 fatty acids on adhesion molecule expression at the transcriptional level were obtained by Northern analysis. This method uses agarose gels for detection of PCR amplification at the end stage (plateau) of the PCR reaction. Real-time RT-PCR, on the other hand, as used here, is a much more precise quantification method allowing quantification of relative gene expression at a particular time. Moreover, to ensure accuracy in the quantification, data is normalized by the amount of RNA from a housekeeping gene measured in the same sample. Since I used a real-time RT-PCR method to assess differences of adhesion molecule expression at the transcriptional level, my results are likely to be more precise. The only limitation in the present study is sample size. Thus further work is highly recommended to confirm my

findings and subsequently to gain clearer picture of the effects of different fatty acids on inflammatory markers in relation to atherogenesis.

5.6 Conclusion

n-3 Fatty acids, particularly EPA and DHA have become a focus of interest, and recent research and trial evidence have highlighted their effects, including as anti-inflammatory agents. Despite this progress, to date, the precise mechanisms by which n-3 fatty acids act remain poorly understood. In the present study, with the limitation of DHA data, EPA appears to be the most potent fatty acid inhibitor of adhesion molecule expression at the mRNA level as assessed by reverse transcription and real-time PCR. However, the mechanisms involved have not been extensively investigated (i.e. mRNA stability, transcription regulation, etc.). Previous studies documented that this effect (anti-inflammatory) may occur via the attenuation of the activated NF-κB transcription factor pathway (Wahle & Rotondo 1999; Hennig & Toborek 2000; Calder 2002). Studies that report effects of n-3 PUFAs on the NF-κB pathway will be described further in Chapter 6. My results also do not support the theory that the presence simply of a double bond within the structure of a fatty acid is a necessary requirement for alteration in endothelial function as suggested by De Caterina et al. (1998). Interestingly, SA (a saturated fatty acid), can act as both anti- and pro-inflammatory depending on the cell type. I also showed that both OA (a monounsaturated fatty acid) and arachidonic acid (an n-6 fatty acid) appear to inhibit some of the adhesion molecule expression at the transcriptional level. n-3 PUFAs were generally inhibitory and of all fatty acids tested EPA was the most potent.

As I had seen in Chapter 4 for adhesion molecule protein expression, the effects of fatty acids on the adhesion molecule expression at the transcriptional level are fatty acid, adhesion molecule and endothelial cell specific. In general, the inhibitory effects of fatty acids are more pronounced in vein endothelial cells (i.e. HUVECs and HSaVECs) than arterial endothelial cells. More needs to be understood about the mechanisms by which fatty acids affect adhesion molecule gene and protein expression and to examine whether these effects seen in cell culture also occur *in vivo*.

Chapter 6

General Discussion

6.1 Overview

The present thesis was primarily aimed at examining the effects of long chain n-3 fatty acids, particularly EPA and DHA, on processes and outcomes relevant to cardiovascular disease (CVD). The focus of the human studies conducted was the most widely recognised conventional risk factor, namely the blood lipid profile and the newly recognised (emerging) risk factor inflammation. With regard to markers of inflammation I was especially interested in blood adhesion molecules. This is because leukocyte-endothelial adhesive interaction is amongst the earliest events in the atherosclerotic process, yet remains important throughout the progression of atherosclerosis. It has been reported that supplementation with fish oil results in reduced atherosclerosis in animal models (Weiner et al. 1986; Davis et al. 1987; Mortensen et al. 1998). Furthermore, large studies in humans (GISSI 2001; Yokoyama et al. 2007) reveal a cardio-protective effect of long chain n-3 PUFAs which might be attributable to a combination of lipid-lowering and decreased inflammation (Calder 2002). Having studied inflammatory markers in healthy volunteers and in patients with advanced carotid atherosclerosis and observed some effects on soluble adhesion molecules suggesting an effect of the endothelium, I moved my focus to in vitro studies of human endothelial cells in order to identify whether individual fatty acids have different effects and whether cells of different origin respond differently to fatty acids. Thus, overall I conducted four separate but related studies:

1) the effects of moderate dose of long-chain n-3 PUFAs (about 2.1 g/day of EPA plus DHA) on risk factors for cardiovascular disease in particular blood lipids and inflammatory markers in healthy, middle-aged males;

2) the effects of 1.8 g/day EPA plus DHA in the form of ethyl esters (Omacor) on blood lipids and inflammatory markers in patients awaiting carotid endarterectomy;

3) the effects of selected fatty acids on adhesion molecule expression (at the protein level) in cultured human endothelial cells of different origins;

4) the effects of selected fatty acids on adhesion molecule mRNA expression (at the transcriptional level) in cultured human endothelial cells of different origins.

6.2 The effects of Fish Oil Supplementation *in vivo*

6.2.1 Fatty Acid Incorporation into Plasma or Plaque Phosphatidylcholine

In order for long chain n-3 fatty acids from fish oil to affect the plasma lipid profile and inflammatory markers, those fatty acids must appear in relevant pools such as plasma lipids. My findings from human studies showed that EPA and DHA were incorporated into plasma phosphatidylcholine (PC), whereby remarkably increased levels of these fatty acids at the end of supplementation were revealed. In the first study, 2.1 g/day EPA plus DHA resulted in a 363% increase in EPA and only a 13% increase in DHA. In this study, DHA was not significantly increased in any of the lipid fractions studied probably because the dose of DHA was only 300 mg/day compared with 1800 mg/day of EPA administered. In the second study, a dose of 1.8 g/day of EPA plus DHA (818 mg of EPA plus 675 mg of DHA) resulted in significantly increased EPA and DHA in plasma PC by 161% and 70%, respectively. Using the data from Rees et al. (2006) I plotted the percent change in EPA and DHA content of plasma PC as a function of supplemental EPA observed in both studies and found that the change in EPA I observed fits well on the dose-response curve. The increase in the proportion of EPA in plasma PC was accompanied by a significant decrease in proportion of n-6 fatty acids and it seems that EPA consistently more easily replaces dihomo-γ-linolenic acid (DGLA) and linoleic acid (LA) than arachidonic acid (ARA), in plasma phospholipids. It has been reported that increased dietary intake of LA leads to oxidation of low-density lipoprotein (LDL), and platelet aggregation, and interferes with the incorporation of essential fatty acids (EFA) in cell membrane phospholipids (Simopoulos 2008). Thus the decrease in the proportion of LA is most likely of importance. For DHA incorporation, the data obtained did not fit well onto the dose-response curve from Rees et al. (2006). However if supplemental DHA was normalised according to EPA, then a dose-response incorporation of DHA was observed. I also analysed the incorporation of PUFAs into atherosclerotic plaques since it is believed that if PUFAs are to affect plaque stability (Thies et al., 2005), they must be first incorporated into the plaque. It was found that there were no significant differences between groups for any fatty acid in plaque PC apart from EPA which was demonstrated to be significantly higher in the Omacor (n-3 fatty acid) group as compared to the placebo (0.71 vs. 0.40 g/100 g fatty acid). Thus, the dose of EPA

in the Omacor group was enough to enable incorporation into plaque PC. It appears that the dose of DHA however, is insufficient, since successful incorporation was not demonstrated.

6.2.2 The Effects of Fish Oil Supplementation on Blood Pressure and Lipid Profile

In my first study involving normotensive, healthy subjects, there was no effect of fish oil supplementation on blood pressure. This is in agreement with a meta-analysis that identified that the blood pressure lowering effects of long chain n-3 fatty acids were greater in subjects that were older or hypertensive than those who were younger or normotensive (Geleijnse et al. 2002). In addition, Mori et al. (2000) reported that DHA was more effective compared with EPA in lowering blood pressure. Thus, the lack of blood pressure-lowering in my study might due to insufficient dose of DHA. On the other hand, in the second study in which subjects were hypertensive and also on anti-hypertensive medication, both control and Omacor groups exhibited significant decreases in systolic and diastolic blood pressures. The effect of Omacor was similar to the effect of placebo, therefore ruling out any specific effect of EPA plus DHA on blood pressure.

In terms of the blood lipid profile, results from my study in healthy subjects were consistent with other studies demonstrating that fish oil supplementation does not lower the concentration of blood cholesterol, and indeed may have slight total cholesterol, LDL-c and HDL-c raising effects (Katan et al. 1995; Harris et al. 1996). However, in the Omacor group whereby subjects also taking statins and lipid-lowering drugs, total cholesterol and TAG were significantly decreased by 13% and 20%, respectively. These effects were much more apparent in the Omacor group as compared to those seen in the placebo group. The reduction in total cholesterol with long chain n-3 PUFAs as demonstrated in my second study in carotid endarterectomy patients suggests that it may be a useful adjunct to lipid-lowering therapy even though fish oil supplementation has been reported by others to increase total cholesterol by 5-10% (Harris 1997). My findings also in accordance with previous studies showing the beneficial effects of co-administration of statins and fish oil on the lipid profile (Durrington et al. 2001; Davidson et al. 2007; Mayer et al. 2007). Hence

it is suggested that fish oil supplementation be used in combination with statin treatment as an optimal lipid lowering strategy.

6.2.3 The Effects of Fish Oil Supplementation on Plasma Inflammatory Markers

In terms of plasma inflammatory markers, again the anticipated effects of fish oil in healthy subjects were not as apparent as in carotid endarterectomy patients. The only beneficial effect of long chain n-3 fatty acids in the first study in healthy subjects was a significant difference between the fish oil and placebo groups in percent change from baseline for sICAM-1, with a reduction (about 10% from baseline) in the fish oil group. There was an inverse association between the levels of sICAM-1 and plasma PC DHA. This is the first time this relationship has been reported and it suggests that long chain n-3 PUFAs, particularly DHA, are of importance in controlling inflammatory responses at the level of the vascular endothelium. The recent study of Paulo et al. (2008) on the effects of 1.4 g/day n-3 PUFAs in young, healthy subjects also reported no significant effects on sICAM-1. In contrast, following Omacor supplementation, sE-selectin and sVCAM-1 were decreased significantly by 23% and 25%, respectively. Interestingly, the beneficial effect of Omacor was more pronounced in male subjects whereby IL-6 was also decreased compared to what was seen in of females. Based on correlation analysis between mRNA expression of inflammatory markers in plaque and plasma concentrations, it seems that soluble inflammatory markers cannot be used to reflect the expression of these molecules at the cell surface, i.e. in the vasculature or in the plaque. Integrating the findings of these two studies indicates that supplementation with the long chain n-3 PUFAs EPA and DHA reduces sICAM-1 (in healthy subjects), and sVCAM-1 and sE-selectin (both in carotid endarterectomy patients). Further *in vitro* experiments were performed using several fatty acids and different cell types in order to progress mechanistic understanding of the effects of long chain n-3 fatty acids.

6.3 The Effects of Fatty Acids on Adhesion Molecule Expression on Cultured Human Endothelial Cells

In order to systematically evaluate the effects of a range of fatty acids on endothelial adhesion molecules studies were conducted on five different cell lines. This was done in order to establish whether fatty acids behaved differently with arterial and venous cells; CVD is an arterial disease but most studies of fatty acids and adhesion molecules have used venous cells as models. Also it was thought possible that some of the discrepancies in the literature might be due to use of different cell lines. This is the first study comparing the effects of fatty acids on adhesion molecule expression using different endothelial cell types. Previously, work of this type focused upon human venous endothelial cells, including saphenous (De Caterina et al. 1994; De Caterina 1996; De Caterina & Libby 1998) or umbilical (Khalfoun et al. 1996; Collie-Duguid & Wahle 1996; Carluccio et al.1999). To date, there were no studies on the effects of long chain fatty acids on adhesion molecule expression performed using either arterial or aortic endothelial cells. Hence, this is the first comparative study of the effects of fatty acids on adhesion molecule expression (at protein and mRNA levels) under similar experimental conditions using several types of endothelial cells.

De Caterina et al. (1998) reported that the saturated fatty acids palmitate and stearate have no effects on endothelial activation. In relation to inflammation, *in vitro* and animal feeding studies also suggest that saturated fatty acids have limited impact on T lymphocyte proliferation, Th1-type and Th2-type cytokine production and NK cell activity (Calder 1998). The lack of effect of dietary saturated fatty acids on NK cell activity is supported by human studies (Hebert et al. 1990; Rasmussen et al. 1994). In this study, however, I found that stearic acid can affect adhesion molecule expression, but it had different effects on different cell types and on different adhesion molecules. The effects seen at the protein level were not always consistent with the effects seen at the transcriptional level. In HUVECs and HUAECs, I found that the effect of stearic acid on VCAM-1 expression was consistent, at both protein and transcriptional levels. On the other hand, my results revealed that in the other endothelial cells studied, stearic acid significantly enhanced VCAM-1 expression at protein level. However this effect became non-significant when it was

analysed by real-time RT-PCR for gene expression. This is because the outcomes from real-time RT-PCR are more accurate compared with ELISA due to method sensitivity, and in the real-time technique, data were also normalised to a housekeeping gene. Of note, consistent results (at protein and at mRNA levels) were obtained from endothelial cells that are from umbilical cord, irrespective of whether the cells were from vein or artery. Moreover, for ICAM-1 expression, only HAOECs exhibited similar results for both protein and gene expression. Therefore, saturated stearic acid can have down-regulatory effects on the expression of some inflammatory genes in some cells, while having neutral effects or even up-regulatory effects on genes associated with inflammation in other cells.

For oleic acid which represents monounsaturated fatty acids, the present study also showed inconsistent results. Results revealed that the inhibitory effect was almost similar to that of stearic acid. In addition, not only VCAM-1 attenuation effects with oleic acid as demonstrated in HUVECs and HUAECs were observed, but decreased ICAM-1 mRNA expression was also demonstrated in both endothelial cell lines. Interestingly, my results also showed that the effects of stearic and oleic acid on E-selectin expression were similar amongst the endothelial cells studied, whereby in HUVECs, HUAECs and HCAECs, both these fatty acids resulted in increased E-selectin expression at protein levels.

It has been known that in inflammatory conditions, 20-carbon PUFAs present in cellular membranes are converted to eicosanoids and the activity of these molecules depends on the nature of their precursors: when arachidonic acid (n-6) is present, strongly pro-inflammatory molecules are released, meanwhile EPA-derived eicosanoids are weakly inflammatory. In relation to this, n-3 PUFAs increase the content of EPA-eicosanoids and decrease arachidonic acid in immune and endothelial cells leading to a decrease inflammatory activity. Likewise, oleic acid exhibits anti-inflammatory effects by preventing the release of particular chemotactic molecules (Garcia et al. 2006). Previously, Massaro & De Caterina (2002) reported decreased expression of a number of major pro-inflammatory proteins, such as endothelial leukocyte adhesion molecules by incorporation of oleic acid into total cell lipids. The mechanisms by which the effects of oleic acid on vascular dysfunction occur are believed through modulation of the endothelial activity that involves the release of nitric oxide, eicosanoids (prostaglandins and leukotrienes) and adhesion

molecules, and indeed in most cases by activation of nuclear factor κB (NFκB) by reactive oxygen species (Perona et al. 2006).

My study revealed that the effect of n-6 arachidonic acid (ARA) on adhesion molecule expression, particularly on VCAM-1 was more pronounced as compared to that of stearic and oleic acids. VCAM-1 expression at the protein level was decreased significantly in all endothelial cells studied apart from HAOECs, even though no significant effect was observed using real-time RT-PCR for mRNA expression except from HUAECs. The decreased ICAM-1 expression at the protein level was only demonstrated in HUVECs, whereas E-selectin was attenuated at both protein and mRNA levels.

Interest in the effects of ARA in modulating inflammation is based upon the roles of mediators derived from ARA which can be inhibited by the long chain n-3 PUFAs found in fish oils (Calder et al. 2002). It has been documented that n-6 fatty acids influence gene expression and tend to be pro-inflammatory. However, contrary to the pro-atherosclerotic effects of ARA and LA, there is also broad evidence that 15-LOX metabolites of these fatty acids have anti-inflammatory effects. The 15-LOX arachidonic acid metabolite 15-HETE inhibits superoxide production and polymorphonuclear neutrophil (PMN) migration across cytokine-activated endothelium and subsequently can be further metabolized to the anti-inflammatory lipoxins. These lipoxins promote vasorelaxation in the aorta and counteract the action of most other pro-inflammatory factors like leukotrienes and prostanoids (Wittwer & Hersberger 2007).

Amongst the fatty acids studied in the present study, n-3 PUFAs are of great interest particularly EPA and DHA. These fatty acids are the major bioactive n-3 fatty acids that have long been recognized to modulate inflammatory responses (Calder 2001). In the present study, DHA had the greatest and the most consistent effects on adhesion molecule expression at the protein level. However, interpretation of this study at the mRNA level was hindered by limited DHA data available, leading to a conclusion from the data available that EPA is the most potent fatty acid inhibitor. Because inflammation is believed to be the root of many chronic diseases, including cardiovascular disease, increased dietary intake of

n-3 fatty acids, particularly EPA and DHA has been recommended (Ruxton et al 2004; Simopoulos 2008).

The mechanism by which n-3 PUFAs affect the expression of inflammatory genes could be through direct actions on the intracellular signaling pathways that lead to inactivation of one or more transcription factors such as NF-κB (Ross et al. 1999; von Schacky et al. 2007). NF-κB is composed of homo- or heterodimeric complexes of at least five distinct subunits, such as p50, p52, p65 (RelA), c-Rel and Rel-B; however, the p50/p65 heterodimer is the predominant form of this transcription factor (Baeuerele 1998). NF-κB binding sites were identified in the promoter regions of genes encoding for adhesion molecules VCAM-1 (Wang et al. 2008; Massaro et al. 2008), ICAM-1, and E-selectin (Massaro et al. 2008) and inflammatory cytokines (such as TNF-α, IL-1β, IL-6 or IL-8), growth factors and chemokines. Although other transcription factors are also required for expression of these genes, NF-κB constitutes an important component of their transcriptional regulation. Berliner et al. (1995) demonstrated that generation of inflammatory cytokines is dependent on activated NF-κB, and in turn, these cytokines can stimulate activation of this transcription factor. In relation to this, fish oil has been reported to decrease endotoxin induced activation of NF-κB in human monocytes (Lo et al. 1999), and this was linked with decreased IκB phosphorylation (Novak et al. 2003). The inhibition of NF-κB activation can be caused by mechanisms that produce the activation of PPAR or inhibition of Toll-like receptors (Torrejon et al. 2007). Evidence from *in vitro* studies (De Caterina & Massaro, 2005) indicates that both EPA and DHA decrease agonist-induced activation of NF-κB and increase PPAR. In contrast, Goua et al. (2008) reported that reduction of ICAM-1 and VCAM-1 protein expression by n-3 PUFA was less dependent on the NF-κB pathway than reduction by conjugated linoleic acid which reflected the parallel attenuation of NF-κB activity. However, they suggested involvement of other transcription factors (i.e. activator protein-1, AP-1) in the fatty acid regulation of adhesion molecule expression.

Besides the transcription factor NF-κB, AP-1 also appears to be most important in regulating the inflammatory process. Activation of AP-1 enhances the expression of inflammatory genes. AP-1 binding sites were identified in the promoter regions of genes encoding for VCAM-1 (Ahmad et al. 1998; Goua et al. 2008), ICAM-1 (Stade et al. 1990;

Goua et al. 2008) and E-selectin (Lee et al. 2004). The schematic diagram is proposed (Figure 6.1), in which n-3 fatty acids particularly EPA and DHA would act downstream to receptors and likely at the level of reactive oxygen species (ROS). This ROS appears to be a critical mediator of NF-κB activation and would activate the NF-κB system transcription factors, and most likely via IκB degradation and subsequent translocation of free active NF-κB heterodimer (relA-p50) into the nucleus and further binding to specific consensus sequences in a number of NF-κB-responsive genes, including genes for VCAM-1, ICAM-1 and E-selectin (De Caterina & Massaro 2005).

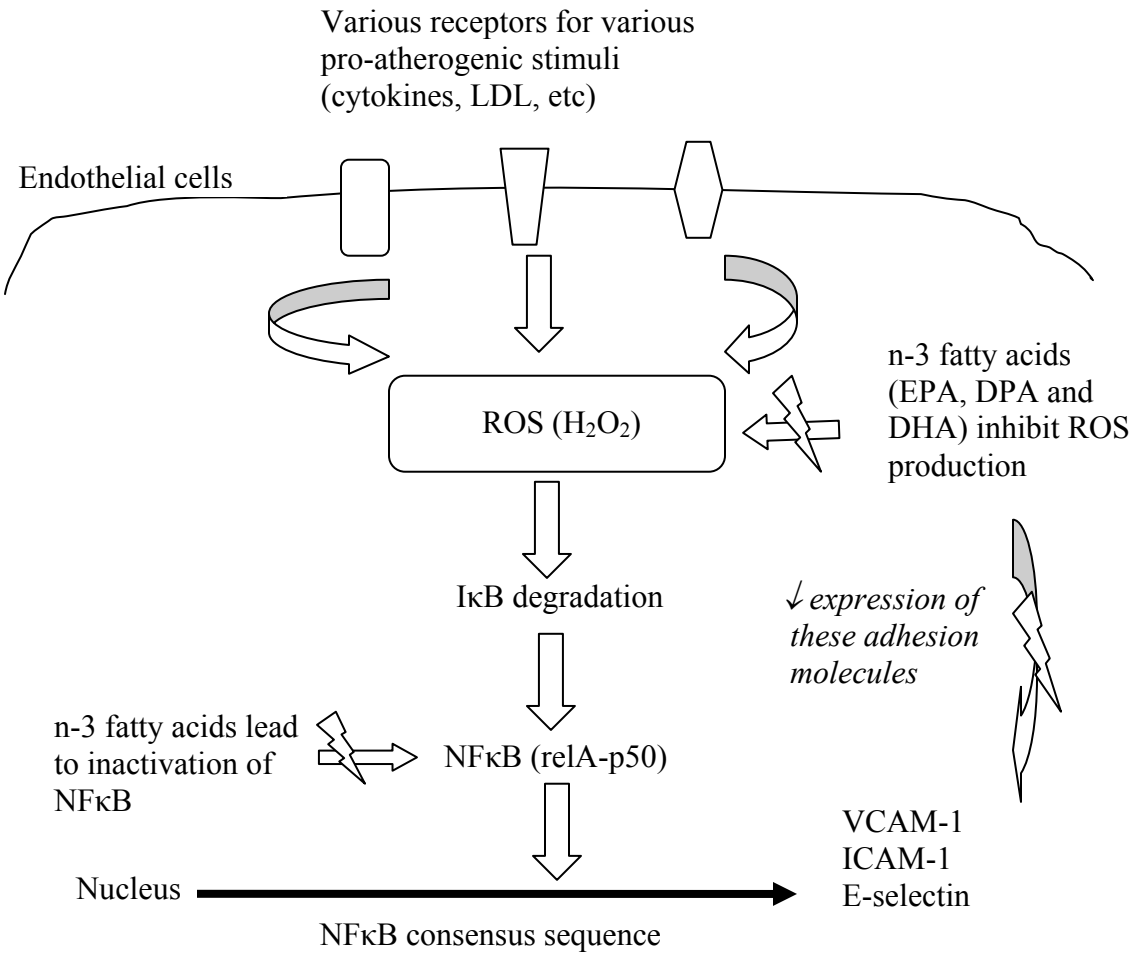


FIGURE 6.1: Schematic Diagram of Anti-inflammatory Pathway Induced by n-3 Fatty Acids

In the present work both *in vivo* and *in vitro* studies demonstrated that the beneficial effects of EPA and DHA are more pronounced in inflammatory conditions than in normal or 'healthy' conditions. This is not surprising since fish oil supplementation has been shown to be more effective in those who already manifested the disease outcomes. However, even though no significant enhancement of immune function in relation to adhesion molecule expression has been shown in normal cells, still it is very difficult to conclude whether any enhancement when cells are healthy is a good sign. Based on these in vivo and in vitro studies, I conclude that fish oil supplementation is likely to be more beneficial for patients who already have developed a disease involving inflammation than for healthy individuals. Therefore I do not think that there is a need to increase the current recommendation of EPA and DHA intake to a higher level especially to those who are healthy.

6.4 Study Limitations and Suggestions for Further Work

Based on the current studies, it is important to further examine whether the beneficial effects seen *in vitro* also occur *in vivo* and if so, what the minimum dose of EPA or DHA required to induce these effects is. One of the limitations of my *in vitro* experiments is sample size. Therefore, future work is warranted to increase the sample size to improve confidence in the findings. There is an opportunity to study more fatty acids than studied here. Moreover, further work can be carried out in order to investigate whether the effect of fatty acids on adhesion molecule expression is mediated via an effect on the NF-κB pathway or on other pathways. The precise underlying mechanisms remain to be clarified, and it has been proposed that these might involve PPAR, NF-κB, AP-1 and/or the eicosanoid system.

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