UNIVERSITY OF SOUTHAMPTON

The Effect of Dietary n-3 and n-6 PUFA Intake on Atheromatous Plaque Lipid Composition

Jennifer Mary Christina Garry BSc (Hons)

Doctor of Philiosophy

Institute of Human Nutrition

May 2001

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE, HEALTH AND BIOLOGICAL SCIENCES

INSTITUTE OF HUMAN NUTRITION

Doctor of Philosophy

EFFECT OF DIETARY N-3 AND N-6 PUFA INTAKE PUFA INTAKE ON ATHEROMATOUS PLAQUE LIPID COMPOSITION

By Jennifer Mary Christina Garry

Cardiovascular diseases are the main cause of mortality in Western countries. The development of atheromatous plaques plays a key role in the progression of such diseases. The extent of lipid accumulation within the plaque is a major determinant of its stability and is a potent risk factor for myocardial infarction and stroke. Recent studies have indicated that the unsaturated fatty acid composition of lipid within the plaque may have an impact on its stability. The relationship between the fatty acid composition of dietary fat and of plaque lipid is unknown, although dietary intake influences the fatty acid composition of the lipoprotein pool from which plaque lipid is derived. The aims of this study were to determine the effects of moderate supplementation with n-3 and n-6 polyunsaturated fatty acids (PUFA) on the fatty acid profile and oxidisability of plasma low density lipoprotein (LDL) and of lipid fractions isolated from atherosclerotic plaques from patients undergoing carotid endarterectomy.

Patients (n = 54/group) were given placebo, sunflower oil (SO) or fish oil (FO) in capsules (6 g of oil/day) for at least two weeks (mean eight weeks) before undergoing surgery. The FO capsules provided 0.86 g eicosapentaenoic acid (EPA) and 0.5 g docosahexaenoic acid (DHA) per day. Blood samples were taken before and after supplementation. Copper-induced oxidation of isolated LDL (conjugated diene formation) and the fatty acid composition of lipid classes from LDL and from plaques were determined, as well as LDL and plasma triacylglycerol (TAG), cholesterol and lipid peroxide concentrations. The cell populations involved in the inflammatory activity in the carotid atherosclerotic lesions were characterised with cell specific monoclonal antibodies.

FO supplementation increased the amount of EPA and DHA in LDL and plaque, phospholipid (PL), TAG and cholesteryl ester (CE) fractions, and in adipose tissue. Supplementation with SO resulted in increased levels of linoleic acid in LDL PL and CE fractions. Plasma TAG concentration decreased by 29% and the lag time of copper induced-LDL oxidation was significantly decreased (-26%) after FO supplementation. Peroxide levels in LDL were not affected by any of the supplements.The incorporation of n-3 PUFA into plaques led to decreased levels of macrophages.

The results of this study show that a moderate supplementation of fish oil in patients with carotid atherosclerosis results in incorporation of the long chain n-3 PUFA (EPA and DHA) into LDL fractions, adipose tissue and atherosclerotic plaques. Plasma TAG concentrations were decreased but LDL was more susceptible to oxidation. However, the incorporation of n-3 PUFA into atherosclerotic plaques led to a lower macrophage content which may make these plaques more stable and less likely to rupture.

Table of Contents

1 GENERAL INTRODUCTION 15

1.1 FATTY ACIDS 15

1.1.1 Structure of fatty acids 15

1.1.2 Synthesis of polyunsaturated fatty acids 17

1.1.3 Fatty acids and the UK diet 20

1.1.4 Lipid transport and lipoprotein metabolism 22

1.1.4.1 Chylomicron metabolism: the exogenous pathway 23

1.1.4.2 VLDL and LDL metabolism 24

1.1.4.3 HDL metabolism 27

1.2 ATHEROSCLEROSIS 29

1.2.1 Morphology of atherosclerotic lesions 30

1.2.2 Pathogenesis of Atherosclerosis 34

1.3 RISK FACTORS FOR CARDIOVASCULAR DISEASE 40

1.3.1 Promoting factors 41

1.3.1.1 Hypercholesterolaemic fatty acids 41

1.3.1.2 Thrombogenic fatty acids 42

1.3.2 Protective factors 42

1.3.2.1 n-6 PUFA 42

1.3.2.2 MUFA 44

1.3.2.3 n-3 PUFA 45

1.3.2.4 Fibre and antioxidants 52

1.4 SUMMARY OF INTRODUCTION AND AIMS OF THIS STUDY 52

2 STUDY DESIGN AND CHARACTERISTICS OF PATIENTS 55

2.1 INTRODUCTION 55

2.1.1 Carotid atherosclerosis and carotid endarterectomy 55

2.1.2 Aims 56

2.2 METHODS 56

2.2.1 Patients and study design 56

2.2.2 Composition of capsules and assessment of dietary fatty acid intake 58

2.2.3 Measurement of plasma and LDL lipid concentrations 59

2.2.3.1 LDL Preparation 59

2.2.3.2 Total Cholesterol 60

2.2.3.3 HDL Total Cholesterol 62

2.2.3.4 Free Cholesterol 62

2.2.3.5 Esterified Cholesterol 64

2.2.3.6 Triacylglycerols 64

2.2.4 *Adipose tissue fatty acid composition* *66*
2.2.5 *Statistical analysis* *67*
2.3 RESULTS 68
2.3.1 *Patient characteristics* *68*
2.3.2 *Nutrient intakes of patients* *69*
2.3.2.1 Adipose tissue fatty acid composition 73
2.3.3 *Plasma, LDL and HDL lipid concentrations* *76*
2.3.3.1 Plasma lipid concentrations 76
2.3.3.2 LDL and HDL lipid concentrations 78
2.4 DISCUSSION 80
3 LDL OXIDISABILITY AND FATTY ACID COMPOSITION 87
3.1 INTRODUCTION 87
3.1.1 *LDL oxidation* *87*
3.1.2 *Lipid peroxidation* *87*
3.1.3 *Markers of lipid peroxidation* *89*
3.1.4 *PUFA and LDL oxidation* *90*
3.1.5 *Aims* *92*
3.2 METHODS 93
3.2.1 *Measurement of plasma and LDL lipid concentrations* *93*
3.2.2 *LDL Oxidation* *93*
3.2.2.1 Lipid Peroxides 93
3.2.2.2 Conjugated Diene Formation 94
3.2.3 *LDL fatty acid composition* *97*
3.2.4 *LDL Vitamin E content* *97*
3.3 RESULTS 99
3.3.1 *Treatment period* *99*
3.3.2 *Plasma Cholesterol and triacylglycerol concentrations* *99*
3.3.3 *LDL and HDL cholesterol and triacylglycerol concentrations* *102*
3.3.4 *Fatty acid composition of LDL* *104*
3.3.5 *LDL Oxidation* *113*
3.3.5.1 Conjugated Diene Formation 113
3.3.5.2 Lipid Peroxides 115
3.3.5.3 LDL Vitamin E Concentrations 116
3.4 DISCUSSION 118
3.4.1 *Plasma and LDL cholesterol and TAG* *118*
3.4.2 *LDL fatty acid composition* *120*
3.4.3 *LDL oxidation* *121*

4 PLAQUE AND ADIPOSE TISSUE FATTY ACID COMPOSITION AND MEASURES OF PLAQUE MORPHOLOGY ... 124

4.1 INTRODUCTION ... 124

4.1.1 Plaque lipid composition ... 124

4.1.1.1 Dietary PUFA and the composition of aortic plaques ... 124

4.1.1.2 Dietary EPA and DHA from fish oil and their incorporation into atherosclerotic plaques ... 126

4.1.2 Plaque lipid composition and morphology and how it affects plaque stability ... 127

4.1.3 Adipose tissue composition ... 129

4.1.4 Aims ... 130

4.2 METHODS ... 131

4.2.1 Collection of samples ... 131

4.2.2 Plaque fatty acid composition ... 131

4.2.3 Immunohistochemistry on plaque sections ... 132

4.2.4 Adipose tissue fatty acid composition ... 135

4.2.5 Statistical analysis ... 135

4.3 RESULTS ... 136

4.3.1 Plaque fatty acid composition ... 136

4.3.2 Immunohistochemistry on plaque sections ... 146

4.3.3 Adipose tissue fatty acid composition ... 152

4.4 DISCUSSION ... 155

4.4.1 Plaque fatty acid composition ... 155

4.4.2 Immunohistochemistry on plaques ... 157

4.4.3 Adipose tissue fatty acid composition ... 158

5 GENERAL DISCUSSION ... 160

6 REFERENCES ... 165

7 APPENDIX ... 193

Table of Figures

FIGURE 1.1: STRUCTURE OF LINOLEIC AND α-LINOLENIC ACIDS 17
FIGURE 1.2: METABOLIC PATHWAYS FOR THE SYNTHESIS OF PUFA 19
FIGURE 1.3 THE METABOLISM OF LIPOPROTEINS 26
FIGURE 1.4 HDL METABOLISM AND REVERSE CHOLESTEROL TRANSPORT 28
FIGURE 1.5 MORPHOLOGY OF ATHEROSCLEROTIC LESIONS 33
FIGURE 1.6: MULTIFACTORIAL HYPOTHESIS OF THE ORIGIN OF ATHEROSCLEROSIS 36
FIGURE 1.7 ENDOTHELIAL CELL DYSFUNCTION 39
FIGURE 1.8: PROSTANOIDS FORMED FROM DIFFERENT FATTY ACIDS 43
FIGURE 2.1 MICROSCOPIC IMAGE OF A LESION FROM A CAROTID ARTERY 55
FIGURE 2.2 STUDY PROTOCOL 57
FIGURE 2.3 RELATIONSHIP BETWEEN INTAKE OF 18:2N-6 AND THE PROPORTION OF 18:2N-6 IN ADIPOSE TISSUE 75
FIGURE 3.1 THE PROCESS OF LIPID PEROXIDATION 89
FIGURE 3.2 KINETICS OF OXIDATION OF LDL MEASURED BY CONTINUOUS MONITORING OF THE ABSORBANCE AT 234NM. THREE PHASES OF OXIDATION ARE EVIDENT LAG, PROPAGATION AND DECOMPOSITION 95
FIGURE 3.3 TIME-COURSE OF THE CONSUMPTION OF ANTIOXIDANTS AND FORMATION OF CONJUGATED DIENES DURING OXIDATION OF LDL (FROM ESTERBAUER *ET AL.*, 1989) 95
FIGURE 3.4 EFFECT OF LENGTH OF SUPPLEMENTATION WITH FO ON EPA ACCUMULATION IN LDL PL 107
FIGURE 3.5 EFFECT OF LENGTH OF SUPPLEMENTATION WITH FO ON DPA ACCUMULATION IN LDL PL 107
FIGURE 3.6 EFFECT OF LENGTH OF SUPPLEMENTATION WITH FO ON DHA ACCUMULATION IN LDL PL 108
FIGURE 3.7 EFFECT OF LENGTH OF SUPPLEMENTATION WITH FO ON EPA ACCUMULATION IN LDL TAG 110
FIGURE 3.8 EFFECT OF LENGTH OF SUPPLEMENTATION WITH FO ON DHA ACCUMULATION IN LDL TAG 110
FIGURE 3.9 EFFECT OF LENGTH OF SUPPLEMENTATION WITH FO ON EPA ACCUMULATION IN LDL CE 112
FIGURE 3.10 EFFECT OF LENGTH OF SUPPLEMENTATION WITH FO ON DHA ACCUMULATION IN LDL CE 112
FIGURE 4.1 EFFECT OF LENGTH OF SUPPLEMENTATION WITH FO ON THE PROPORTION OF EPA IN PLAQUE CE 140
FIGURE 4.2 EFFECT OF LENGTH OF SUPPLEMENTATION WITH FO ON THE PROPORTION OF DHA IN PLAQUE CE 140
FIGURE 4.3 RELATIONSHIP BETWEEN EPA IN PLAQUE CE AND LDL CE 141
FIGURE 4.4 EFFECT OF LENGTH OF SUPPLEMENTATION WITH FO ON THE PROPORTION OF EPA IN PLAQUE PL 143
FIGURE 4.5 EFFECT OF LENGTH OF SUPPLEMENTATION WITH FO ON THE PROPORTION OF DHA IN PLAQUE PL 143
FIGURE 4.6 EFFECT OF LENGTH OF SUPPLEMENTATION WITH FO ON THE PROPORTION OF EPA IN PLAQUETAG 145

Figure 4.7 Effect of length of supplementation with FO on the proportion of DHA in plaque TAG 145
Figure 4.8 Microscopic image of CD3 T-cell staining on a carotid plaque 149
Figure 4.9 Microscopic image of VCAM-1 staining on a carotid plaque 149
Figure 4.10 Microscopic image of ICAM-1 staining on a carotid plaque 150
Figure 4.11 Microscopic image of KP-1 macrophage staining on a carotid plaque 150
Figure 4.12 Microscopic image of PGM1 macrophage staining on a carotid plaque 151
Figure 4.13 Effect of length of supplementation with FO on EPA accumulation in adipose tissue 154
Figure 4.14 Effect of length of supplementation with FO on DHA accumulation in adipose tissue 154

Table of Tables

TABLE 1.1: SOME COMMONLY FOUND FATTY ACIDS AND THEIR NOMENCLATURE 16
TABLE 1.2 : THE ACTUAL AND RECOMMENDED FATTY ACID INTAKES OF THE UK POPULATION........ 21
TABLE 1.3: CHARACTERISTICS OF THE MAJOR LIPOPROTEIN CLASSES 23
TABLE 1.4: EFFECTS OF N-3 PUFA ON BLOOD LIPID CONCENTRATIONS........ 49
TABLE 1.5: KEY RANDOMISED TRIALS AND EPIDEMIOLOGICAL STUDIES ON N-3 PUFA AND CARDIOVASCULAR DISEASE 51
TABLE 2.1 FATTY ACID COMPOSITION OF CAPSULES USED AND THE AVERAGE UK DIET........ 59
TABLE 2.2 INTRA-ASSAY VARIABILITY OF THE TOTAL CHOLESTEROL ASSAY 61
TABLE 2.3 INTRA-ASSAY VARIABILITY OF THE FREE CHOLESTEROL ASSAY........ 64
TABLE 2.4: INTRA-ASSAY VARIABILITY OF THE TRIGLYCERIDE ASSAY 66
TABLE 2.5 CHARACTERISTICS OF PATIENTS BY TREATMENT GROUP........ 68
TABLE 2.6 BMI DISTRIBUTIONS BY TREATMENT GROUP 69
TABLE 2.7 DAILY ENERGY AND NUTRIENT INTAKES OF PATIENTS BY TREATMENT GROUP 70
TABLE 2.8: FATTY ACID INTAKES OF PATIENTS BY TREATMENT GROUP (G/DAY)........ 71
TABLE 2.9: FATTY ACID INTAKES OF PATIENTS BY TREATMENT GROUP (% ENERGY)........ 72
TABLE 2.10 FATTY ACID COMPOSITION OF ADIPOSE TISSUE FROM PATIENTS IN THE DIFFERENT DIETARY GROUPS BEFORE SUPPLEMENTATION........ 73
TABLE 2.11 RELATIONSHIP BETWEEN ADIPOSE TISSUE FATTY ACID COMPOSITION, ... 75
TABLE 2.12: PLASMA LIPID CONCENTRATIONS IN THE DIFFERENT TREATMENT GROUPS BEFORE SUPPLEMENTATION 76
TABLE 2.13 PLASMA LIPID CONCENTRATIONS IN THE DIFFERENT TREATMENT GROUPS ACCORDING TO STATIN TREATMENT BEFORE SUPPLEMENTATION 77
TABLE 2.14: LDL AND HDL LIPID CONCENTRATIONS IN THE DIFFERENT TREATMENT GROUPS BEFORE SUPPLEMENTATION 78
TABLE 2.15 LDL AND HDL LIPID CONCENTRATIONS IN THE DIFFERENT TREATMENT GROUPS ACCORDING TO STATIN TREATMENT BEFORE SUPPLEMENTATION 79
TABLE 3.1: INTRA-ASSAY VARIABILITY OF CU-INDUCED OXIDATION OF LDL 96
TABLE 3.2 PLASMA CHOLESTEROL AND TRIACYLGLYCEROL CONCENTRATIONS OF PATIENTS IN THE DIFFERENT TREATMENT GROUPS BEFORE AND AFTER SUPPLEMENTATION 100
TABLE 3.3 THE EFFECT OF GENDER ON PLASMA TAG CONCENTRATIONS IN THE FISH OIL GROUP 100
TABLE 3.4 PLASMA CHOLESTEROL AND TRIACYLGLYCEROL CONCENTRATIONS IN THE DIFFERENT TREATMENT GROUPS ACCORDING TO STATIN TREATMENT BEFORE AND AFTER SUPPLEMENTATION 101
TABLE 3.5 LDL AND HDL CHOLESTEROL AND TRIACYLGLYCEROL CONCENTRATIONS OF PATIENTS IN THE DIFFERENT TREATMENT GROUPS BEFORE AND AFTER SUPPLEMENTATION 102
TABLE 3.6 LDL AND HDL CHOLESTEROL AND TAG CONCENTRATIONS IN THE DIFFERENT TREATMENT GROUPS ACCORDING TO STATIN TREATMENT BEFORE AND AFTER SUPPLEMENTATION 103

Table 3.7 Fatty acid composition of LDL phospholipids of patients in the different treatment groups before and after supplementation 106
Table 3.8 Fatty acid composition of LDL TAG of patients in the different treatment groups before and after supplementation 109
Table 3.9 Fatty acid composition of LDL CE of patients in the different treatment groups before and after supplementation 111
Table 3.10 Oxidisability of LDL from patients in the different treatment groups before and after supplementation 113
Table 3.11 Lag time of copper-induced LDL oxidation with and without addition of α-tocopherol 114
Table 3.12 Lipid peroxide concentrations in LDL from patients in the different treatment groups before and after supplementation 115
Table 3.13 Vitamin E concentrations in LDL from patients in the different treatment groups before and after supplementation 116
Table 3.14 LDL CE PUFA content and Vitamin E concentrations from patients in the different treatment groups before and after supplementation 117
Table 3.15 LDL PL PUFA content and Vitamin E concentrations from patients in the different treatment groups before and after supplementation 117
Table 4.1 Description of primary and secondary antibodies used 134
Table 4.2 Fatty acid composition of plaque CE of patients in the different treatment groups 139
Table 4.3 Fatty acid composition of plaque PL of patients in the different treatment groups 142
Table 4.4 Fatty acid composition of plaque TAG of patients in the different treatment groups 144
Table 4.5 Immunohistochemistry scores 148
Table 4.6 Fatty acid composition of adipose tissue from patients in the different treatment groups before and after supplementation 153

Acknowledgements

The work described in this thesis was supported by MAFF. I would like to thank everybody involved in this project especially Professor Bob Grimble for co-ordinating the project, Mr Cliff Sherman, Jenny Williams, Kittipan Rerkasem and Adhisabandh Chulakadabba for recruiting the patients and collecting the samples. I would also like to thank all the volunteers who took part in this study as without them none of this work would have been possible.

I would like to thank my supervisor Dr. Philip Calder for giving me the opportunity to carry out this work and for his unfailing support. I am also very grateful to Dr Parveen Yaqoob for teaching me several of the techniques necessary to carry out the analysis, to Dr Frank Thies for all his help with the analysis, to Dr Patrick Gallagher for his advice on the histochemical analysis of the plaques, to Ron for his help with the sectioning of plaques and to Dr Chris van der Loos for allowing me to spend time in his laboratory learning immunohistochemical techniques.

I would like to thank the many friends I made in Southampton especially Ria Banerjee, Sam Kew, Drs Fiona Wallace, Liz Miles and Chris Donnellan, for all their help and encouragement along the way. My thanks also to all the Monte and Stoneham Wardens.

Finally, I would like to thank my family for all their support. To Aiveen, Marian and Caitriona for always being there at the end of the phone and to Clare for having a parallel life. However my biggest thank you has been saved for Paul. For giving me the strength and courage to believe in myself. For your endless support and help and for making my life a lot more tolerable over the last few months. I could not have got here without you.Thank you.

Abbreviations

AA	Arachidonic acid
AAP	Aminoantipyrine
ACAT	Acyl:CoA-cholesterol acyltransferase
ADP	Adenosine-5-diphosphate
Apo	Apolipoprotein
ATP	Adenosine triphosphate
BMI	Body mass index
C	Control oil
CE	Cholesteryl ester
CETP	Cholesteryl ester-transfer protein
CHD	Coronary heart disease
CVD	Cerebrovascular disease
DHA	Docosahexaenoic acid
DHLA	Dihomo-γ-linolenic acid
DPA	Docosapentaenoic acid
ELAMS	Endothelial leukocyte adhesion molecules
EPA	Eicosapentaenoic acid
ESPA	Sodium N-ethyl-N-(3-sulfopropyl)m-anisidine
FC	Free cholesterol
FID	Flame ionising detector
FO	Fish oil
GC	Gas chromatography
GK	Glycerol kinase
GPO	Glycerol phosphate oxidase
H_2O_2	Hydrogen peroxide
HDL	High density lipoprotein
HMG-CoA	Hydroxymethylglutaryl Coenzyme A
ICAM-1	Intercellular adhesion molecule-1
IL-1	Interleukin-1
LCAT	Lecithin-cholesterol acyltransferase

LDL	Low density lpoprotein
Lp(a)	Lipoprotein (a)
LPL	Lipoprotein lipase
MCSF	Macrophage colony stimulating factor
MDA	Malondialdehyde
MUFA	Monounsaturated fatty acid
NGS	Normal goat serum
Ox-LDL	Oxidised LDL
PGI_2	Prostacyclin I_2
PL	Phospholipid
PUFA	Polyunsaturated fatty acid
PVD	Peripheral vascular disease
SFA	Saturated fatty acid
SO	Sunflower oil
TAG	Triacylglycerol
TBARS	Thiobarbituric acid-reactive substances
TC	Total cholesterol
T-cells	T-lymphocytes
TLC	Thin-layer chromatography
TNFα	Tumour necrosis factor α
TXA_2	Thromboxane A_2
VCAM-1	Vascular cell adhesion molecule-1
VLDL	Very low density lipoprotein

Chapter 1
General Introduction

1 General Introduction

1.1 Fatty acids

1.1.1 Structure of fatty acids

The most important component of dietary fat is triacylglycerol (TAG) and in the United Kingdom TAG typically represents >95% of dietary fat. Dietary fat also contains phospholipids, glycolipids, sterols and their esters and fat-soluble vitamins. Each triacylglycerol molecule is composed of three fatty acids attached to a glycerol backbone; phospholipids, glycolipids and sterol esters also include fatty acids in their structure. Thus, fatty acids are an important component of the diet. Fatty acids are hydrocarbon chains of alternating -CH_2- units with a methyl group at one end and a carboxyl group at the other; the carboxyl group is esterified to the glycerol backbone in triacylglycerols, phospholipids and glycolipids and to cholesterol in cholesterol esters. Saturated fatty acids (SFA) are the most abundant fatty acids in the typical Western diet (see section 1.1.3) and these have straight chains with an even number of carbon atoms. Fatty acids can contain double bonds these and are termed unsaturated fatty acids. The number and position of the double bonds differs among different unsaturated fatty acids; if there are two or more double bonds the fatty acid is termed a polyunsaturated fatty acid (PUFA).

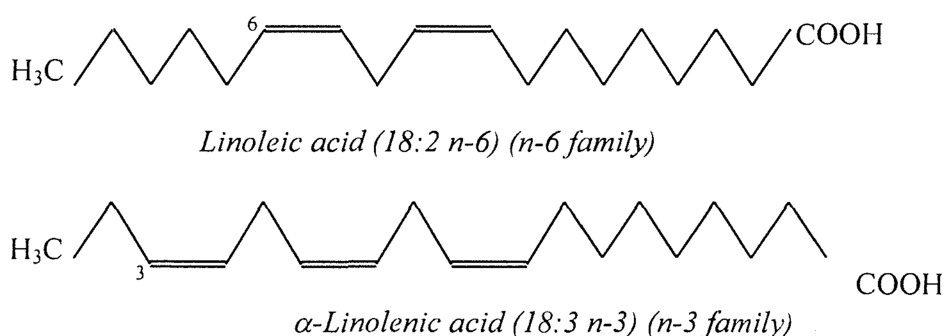
Fatty acids have systematic names but most also have common names and are described by a shorthand nomenclature (Table 1.1). This indicates the number of carbon atoms in the chain, the number of double bonds and the position of the first double bond from the methyl terminus of the chain (Figure 1.1). The position of the first double bond in the hydrocarbon chain is indicated by the n-7, n-9, n-6 or n-3 part of the shorthand notation. Therefore an n-3 fatty acid has the first double bond on carbon 3 counted from the methyl terminus and an n-6 fatty acid has the first double bond on carbon number 6 from the methyl terminus (Figure 1.1).

Table 1.1: Some commonly found fatty acids and their nomenclature

Systematic name	Common name	Shorthand
Saturated fatty acids (SFA)		
Octanoic	Caprylic	8:0
Decanoic	Capric	10:0
Dodecanoic	Lauric	12:0
Tetradecanoic	Myristic	14:0
Hexadecanoic	Palmitic	16:0
Octadecanoic	Stearic	18:0
Monounsaturated fatty acids (MUFA)		
9-Hexadecenoic	Palmitoleic	16:1n-7
9-Octadecenoic	Oleic	18:1n-9
trans 9-Octadecenoic	Elaidic	18:1n-9
11-Eicosaenoic	Gadoleic	20:1n-9
13-Docasenoic	Erucic	22:1n-9
15-Tetracosaenoic	Nervonic	24:1n-9
Polyunsaturated fatty acids (PUFA)		
9,12-Octadecadienoic	Linoleic	18:2n-6
9,12,15-Octadecatroenoic	α-Linolenic	18:3n-3
6,9,12-Octadecatrienoic	γ-Linolenic	18:3n-6
11,14,17-Eicosatrienoic	Mead	20:3n-9
8,11,14,17-Eicosatetraenoic	Dihomo-γ-linolenic	20:3n-6
5,8,11,14-Eicosatetraenoic	Arachidonic	20:4n-6
5,8,11,14,17-Eicosapentaenoic	Eicosapentaenoic	20:5n-3
4,7,10,13,16-Docosapentaenoic	Docosapentaenoic	22:5n-3
4,7,10,13,16,19-Docosahexaenoic	Docosahexaenoic	22:6n-3

All double bonds are in the cis configuration unless otherwise stated.

Figure 1.1: Structure of linoleic and α-linolenic acids



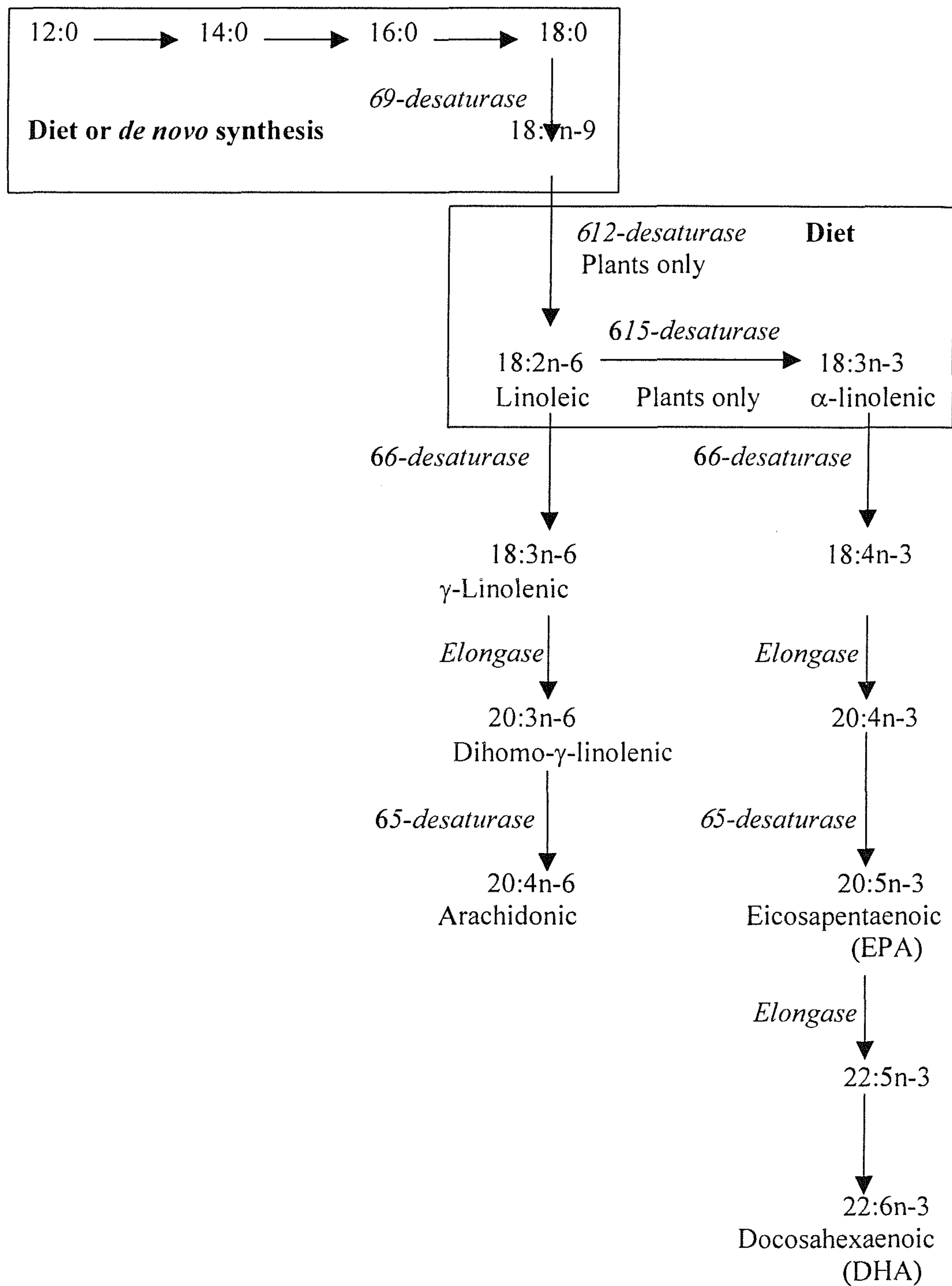
1.1.2 Synthesis of polyunsaturated fatty acids

Mammals can synthesise saturated fatty acids (SFA; Table 1.1) *de novo* from simple precursors such as glucose and amino acids. Monounsaturated fatty acids (MUFA; table 1.1) such as oleic acid can also be synthesised *de novo* in mammals, due to the presence of the 69-desaturase enzyme (Figure 1.2). Liver and adipose tissue are the main sites of *de novo* fatty acid synthesis. However, in Western countries there is little requirement for synthesis of SFA and MUFA as they are supplied in excess by the diet. Mammals cannot insert double bonds between carbon number 9 and the methyl terminus of oleic acid. Thus, mammals cannot convert oleic acid (18:1n-9) into linoleic acid (18:2n-6). The enzyme which does this is called 612-desaturase and this is found only in plants. Likewise, mammals cannot convert linoleic acid into α-linolenic acid (18:3n-3). The enzyme which does this is called 615-desaturase and again this is found only in plants. In mammals linoleic and α-linolenic acids are required for normal growth and physiological integrity and therefore must be supplied by the diet and are thus termed 'essential' fatty acids. Also, because mammalian tissues do not contain the 615-desaturase they cannot interconvert n-6 and n-3 fatty acids.

Four distinct families of unsaturated fatty acids result from the process of desaturation, the n-6 and n-3 families are shown in Figure 1.2. Members of each family compete for the desaturase and elongase enzymes and therefore one family influences the metabolism of other families. Substrates which are members of different families compete

for the 66-desaturase, but this enzyme has different affinities for different substrates (α-linolenic acid > linoleic acid > oleic acid) (Hrelia *et al.*, 1990). However, because the Western diet contains more linoleic acid than α-linolenic acid (Table 1.2) the most important conversion in quantitative terms is that involving the n-6 family, which results in the conversion of linoleic acid into arachidonic acid (Figure 1.2). This probably accounts for the high proportion of arachidonic acid in human tissues. Plant tissues and plant oils tend to be rich sources of linoleic acid and α-linolenic acid. For example, linoleic acid contributes over 50% and often up to 80% of the fatty acids found in corn, sunflower, safflower and soybean oils. Rapeseed and soybean oils are also good sources of α-linolenic acid and this fatty acid contributes between 5 and 15% of the fatty acids present. However, the richest source of α-linolenic acid is linseed oil, and this fatty acid can contribute as much as 60% of the fatty acids present. If the dietary intake of linoleic acid is low, other C18 substrates are able to compete effectively for the 66-desaturase, especially oleic acid if it is abundant. Desaturation of oleic acid by 66-desaturase and the subsequent elongation and desaturation of 18:2n-9 results in the formation of mead acid (Table 1.1). This is believed to be an indicator of essential fatty acid deficiency. Using the same pathway (Figure 1.2) dietary α-linolenic acid can be converted into EPA and DHA. Alpha-linolenic acid (18:3 n-3) is the principal PUFA that occurs in the green tissue of plants. These fatty acids therefore occur in the meat of animals which eat plants, though in ruminants the amounts are diminished by hydrogenation in the rumen. Marine plants, in particular unicellular algae and phytoplakton, contain high activities of the enzymes enabling them to carry out chain elongation and desaturation of α-linolenic acid to yield EPA and DHA. These very long chain n-3 PUFA are then transferred through the food chain to the tissues of some marine mammals (e.g. whales, seals) and fish (e.g. herring, mackerel, tuna: known as oily fish). Therefore the only concentrated edible sources of these PUFA are oily fish and the oils derived from them.

Figure 1.2: Metabolic pathways for the synthesis of PUFA



1.1.3 Fatty acids and the UK diet

The average intake of fat in Western countries is 75 to 150 g per day and fat generally contributes 35 to 45% of dietary energy (Table 1.2; Department of Health, 1991). The major fatty acids in the diet of adults in the United Kingdom are medium and long chain SFA, especially myristic, palmitic and stearic, the MUFA oleic and the PUFA linoleic and α-linolenic (Table 1.2). According to the Adult Survey (Gregory *et al.*, 1990) the daily diet of the average adult male in the United Kingdom contains 42 g SFA, 31 g MUFA and 15.8 g PUFA. The main PUFA in the diet are linoleic acid (intake is approximately 13.8 g/day for adult males) and α-linolenic acid (about 2 g/day). Six per cent of dietary energy in the diet of the UK population comes from n-6 PUFA and 0.7% comes from n-3 PUFA. Since 1970 the absolute consumption of SFA in the United Kingdom has declined by 40%, while the consumption of MUFA has declined by 30%. The consumption of PUFA increased by 25% over this period of time (Department of Health, 1991). This was largely due to increased consumption of linoleic acid which became generally available in margarines and cooking oils. Total PUFA intake increased from 5% of dietary energy in 1980 to 7% in 1992 (Department of Health, 1994) and the long-term safety of diets rich in n-6 PUFA has been questioned (Ulbricht and Southgate, 1991). An estimate of the change in intake of n-3 fatty acids for the population as a whole over the past few years is available from the Total Diet Surveys for 1991 and 1995 (Ministry of Agriculture Fisheries and Food, 1997a). In 1991, total n-3 fatty acid intake was 1.61 g per day. By 1995, this had risen to 1.8 g per day. During this same period, total intake of n-6 fatty acids fell from 10.66 g per day to 10.2 g per day. One current recommendations for dietary PUFA is as follows: linoleic acid should supply 6% of energy, α-linolenic acid 1% of energy, long chain n-3 PUFA 0.5% energy and total PUFA intake should be 7.5% of dietary energy (Table 1.2; British Nutrition Foundation, 1992 ,1999).

Table 1.2 : The actual and recommended fatty acid intakes of the UK population

	Average intake		Recommended intake (1991)		Recommended intake (1999)	
	% Energy	g/day (men)	% Energy	g/day (men)	% Energy	g/day (men)
SFA	15.4	39	11	27	-	-
MUFA	11.6	28	13	33	-	-
Total PUFA	5.8	15	6.5	15	7.5	19
n-6 PUFA	5.1	13	6	15	6	15
n-3 PUFA	0.8	2	-	-	1.0	3
Very long chain n-3 PUFA	<0.1	0.25	-	-	0.5	1
Total fat	Approx. 40	100	35	88	35	88

Data are from the British Nutrition Foundation (1992) (based on Department of Health, 1991) and the British Nutrition Foundation (1999)

1.1.4 Lipid transport and lipoprotein metabolism

Non-esterified fatty acids are carried in the bloodstream bound to albumin. In contrast, the transport of both TAG and cholesterol occurs in specialised structures known as lipoproteins. Because TAG and cholesterol are carried by the same system, their metabolism is closely interrelated. Lipoproteins are particles with a highly hydrophobic core and a relatively hydrophilic outer surface. A typical lipoprotein particle contains a core of TAG and cholesteryl ester, with an outer layer of phospholipid and free cholesterol. Each particle has associated with it one or more protein molecules called apolipoproteins, which have hydrophobic domains, which 'dip into' the core and anchor the protein to the particle, and hydrophilic domains which are exposed at the surface.

Lipoproteins are separated into different classes based on the density at which they float during ultracentrifugation. These classes are further subdivided by particle size, chemical composition, physical properties and metabolic function, but are linked by the common role of lipid transport. The characteristics of the major lipoprotein fractions are listed in Table 1.3. Chylomicron and very-low-density lipoprotein (VLDL) particles are relatively rich in TAG and are often referred to as the TAG–rich lipoproteins; their main function is transporting TAG in the blood. The smaller low-density lipoprotein (LDL) and high-density lipoprotein (HDL) particles are more involved with the transport of cholesterol between the liver and extra-hepatic tissues.

Table 1.3: Characteristics of the major lipoprotein classes
(Based on Frayn, 1996)

Fraction	**Chylomicrons**	**VLDL**	**LDL**	**HDL**
Density range (g/ml)	<0.950	0.950-1.006	1.019-1.063	1.063-1.090
Diameter (nm)	80-1000	30-80	20-22	9-15
Major lipids	Dietary TAG	Endogenous TAG	Cholesterol and CE	CE and PL
Major apolipoproteins	B48, A1, A2, C, E	B100, C, E	B100	A1, A2, C, E
Protein (% weight)	1	10	20	50
TAG (% weight)	90	65	10	2
Cholesterol (% weight)	5	13	45	18
PL (% weight)	4	13	23	30

PL= phospholipid, CE= cholesteryl ester

1.1.4.1 Chylomicron metabolism: the exogenous pathway

The metabolism of chylomicrons is often called the exogenous pathway of lipoprotein metabolism. This pathway transports dietary fat and this is shown in Figure 1.3. TAG and cholesterol are absorbed and re-esterified in the enterocytes and are secreted as chylomicron particles, via the lymphatics, into the circulation. The newly secreted chylomicron particles consist of a core of cholesteryl ester and TAG with a surface of unesterified cholesterol and phospholipid and the apolipoproteins B48 and A1 (apo-B48 and apo-A1).

Once in the circulation chylomicrons acquire apo-C2 from HDL, which makes them substrates for lipoprotein lipase (LPL) as they pass through capillaries of tissues that express this enzyme, such as adipose tissue and muscle. The TAG is hydrolysed and the particles shrink as they pass through a number of capillary beds losing more TAG each time. Chylomicrons also lose some surface coat by dissociating some unesterified cholesterol, phospholipid and apolipoproteins which are taken up by other particles, such as HDL. The smaller remaining particles are called chylomicron remnants; they are enriched in cholesteryl ester since they have lost TAG. When they reach a certain size they become ligands for a receptor ($\alpha 2$ - macroglobulin) on hepatocytes. Thus, dietary TAG is delivered to the tissues, some unesterified cholesterol enters the HDL fraction and some cholesteryl ester is delivered in the remnant particles to the liver.

1.1.4.2 VLDL and LDL metabolism

1.1.4.2.1 VLDL metabolism: the endogenous pathway

The endogenous pathway of lipoprotein metabolism distributes TAG from the liver to other tissues and it is summarised in Figure 1.3 The liver secretes VLDL particles which contain TAG, cholesteryl ester, apo-B100 and small amounts of apo-C and E. Like all lipoproteins, VLDL have a surface coat of phospholipids and unesterified cholesterol.

VLDL particles are substrates for LPL in capillary beds and deliver TAG from the liver to other tissues, thus distributing lipid energy to the tissues. LPL hydrolyses the TAG core leading to redundant surface material which is passed to other particles especially HDL. The cholesteryl ester-enriched particles that result have two possible fates. Firstly, they may be taken up by a receptor on hepatocytes and other tissues, which binds a homologous region in apo-B100 and apo-E. This is called the LDL receptor, which thus serves to deliver cholesteryl ester to tissues. Secondly, these particles may remain in the circulation until they have lost all surface components except apo-B100, a shell of phospholipid and free cholesterol and have a core enriched in cholesteryl ester. These VLDL remnants are otherwise known as LDL.

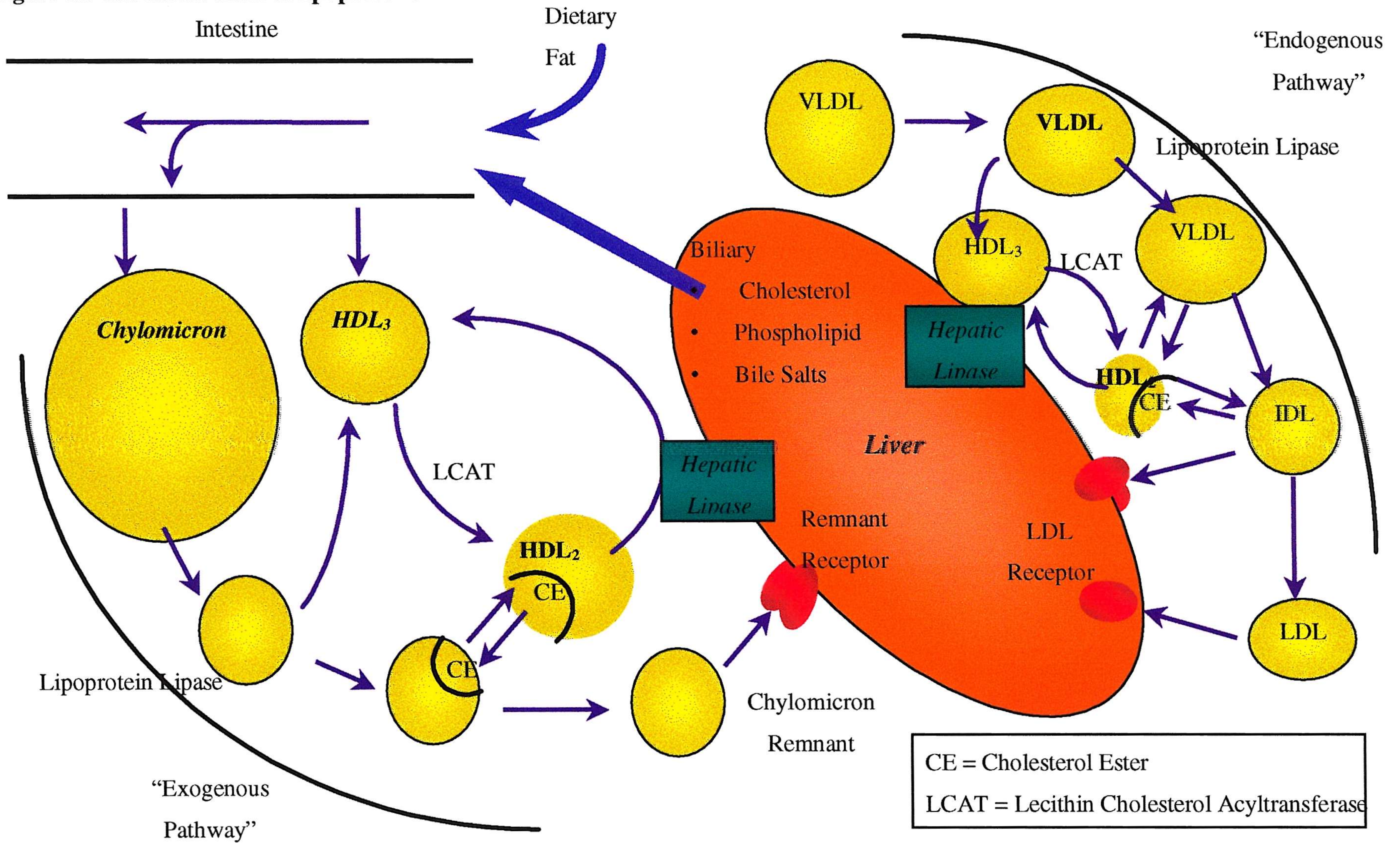
1.1.4.2.2 LDL metabolism and regulation of cellular cholesterol content

LDL particles have a relatively long half-life, about 3 days, in the circulation. They leave the circulation through uptake into tissues by the LDL receptor. This increases the cellular cholesterol content of these tissues and has two effects. Firstly, biosynthesis of cholesterol in these cells is suppressed by inhibition of the enzyme hydroxymethylglutaryl-CoA reductase (HMG-CoA reductase) (Brown & Goldstein, 1986). Secondly, the synthesis of new LDL receptors is suppressed and the number of receptors expressed on the cell surface is decreased (Goldstein & Brown, 1977). Therefore, the increase in cellular cholesterol content caused by the uptake of LDL cholesterol by the LDL receptor is self limiting.

Lipids present on LDL particles are prone to oxidation. This can ultimately result in the addition of an aldehyde group to lysine residues of apo-B, thus modifying its structure. This abolishes the ability of apo-B bind to the LDL receptor (Quinn *et al.*, 1987). The modified LDL is recognised and taken up by scavenger receptors on the surface of certain cells, especially macrophages on the arterial wall (Steinberg *et al.*, 1989; Witztum & Steinberg, 1991). These receptors are not regulated by intracellular cholesterol levels (Brown & Goldstein, 1983) and therefore, especially in people with a high LDL cholesterol concentration, the macrophages may become excessively cholesterol-laden. This may result in the development of 'foam cells' and ultimately atherosclerosis (see section 1.2).

There is a positive correlation between serum LDL levels and the risk of developing coronary heart disease, particularly atherosclerosis (Rose & Shipley, 1986). This correlation is particularly evident for a LDL sub-population known as Lp(a) (Scott, 1991). Lp(a) is smaller and more dense than other LDL particles, as it contains an additional protein to apo-B, apo-(a) (Groener & Kostner, 1987). Lp(a) concentrations were initially thought to be genetically predetermined and independent of diet, but the addition of trans-fatty acids (Mensink *et al.*, 1992) and certain SFA (i.e. lauric and myristic acids; Tholstrup *et al.*, 1995) to the diet has been shown to increase LP(a) concentrations by as much as 30%. Lp(a) concentrations may be decreased by fish oil supplementation (1.3.2.2.3).

Figure 1.3 The metabolism of lipoproteins



1.1.4.3 HDL metabolism

LDL particles regulate the cholesterol content of cells by delivering cholesterol to tissues, whereas HDL particles remove cholesterol which is then transported to the liver for excretion.

1.1.4.3.1 HDL and reverse cholesterol transport

HDL particles begin as discoidal HDL which are secreted by the liver and they consist mainly of phospholipid and apo-A1. They receive unesterified cholesterol during the action of LPL on TAG-rich lipoproteins. They also pick up unesterified cholesterol by interaction with cells, thereby removing cholesterol from cells. The unesterified cholesterol is esterified by the plasma enzyme lecithin-cholesterol acyltransferase (LCAT), which is activated by apo-A1. The particles then develop a core of hydrophobic cholesteryl esters and become spherical rather than discoidal. These large spherical cholesteryl ester-enriched particles are known as HDL_2 (Figure 1.4). Their cholesteryl ester can be taken up by hepatocytes in a number of ways:

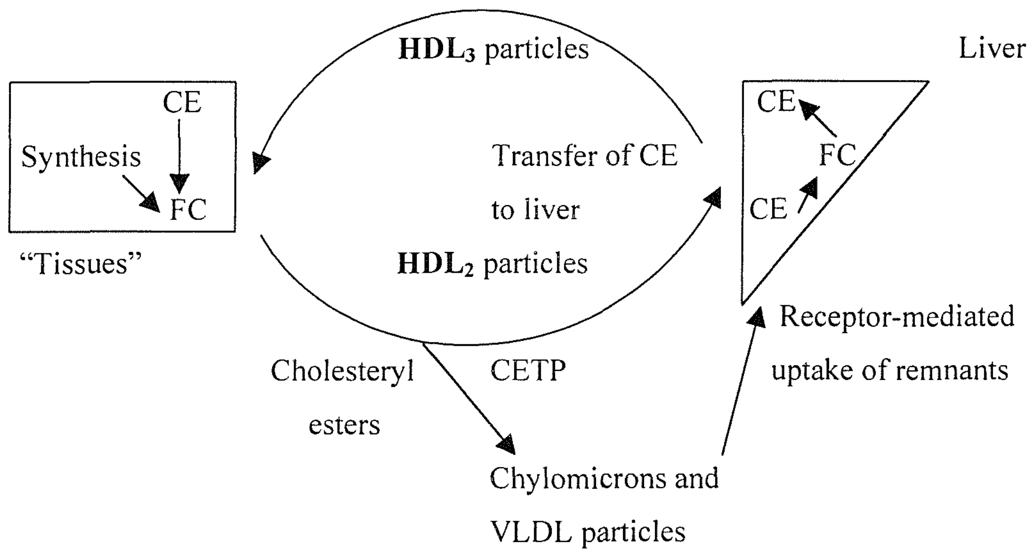
- receptor-mediated uptake of large HDL particles which contain apo-E
- uptake of cholesteryl ester from the larger HDL particles
- hydrolysis of the cholesteryl ester by hepatic lipase
- transfer of cholesteryl ester from larger HDL particles to TAG-rich lipoproteins

The smaller HDL particles that result are known as HDL_3 and are ready to accept more cholesterol from peripheral tissues. By these mechanisms cholesterol is transferred from peripheral tissues to the liver, and it can then be excreted as cholesterol and as bile salts in the bile. This process of removing cholesterol from tissues, transport to the liver and excretion from the body is the opposite of the delivery of cholesterol to tissues by LDL and is known as reverse cholesterol transport (Figure 1.4).

1.1.4.3.2 Cholesteryl ester-transfer protein

Cholesteryl ester-transfer protein (CETP) catalyses the exchange of hydrophobic lipids (i.e. cholesteryl esters and TAG) between lipoprotein particles by diffusion along concentration gradients. When plasma TAG concentration is high, for example after a meal, CETP catalyses the exchange of cholesteryl ester from HDL to chylomicrons, while TAG moves in the opposite direction. The cholesteryl esters remain with the chylomicron particle until it is taken up by the liver as a chylomicron remnant. HDL is now enriched with TAG and can be hydrolysed by hepatic lipase to give smaller cholesteryl ester-depleted HDL_3 particles which can then pick up more cholesterol from cells as described in the previous section (Figure 1.4).

Figure 1.4 HDL metabolism and reverse cholesterol transport



CE = cholesteryl esters, FC = free cholesterol

1.2 Atherosclerosis

The principal cause of mortality and morbidity in the Western World is cardiovascular disease and the underlying cause for most forms of cardiovascular disease is atherosclerosis. Atherosclerosis can affect any vascular bed: myocardial infarction (MI) and stable and unstable angina stem from coronary artery atherosclerosis while ischaemic stroke and peripheral vascular disease (PVD) are peripheral artery related. Coronary heart disease (CHD) and cerebrovascular disease (CVD) are in most instances due to obstruction of coronary or carotid vessels, respectively, by atherosclerosis or thrombosis singly or in combination. Atherosclerosis is the principal cause of heart attack, stroke and gangrene of the extremities and is responsible for 50% of all mortality in the USA, Europe and Japan (Ross, 1993). CHD accounts for 30% of all male deaths in the UK compared with 23% of all female deaths, whereas stroke accounts for 15% of all female deaths and 9% of male deaths (Office of Population Censuses and Surveys, 1982). The tendency for CHD to run in families has been recognised for some time. Several studies now show that familial aggregation of CHD is not exclusively mediated by classical risk factors; risk is related to a strong family history of CHD, as demonstrated in the Framingham study in which the incidence of MI in brothers was significantly related after effects of cholesterol, blood pressure and smoking had been controlled for (Ball, 1988).

There is much evidence to indicate that atherosclerosis is a multifactorial process, commencing during childhood (Stary, 1989) and becoming clinically manifest later in life. Atherosclerosis is characterised by the thickening and hardening of arteries due to accumulation of lipids, carbohydrates, blood products, fibrous tissue and calcium deposits within the sub-endothelial space i.e. plaque formation. The general scientific consensus is that the precursor stage of atherosclerotic plaques are the so-called "fatty streaks", these being macroscopically distinguishable, cushion-like, whiteish, sub-endothelial intimal areas that mainly comprise foam cells. The lesion is normally asymptomatic and may either revert to normal or progress to more severe stages of the disease (Fagiotto and Ross, 1984). The accumulation of monocytes and lipid-filled macrophages can be found in coronary arteries before the age of ten years and increases during adolescence. Juvenile fatty streaks occur in children of all societies, and are estimated to be present in half the population of children aged 10 to 14 (Stary, 1989), including those in which adult

symptoms of atherosclerosis are rare. The general consensus is that these lesions may progress to fibrous plaques during adulthood.

Significant advances have been made in understanding the mechanisms underlying this disease process, and there is considerable evidence to show that plaque composition is a major determinant of the onset and severity of acute coronary syndromes (Zaman *et al.*, 2000). The atherosclerotic process may be a protective response to insults to the endothelium and smooth muscle cells of the arterial wall and it consists of the formation of fatty and fibrous lesions preceded and accompanied by inflammation (Ross, 1993). This process is initiated and maintained by a number of growth factors and chemotactic agents released by the damaged endothelium, inflammatory cells and smooth muscle cells (Strong, 1992).

1.2.1 Morphology of atherosclerotic lesions

Atherosclerotic lesions begin to appear very early in life. Fatty streaks, the first alteration observed, are common findings in children and teenagers all over the world (Montenegro, 1999). Figure 1.5 (A) shows the histology of the inner aspect of the aorta in a 7-month-old child from Brazil, where several cells containing fat (stained red) are present. Figure 1.5 (B) shows the aorta of a teenager where several isolated or confluent red streaks are seen; these are fat deposits also stained red. In certain populations a few years later a second type of lesion, the fibrous plaque begins to appear and the size of these increases sharply with time. Figure 1.5 (C) is from the aorta of a middle-aged white American and shows white prominent fibrous plaques. These plaques (Figure 1.5 (D)) are made up of a loose centre containing abundant fat (stained red) covered by a fibrous cap. Figure 1.5 (E) shows an ulcerated and thrombosed, complicated lesion. Lesions, may also be complicated by calcification. Fatty streaks, fibrous plaques and complicated lesions may occur in large and mid-sized arteries such as the aorta, the carotids, iliacs and femurals, coronaries and cerebral arteries.

There are three main consequences of atherosclerosis. Firstly, the plaques protrude into the lumen of the vessel that becomes stenosed (i.e. narrowed). The amount of blood that passes through the stenotic areas may be adequate for the basal nutrition of the tissues supplied by the artery. However, as soon as there is an increase in demand, such as

during exercise, the flow becomes insufficient and symptoms may appear. For example, patients with certain types of angina pectoris feel severe chest pain during exercise and the pain is relieved either by the use of vasodilator drugs or by ending the exercise. A similar situation occurs when a leg artery is stenosed and the patient suffers severe cramps after walking a few meters; the pain disappears when the exercise is stopped. A second consequence of atherosclerosis is the sudden increase in the size of the plaque. This leads to acute severe reduction or obstruction of the lumen. In the heart the sudden reduction of the flow of blood leads to myocardial ischaemia and necrosis; that is a myocardial infarct (i.e. heart attack), which is one of the leading causes of disease and mortality in the modern world. The same occurs in the cerebral arteries causing cerebral infarcts (i.e. stroke). The third consequence of atherosclerosis is dilation of the arteries with the formation of aneurysms. Nutrition of the wall of large arteries, such as the aorta, depends partly on diffusion of nutrients from the blood through the intima. The thickening of the intima caused by atherosclerosis decreases diffusion and the undernourished media loses elasticity and the vessel dilates. In addition, the atherosclerotic plaques may grow into the media. Both of these factors lead to the formation of aneurysms (Figure 1.5 (F)) (Montenegro, 1999).

The fatty streak represents a balance of the entry and exit of lipoproteins into the intima and is accompanied by a local increase in the size of the extracellular matrix. If there is a decrease in the circulating concentration of lipoprotein as a result of diet or treatment, exit predominates and the lesions remain stable or may even regress. However, if the circulating concentration of lipoprotein increases, the entry of lipids predominates over exit, and so the plaque increases in size and this results in lipid-rich unstable plaques which are separated from the lumen by a cap of connective tissue i.e. the fibrous cap. This fibrous cap may be thick or thin with a variable content of inflammatory cells (Stary *et al.*, 1995). These differences in composition explain the variation in vulnerability to haemorrhage, erosion and rupture of the plaque, with the consequent thrombosis and partial or total acute obstruction. Thin caps found in lipid-rich plaques are much more vulnerable to rupture whereas thick caps are more resistant (Stary *et al.*, 1995). However, other factors such as the presence of inflammatory cells and the local release of their enzymes and cytokines may increase the vulnerability of plaques with thick fibrous caps

to rupture. In the past it was thought that atherosclerotic lesions were relatively stable. However, now it is known that they are dynamic lesions that grow when excess lipid increases, regress when the level decreases and complicate as a result of erosion, haemorrhage or rupture (Fuster *et al.*, 1999).

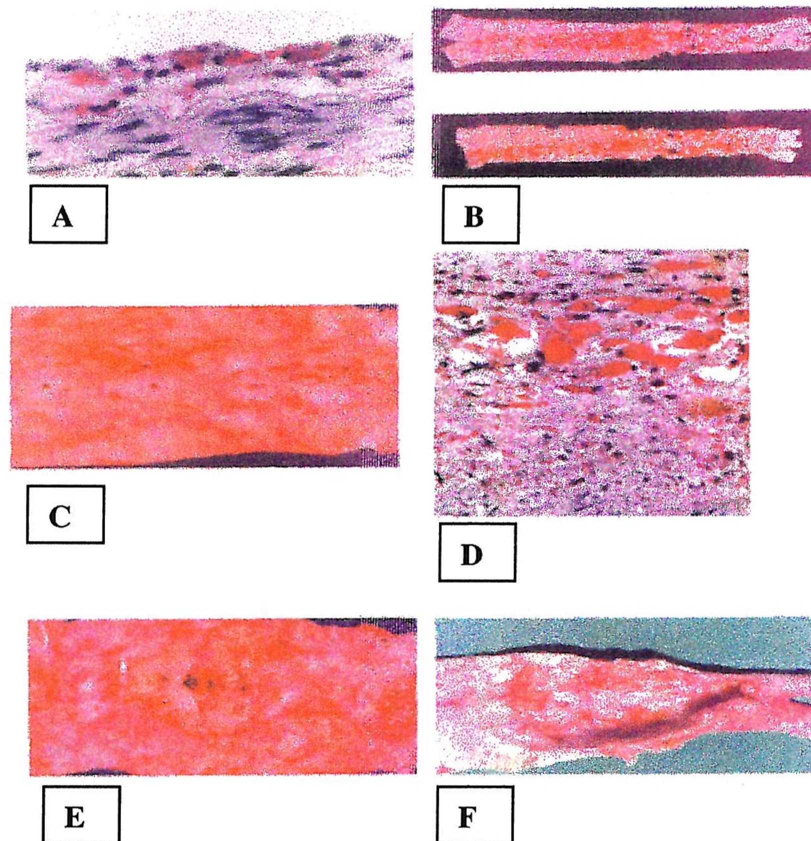


Figure 1.5 Morphology of atherosclerotic lesions

(from Montenegro, 1999). Picture (A) shows the aorta of a 7-month old child with numerous fat-ladden (red) macrophages in a slightly thickened intima. Picture (B) shows aortas of young teenagers with isolated and confluent red fatty streaks. Pictire (C) is the aorta of a middle aged man with several white fibrous plaques. The surface between the plaques is stained red (fat). Picture (D) is the aorta of an old adult with a fibrous plaque, there are several red (fat) macrophages in the center. Picture (E) is the aorta of a middle-aged man. It contains several fibrous plaques and in the center there is a complicated lesion which is ulcerated and thrombosed. Picture (F) is the aorta of an old adult. The aorta is dilated (aneurysm) and the inner surface covered by atherosclerotic fibrous plaques, some of them covered with mural thrombi.

1.2.2 Pathogenesis of Atherosclerosis

Two major hypotheses for the origin of atherosclerosis - the thrombogenic and lipid - were postulated in the nineteenth century (von Rokitansky, 1852). The thrombogenic hypothesis proposed that the organisation of fibrin by fibroblasts, associated with secondary lipid enrichment, leads to intimal thickening. This hypothesis has been reinforced by several authors demonstrating that platelet activation on dysfunctional or injured endothelium triggers the migration and proliferation of smooth muscle cells (Ross, 1993) and the presence of fibrin in atherosclerotic plaques (Bini *et al.*, 1989). The lipid hypothesis proposes that lipid accumulation within arterial walls is the consequence of an increased concentration of plasma lipids resulting from an imbalance between the mechanisms responsible for lipid deposition and removal (Anitschow & Chalatow, 1913). This is supported by experiments demonstrating the ability to induce atherosclerotic-like lesions in several animal species (fed cholesterol-rich diets) and the correlation between high plasma lipid concentrations, especially cholesterol, and incidence of atherosclerotic disease in humans (Rose & Shipley, 1986).

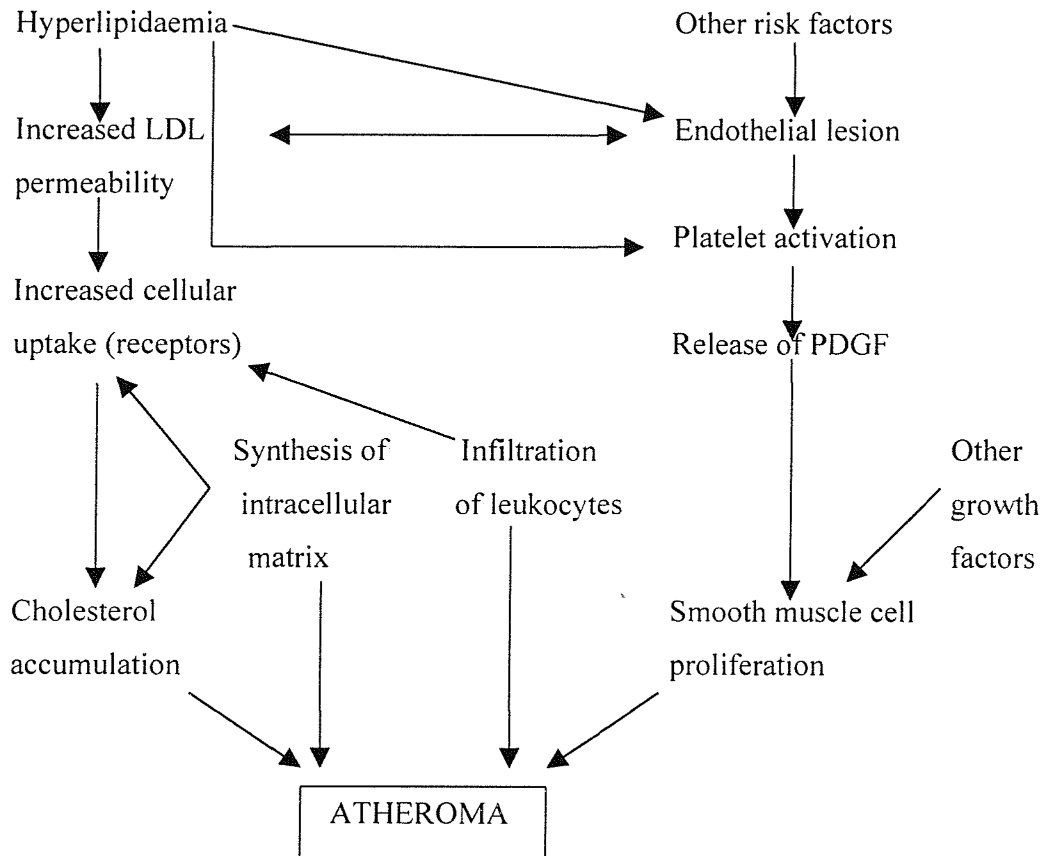
LDL that has been modified by oxidation has been implicated in the development of atherosclerosis. Modification can also be by acetylation or glycosylation. LDL can be modified either in the bloodstream or during the transgression process through the endothelial wall. Once in the subendothelial space, modified LDL acts as a chemoattractant for monocytes that adhere to the endothelium, migrate into the subendothelial space and become macrophages that transform into foam cells upon uptake of modified LDL (Fagiotto and Ross, 1984); these cells constitute the 'fatty streak'. Macrophages may become trapped inside the vascular endothelium and release chemotactic substances, including platelet activating factor, that attract other white cells and platelets. These cells interact with the vascular wall and stimulate the production of growth factors that lead to hyperplasia and migration of smooth muscle cells which leads to thickening of the intimal wall (Ross, 1986).

Modified LDL is taken up by endothelial cells, macrophages and smooth muscle cells, all of which possess the LDL scavenger receptor that exclusively binds modified LDL (Wick *et al.*, 1995). Foam cell formation is also associated with the inhibition of nitric oxide release from endothelial cells. Decreased nitric oxide release leads to reduced

smooth muscle cell relaxation and increased vasoconstriction. Attention has been focused on lipid oxidation as a major mechanism for the modification of LDL since the discovery that LDL incubated with arterial endothelial cells was oxidatively modified and was no longer recognised by the LDL receptor but was bound by the scavenger receptor on macrophages (Quinn *et al.*, 1987). These oxidative changes only seemed to occur if the medium contained trace amounts of transition metal ions. The most likely candidates for lipid oxidation would be the polyunsaturated fatty acids (PUFA) present in the TAG, phospholipid and cholesteryl ester fraction of the lipoprotein.

These two hypotheses can be integrated into a single multifactorial hypothesis involving a single step, that is endothelial dysfunction, to trigger successive events leading to atherosclerotic lesions (Figure 1.6) (Fuster *et al.*, 1992, 1994; Badimon *et al.*, 1993). A normal functioning endothelium is essential in limiting the development of atherosclerosis. This is illustrated by the strong correlation between risk factors for atherosclerosis (smoking, hyperlipidaemia, hypertension, obesity, diabetes, infection / inflammation) and endothelial dysfunction. The endothelium has a critical role in vascular haemostasis. It modulates the permeability of plasma lipoproteins, adhesion of leukocytes, and release of prothrombotic and antithrombotic factors, growth factors and vasoactive substances (Vanhoutte, 1989; Rubanyi, 1993). Alteration of these functions is believed to play a central role in the development of atherosclerosis (Ross, 1993). Endothelial cells are exposed to three types of mechanical forces: pressure from the hydrostatic forces of blood within the vessel, circumferential stress resulting from vasomotion of the vessel and shear stress resulting from blood flow within the vessel (Traub and Berk, 1998). There is a strong correlation between endothelial cell dysfunction and areas of low shear stress and oscillatory flow with flow reversal (Traub and Berk, 1998). At branch points of the arterial tree where flow reversal and low shear stress are present there is an increased incidence of atherosclerotic lesion development (Ku *et al.*, 1985). These sites have increased uptake of lipoproteins, presence of leukocyte adhesion molecules, and secretion of growth factors and chemotactic factors which cause proliferation of monocyte/macrophages and smooth muscle cells (Traub and Berk, 1998).

Figure 1.6: Multifactorial hypothesis of the origin of atherosclerosis
(modified from Zaman *et al.*, 2000)



Injury to the arterial endothelium is induced in various ways such as hypercholesterolaemia, mechanical stress by high blood pressure, free radical production and smoking (Ross, 1993). Endothelial damage has been shown to increase the expression of endothelial leukocyte adhesion molecules (ELAMS) (Chobanian, 1992, Van der Wal *et al.,* 1992, Wood *et al.,* 1993). These glycoproteins provide a site of attachment for macrophages and T lymphocytes. Once attached, the leukocytes migrate between the endothelial cells, under the influence of chemoattractants released by the endothelium, leukocytes or smooth muscle cells (Ross and Agius, 1992, Collins and Fox, 1992, Kisanuki *et al.,* 1992, Clark, 1992). Simultaneous changes in endothelial permeability allow LDL and other lipids to reach a sub-endothelial position. The macrophages then ingest large quantities of lipid/LDL, become foam cells and together with the T

lymphocytes form the fatty streak. The fatty streak can then progress to an intermediate fibrofatty lesion and ultimately to a fibrous plaque. As the lesions accumulate more cells and the macrophages scavenge the lipid, some of the lipid-laden macrophages may emigrate back into the bloodstream by pushing apart the endothelial cells. On doing so those sites such as branches and bifurcations of the blood vessel, may become thrombogenic sites that lead to formation of platelet mural thrombi. Such thrombi can result in release of many potent growth-regulatory molecules from the platelets that can act along with those released by the activated macrophages, and possibly by smooth muscle cells, within the lesion into the artery wall. Ultimately the formation and release of numerous growth-regulatory molecules and cytokines from a network established between cells in the lesion leads to progression of the lesions of atherosclerosis to a fibrous plaque or advanced complicated lesion. Each of the stages of lesion formation is potentially reversible (Figure 1.7). Thus, lesion regression can occur if the injurious agents are removed or when protective factors intervene to reverse the inflammatory and fibroproliferative processes.

Recently Ross (1999) has stated that every step of the molecular and cellular responses leading to atherosclerosis is an inflammatory process. He states that endothelial dysfunction alters the normal homeostatic properties of the endothelium, thereby initiating inflammatory cell activation. Continued inflammation results in increased numbers of macrophages and lymphocytes emigrating from the blood into the lesion. Activation of these cells leads to release of hydrolytic enzymes, cytokines, chemokines and growth factors causing overall growth of the plaque and ultimately leading to cellular necrosis in advanced lesions. Initially the artery compensates by dilation but, at some point it can no longer do so and the lesion protrudes into the lumen to alter the flow of blood.

LDL is a major cause of injury to the endothelium and underlying smooth muscle (Griendling and Alexander, 1997; Navab *et al.*, 1996), especially after oxidation, glycation (in diabetics) or incorporation into immune complexes (Navab *et al.*, 1996; Steinberg, 1997). Internalisation of modified LDL by macrophages leads to the formation of lipid peroxides and facilitates the accumulation of cholesterol esters resulting in the formation of foam cells. Removal and sequestration of modified LDL are important parts

of the initial protective role of the macrophage in the inflammatory response (Han *et al.*, 1997; Diaz *et al.*, 1997) and minimise the effects of modified LDL on endothelial and smooth muscle cells. This initial inflammatory response mediated by monocytes/macrophages leads to release of further mediators of inflammation such as tumour necrosis factor α (TNFα), interleukin-1 (IL-1) interleukin-6 (IL-6) and macrophage colony stimulating factor (MCSF) which in turn further increase binding of modified LDL to the endothelium and smooth muscle cells. Thus, a vicious circle of inflammation is maintained in the artery by the presence of lipids. If the inflammatory response does not neutralise the offending agent(s), it can continue indefinitely leading to plaque formation and rupture.

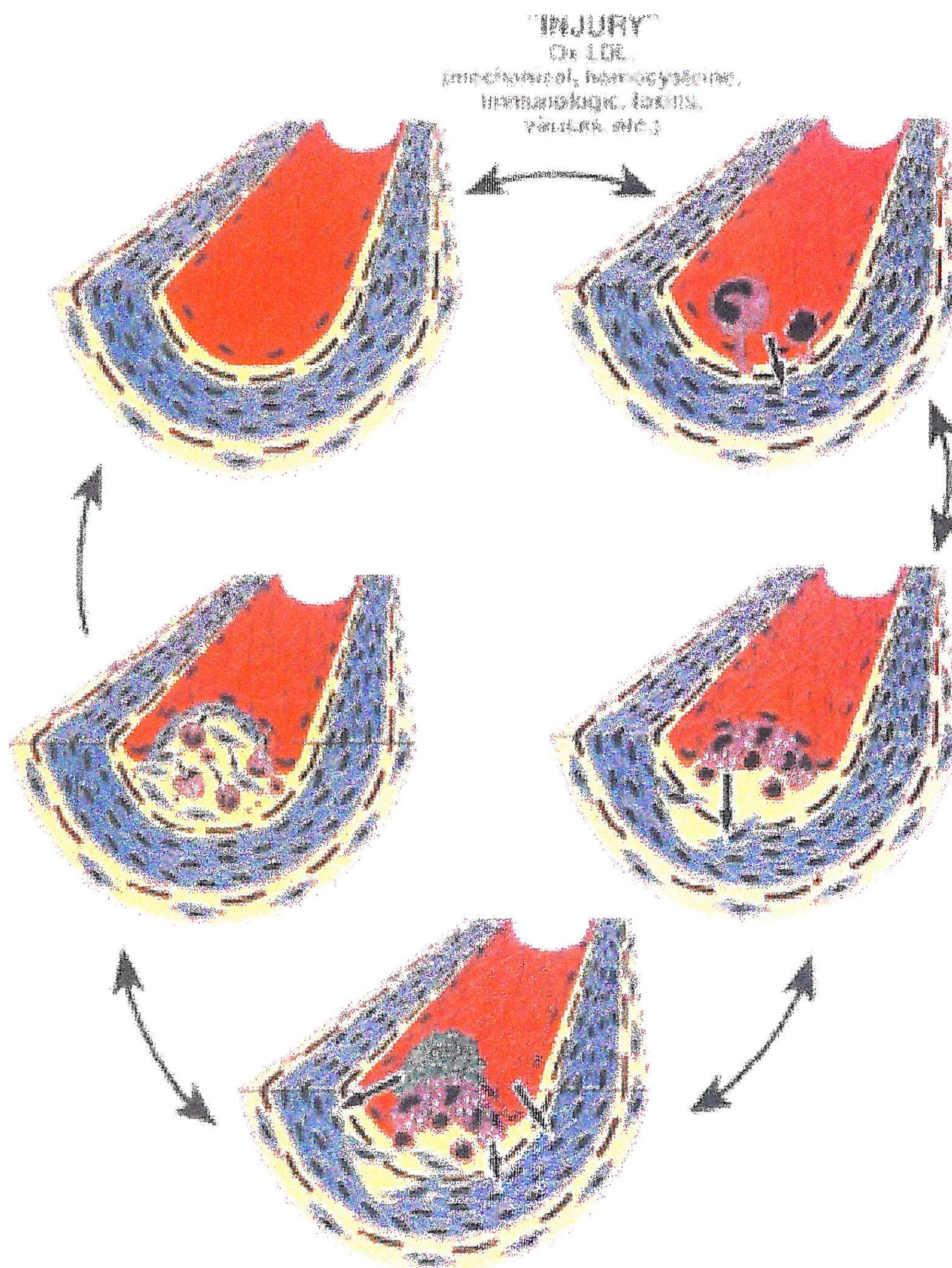


Figure 1.7 Endothelial cell dysfunction

(from Ross, 1993). Cells are colour coded: smooth muscle, blue; endothelium, red;macrophage,violet; T cell, pink; platelet, green.

1.3 Risk Factors for cardiovascular disease

CVD is a disease for which many risk factors have been identified. Family history, age and gender predict prevalence, with heart disease being more common in middle-aged men than middle-aged women, although prevalence rates are similar in older men and women (Department of Health, 1994). In addition, there are a number of characteristics of lifestyle, modification of which can influence risk. The most important of these are smoking and lack of physical activity; however treatment of high blood pressure and blood cholesterol, maintenance of an appropriate body weight, and avoidance of a body distribution centred around the waist are also recognised as important (Department of Health, 1994).

The major risk factors for atheroma are thought to act by providing mechanisms for arterial damage, which changes both endothelial structure and function. Hypertension can damage the endothelium by mechanical trauma, whilst smoking may provide damaging chemical agents. One of the best documented risk factors, is hypercholesterolaemia and in particular elevated plasma levels of LDL and Lp(a) have a proven role in atherogenesis (Chobanian, 1992). LDL and Lp(a) are not thought to act solely as substrates for macrophage ingestion; evidence shows that endothelial cells oxidatively alter LDL to oxidised LDL (Ox-LDL) (Steinberg, 1991). Once formed Ox-LDL has been shown to be significantly more atherogenic than its native form in several ways (Steinberg, 1991). Ox-LDL is chemotactic for circulating monocytes, it is an inhibitor of the motility of resident macrophages, it is cytotoxic for cells in culture and may directly damage the endothelium, it can induce the formation of adhesive surface glycoproteins by the endothelium and it can stimulate the release of a colony stimulating factor and a monocyte chemotactic factor from endothelial cells (Ross, 1993; Steinberg, 1991). Lp(a) may provide a link between atheroma and thrombogenesis. It has been shown to interfere with normal fibrinolysis, by binding to fibrinogen and to endothelial binding sites for plasminogen. It has been suggested that Lp(a) not only promotes thrombosis, but also prevents thrombolysis (Loscalzo, 1990).

Thrombogenesis has a major role to play in atheroma development. Normal intact endothelium provides a surface that is highly resistant to thrombus formation. In atheromatous disease this endothelium is changed in both structure and function. The

endothelial cells express abnormal surface molecules and gaps may appear between the cells exposing the underlying macrophages and T cells. Both of these processes allow platelet adhesion and subsequent thrombogenesis. Once attached, the platelets release various growth factors which can accelerate progression of the atheroma. There is also evidence to suggest that LDL stimulates endothelial cells to release anti-fibrinolytic agents, thus increasing the lifespan of thrombi once formed (Tremoli *et al.,* 1993).

The primary cause(s) or initiator of atherosclerosis is still largely unknown, but epidemiological studies have demonstrated a large number of dietary factors, which influence the course of atherosclerosis:

Promoting factors

- cholesterol-raising SFA
- thrombogenic SFA

Protective factors

- n-6 PUFA
- MUFA
- n-3 PUFA
- fibre
- antioxidants

1.3.1 Promoting factors

1.3.1.1 Hypercholesterolaemic fatty acids

A diet high in SFA is associated with high levels of circulating cholesterol (Keys *et al.*, 1965), which in turn are related to a high incidence of CHD (Rose & Shipley, 1986). The raised levels of cholesterol, especially of LDL cholesterol, appear to be important in atheroma and in the formation of Ox-LDL which is taken up by macrophages and deposited in the plaque. Inhibition of LDL oxidation slows the progression of atherosclerotic lesions (Parthasarathy *et al.*, 1990). Not all SFA are hypercholesterolaemic: 18:0 (stearic acid) does not raise serum cholesterol (Bonanome and Grundy, 1988), and neither do short-chain SFA (C10 and below). The atherogenic

SFA are 12:0 (lauric), 14:0 (myristic), and 16:0 (plamitic) (Ulbricht and Southgate, 1991). However it is thought that 16:0 may not be as potent as either 12:0 or 14:0 (Hayes, 1995)

1.3.1.2 Thrombogenic fatty acids

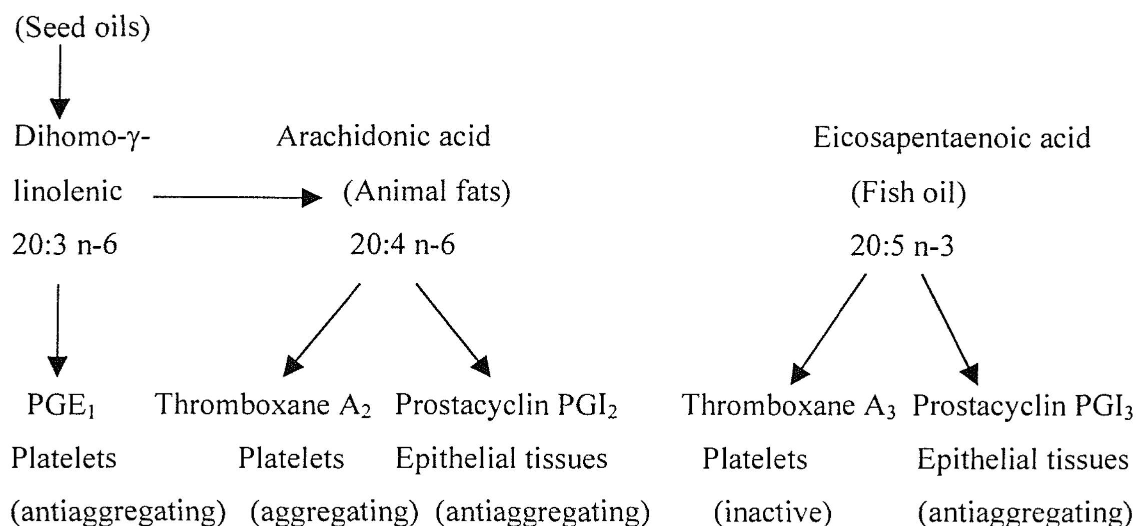
Many studies have shown that SFA can accelerate thrombus formation whereas PUFA and MUFA do not (Hornstra and Lussenberg, 1975; McGregor *et al.*, 1980, MacIntyre *et al.*, 1984, Renaud *et al.*, 1986); it is the longer chain SFA (i.e. 14:0, 16:0 and 18:0) which are thrombogenic and thrombogenicity appears to increase with increasing chain length (Hornstra and Lussenberg, 1975).

1.3.2 Protective factors

1.3.2.1 n-6 PUFA

The n-6 PUFA whose parent member, linoleic acid, is commonly found in vegetable seed oils, have long been known to decrease serum total cholesterol and LDL cholesterol concentrations (Ulbricht and Wheelock, 1989) and it has therefore been widely recommended that SFA in the diet should be partly replaced by n-6 PUFA (Department of Health, 1991). Low levels of adipose tissue linoleic acid (a reflection of long term intake) are related to CHD incidence and mortality (Riemersma *et al.*, 1986). Thus, it has been concluded that an increase in the intake of n-6 PUFA is likely to be beneficial in protecting against atheroma. However, as well as decreasing LDL concentrations, diets high in these PUFA depress the concentration of HDL which is protective against CHD, (Shepherd *et al.*, 1978; Grundy, 1986) and may make LDL more susceptible to oxidation (see Section 3.1.4)

Figure 1.8: Prostanoids formed from different fatty acids
(Ulbricht and Southgate, 1991)



In considering the role of n-6 PUFA in thrombosis one has to bear in mind that there are three series of prostanoids (Figure 1.8) derived from three different 20 carbon PUFA, dihomo-γ-linolenic acid (DHLA), arachidonic acid (AA) and eicosapentaenoic acid (EPA). DHLA and AA are metabolites of linoleic acid. In a normal functioning healthy body vasodilatation has to prevail over vasoconstriction and adhesion to the arterial wall and platelet aggregation are to be prevented. PGE_1 which is formed from DHLA has antiaggregating activity (Ulbricht and Southgate, 1991) and the fact that unstimulated platelets produce more PGE_1 than PGE_2 (Lagarde *et al.*, 1981) indicates that the prostanoids of the first series are predominant in this state. The principal fatty acid released in response to injury is AA, from which both thromboxane A_2 (TXA_2), which strongly aggregates platelets, and prostacyclin (PGI_2), a potent antagonist of platelet aggregation, are formed (Sinclair, 1984). Since PGI_2 is synthesised only by arterial endothelium and PGE_1 from the small amount of its precursor available, the large amount of AA released by platelets as a result of injury, is mainly converted to TXA_2 and PGE_2 (which does not act on arterioles or platelets), leading to platelet aggregation (Ulbricht and Southgate, 1991)

Many studies indicate that long chain unsaturated fatty acids slow down intra-arterial occlusion and platelet aggregation (Hornstra and Lussenberg, 1975, McGregor *et al.,* 1980, MacIntyre *et al.*, 1984, Renaud *et al.*, 1986). Linoleic acid is more anti-thrombotic than MUFA. It has been suggested that diets which have a relatively high content of SFA or a high ratio of n-6 to n-3 PUFA facilitate the production of TXA_2 and the inhibitition of production of PGI_2, thereby increasing the risk of thrombosis (Kinsella *et al.*, 1990).

1.3.2.2 MUFA

Diets high in MUFA are as effective as those high in n-6 PUFA in lowering plasma total and LDL cholesterol concentrations but, in contrast to the effect of n-6 PUFA, MUFA do not lower HDL cholesterol concentrations (Mensink and Katan, 1989; Wardlaw and Snook, 1990). LDL taken from rabbits fed with a high oleic sunflower oil is highly resistant to oxidation (Parthasarathy *et al.*, 1990) and this is also seen in humans (see Section 3.1.4, Reaven *et al.*, 1991; Berry *et al.*, 1992) This suggests that diets sufficiently rich in oleic acid, in addition to their LDL-lowering effect, may slow the progression of atherosclerosis by generating LDL which is resistant to oxidation. As a result of the reduction in the consumption of antioxidants this may make antioxidants more available for other functions.

The antithrombogenic activity of MUFA has been studied much less than that of PUFA. In some systems MUFA inhibit platelet aggregation, though less so than PUFA (MacIntyre *et al.*, 1984; Sinclair, 1984).

1.3.2.3 n-3 PUFA

1.3.2.3.1 n-3 PUFA and primary prevention of atherogenesis and coronary heart disease

The observation that coronary heart disease is rare in Greenland Eskimos is well known, and their low risk has been attributed to a high intake of long-chain n-3 PUFA obtained from the consumption of marine animals and fish (Dyerberg and Bang, 1978). Several studies have been performed investigating the effect of long-chain n-3 PUFA intake and risk of CHD; five of these studies demonstrated an inverse correlation (Miettinen *et al.,* 1982, Kromhout *et al.,* 1985, Shekelle *et al.,* 1985, Norell *et al.,* 1986, Dolecek *et al.,* 1989) and three reported no association (Curb and Reed, 1985, Vollset *et al.,* 1985, Lapidus *et al.,* 1986) between intake of long-chain n-3 PUFA and CHD. In the studies that showed no association, habitual consumption of fish was already high. It is possible therefore that consumption of a moderate amount of fish may be protective against CHD and that continued consumption of large amounts does not provide additional protection.

A study by Daviglus *et al.* (1997) looked at the association between fish consumption and the 30-year risk of death from myocardial infarction (sudden or nonsudden), coronary heart disease, cardiovascular diseases and all causes in the Chicago Western Electric Study. The study participants were 1822 men who were 40 to 55 years old and free of cardiovascular disease at baseline. Fish consumption as determined from a detailed dietary history was stratified (0, 1 to 17, 18 to 34 and $\geq$ 35 g per day). The authors found an inverse association between fish consumption and death from coronary heart disease, especially non-sudden death from myocardial infarction (Table 1.5).

Data from the prospective cohort US Physicians' Health Study (Albert *et al.*, 1998) were examined to determine the relationship between the average amount of fish consumed at baseline and the risk of sudden cardiac death for up to 11 years. Out of 20,551 men 133 sudden deaths occurred. Eating fish at least once a week, compared with less than once a month, was associated with a greater than 50% lower relative risk by multivariate analysis. However the risk of myocardial infarction was not lessened at any level of fish consumption (Table 1.5).

In the EURAMIC study (Guallar *et al.*, 1999), 639 patients after a first myocardial infarction were compared with 700 controls. The proportion of α-linolenic acid in the adipose tissue was found to be lower in patients than in controls suggesting that increased intake of α-linolenic acid might be protective. However, after adjustment for classical risk factors, the effect of α-linolenic acid was no longer significant. No differences were found for adipose tissue content of EPA or DHA between patients and controls.

1.3.2.3.2 n-3 PUFA and progression of atherosclerosis and secondary prevention of coronary heart disease

A randomised controlled trial of fish consumption on mortality was reported by Burr *et al.* (1989). In this study 2033 men who had suffered a recent myocardial infarction were randomly allocated to fish advice or no fish advice groups. Those allocated to the fish group were advised to consume at least two portions of fatty fish per week; patients who could not tolerate fish were given fish oil capsules and asked to take three daily. The patients advised to eat fish had a 29% reduction in two year all-cause mortality compared with those not given this advice. However, the two year incidence of reinfarction plus death from ischaemic heart disease was not significantly affected by any of the dietary regimens. This study concluded that a modest intake of fatty fish might reduce mortality in men who have recovered from an acute myocardial infarction.

The Study on Prevention of Coronary Atherosclerosis by Intervention with Marine Omega-3 fatty acids (SCIMO) was a randomised, double-blind, placebo-controlled single-centre trial (von Schacky *et al.*, 1999, Table 1.5). It was hypothesised that the ingestion of a fish oil concentrate for two years would reduce the progression of coronary atherosclerosis, as assessed by standardised angiography at baseline. A total of 223 patients were randomly assigned to receive either 1.65 g EPA plus DHA per day or a placebo reflecting the average fatty acid composition of the European diet. After two years 162 patients had a repeat angiography. An expert panel evaluated all films pairwise and rated the magnitude of any difference. It was found that patients who received fish oil had less progression and more regression of coronary atherosclerosis. They also had fewer cardiovascular events, but other clinical variables did not differ between the study

groups. The authors concluded that supplementation with n-3 fatty acids modestly mitigates the course of coronary atherosclerosis in humans.

The results of the Lyon Diet Heart Study (de Lorgeril *et al.*, 1994; 1999) support the view that n-3 PUFA decrease cardiovascular and all-cause mortality. Patients who survived a first myocardial infarction were randomly assigned to either usual care or advised to adopt a Mediterranean-type diet. The dietary group showed changes in plasma fatty acids among which the increase of n-3 PUFA (α-linolenic acid and EPA) and the decrease in n-6 PUFA were most prominent. The extended follow-up after 46 months was recently reported (de Logeril *et al.*, 1999): the risk of cardiac death and non-fatal myocardial infarction combined was reduced by 72% in patients on the Mediterranean diet compared with the usual care group. α-Linolenic acid in plasma was significantly associated with improved prognosis and a trend towards protection was also observed for DHA no improvement was reported for EPA.

Two recent randomised trials have shown a reduction in cardiac events after n-3 PUFA supplementation. Treatment with fish oil, mustard oil (containing α-linolenic acid), or placebo for 1 year was compared in 360 patients with suspected myocardial infarction (Singh *et al.*, 1997, Table 1.5). Both n-3 PUFA groups experienced significantly fewer total cardiac events and non-fatal myocardial infarctions, whereas the reduction in cardiac deaths was restricted to the fish oil group. In 1999 results of the Gruppo Italiano per lo Studio della Sopravvivenza nell-Infarto miocardico-Prevenzione (GISSI-P) trial were published. This was a large trial that enrolled 11,324 patients who had survived a myocardial infarction within the previous 3 months. Treatment with either highly purified fish oil containing 850 mg EPA plus DHA, or 300 mg vitamin E, or both for 3.5 years and was compared with no treatment, with respect to all-cause mortality, non-fatal myocardial infarction, and non-fatal stroke (primary combined endpoint). An intention-to-treat comparison of all patients who received fish oil versus all other patients showed a 10% reduction of the primary combined endpoint; patients who only received fish oil versus those who received neither fish oil nor vitamin E showed a 15% reduction. This was due to a relative decrease in mortality: 20% for total death, 30% for cardiovascular death, 45% for sudden death, whereas non-fatal myocardial infarction and stroke were not substantially influenced by fish oil. The combination of fish oil with

vitamin E did not increase the benefit compared with fish oil alone. Patients studied by GISSI and SCIMO received state-of-the-art drug treatment for secondary prevention. In both trials, the concentration of LDL cholesterol slightly increased in the n-3 PUFA treatment groups. Therefore n-3 PUFA seem to be effective in addition to conventional treatment and independently from lowering LDL.

1.3.2.3.3 n-3 PUFA and blood lipids

The impact of n-3 PUFA on blood lipoprotein concentrations has been examined in many studies over the last 15 years in both animals and humans (Harris, 1996). Elevated levels of Lp(a) in plasma appear to be a predictor for CHD events (see section 1.1.3.2.2, Scott, 1991). Hermann *et al.* (1989) studied patients with elevated levels of Lp(a) and found that fish oil supplementation led to a significant reduction. However a subsequent study could not confirm that fish oil decreased Lp(a) concentrations (Gries *et al.*, 1990). A small study in healthy volunteers observed a 15% decrease in Lp(a) levels following EPA plus DHA supplementation (Haglund *et al.*, 1994). However, a large randomised trial in patients with elevated levels of LP(a) undergoing bypass surgery failed to demonstrate a difference in Lp(a) serum levels after 6 months of treatment with 3.4 g EPA plus DHA compared with placebo (Eritsland *et al.*, 1995). Therefore, although Lp(a) concentrations are to a great extent genetically determined by the molecular weight of the apolipoprotein A isoform, n-3 PUFA may lower Lp(a) levels.

The potent triglyceride lowering effect of n-3 PUFA was first demonstrated in human studies and these were followed up with animal studies to determine the mechanism of action. Harris (1996) has reviewed the reported effects of n-3 PUFA on blood lipoprotein concentrations in 72 placebo controlled human trials, lasting at least two weeks and providing 7 g or less of n-3 PUFA/day. He compared trials in normolipidemic patients (triglycerides $<$ 2.0mM; 177 mg/dL) to those in hypertriglyceridemic patients (triglycerides $\geq$ 2.0mM) and noted the changes shown in Table 1.4.

Table 1.4: Effects of n-3 PUFA on blood lipid concentrations.

	Triglycerides < 2.0mM	Triglycerides ≥ 2.0mM
Triglycerides	↓ 25% ($p < 0.0001$)	↓ 28% ($p < 0.0001$)
Total Cholesterol	↑ 2% ($p < 0.009$)	NS
LDL cholesterol	↑ 4% ($p < 0.02$)	↑ 7% ($p < 0.0001$)
HDL cholesterol	↑ 3% ($p < 0.008$)	NS

Summarised from Harris (1996)

Although the effect on triglyceride levels is also observed in rats (Jen *et al.*, 1989; Yaqoob *et al.*, 1995) and pigs (Weiner *et al.*, 1986), it is rarely seen in mice (Renier *et al.*, 1993), rabbits (Kristensen *et al.*, 1988), monkeys (Abbey *et al.*, 1990), dogs (Cahill *et al.*, 1988) or hamsters (Jones *et al.*, 1990). N-3 PUFA have only a minor impact on cholesterol concentrations in humans, although they often markedly lower both total cholesterol and HDL concentrations in animals, especially monkeys (Harris, 1996).

Other factors believed to be involved in the pathogenesis of atherosclerosis also appear to be affected by n-3 fatty acids. These include inhibition of intimal hyperplasia in autologous vein grafts, as demonstrated in dogs (Landymore *et al.,* 1986; Cahill *et al.,* 1988), decreased endothelial cell production of a platelet derived growth factor-like protein (Fox and DiCorleto, 1988), increased production of endothelium-derived relaxing factor (nitric oxide) (Shimokawa *et al,* 1987), decreased production of TXA_2 (Needleman *et al,* 1979) and a potential reduction in the intensity of the inflammatory response through changes in eicosanoid and cytokine production (Lee *et al.,* 1985; Fisher *et al.,* 1986; Calder, 1996; Grimble, 1998).

PUFA differ in their anti-thrombogenic activity. EPA and DHA are particularly effective in inhibiting platelet aggregation and act by inhibiting the conversion of arachidonic acid to the prostanoid TXA_2 by platelets (Rao *et al.*, 1983). A diet high in n-3 PUFA will also facilitate the production of the prostanoid PGI_3, a potent inhibitor of platelet aggregation, whereas the corresponding TXA_3 is a very weak platelet aggregator (Editorial, 1988; British Nutrition foundation, 1992) Therefore, a high oily fish diet will lead to anti-thrombotic changes in prostanoids. Thus, whereas the principal effect of

increasing the intake of n-6 PUFA is believed to be anti-atherogenic (reduction of serum lipid concentrations), that of increasing n-3 PUFA intake is anti-thrombotic (reduction of platelet activity) (Renaud *et al.*, 1986).

Table 1.5: Key randomised trials and epidemiological studies on n-3 PUFA and cardiovascular disease

Reference	Design	Follow-up (years)	Sample	Effect of n-3 PUFA on main outcome measures	Type of intervention	Fish or n-3 PUFA intake per day
Burr *et al.* (1989)	A	2	2033 Men with acute MI	29% Reduction in all-cause mortality	Dietary advice: more fish versus more fibre versus less (saturated) fat	0.34 g EPA
Daviglus *et al.* (1997)	C	30	1822 Men, free from CVD at baseline	38% Reduction in CHD mortality, due to 67% reduction non-sudden death from MI	None (observation)	≥ 35 g fish compared with 0 g fish
Singh *et al.* (1997)	B	1	360 Patients with suspected acute MI	29% Reduction in total cardiovascular events, 49% in non-fatal MI, 48% in cardiac deaths	Purified fish oil versus purified mustard oil versus placebo	1.08 g EPA + 0.72 g DHA
Albert *et al.* (1998)	C	Up to 11	20,551 Men, free from CVD at baseline	52% Reduction in sudden death	None (observation)	≥ 1 Meal fish/ week compared with < 1 meal fish/month
Sacks *et al.* (1995)	B	2.3	59 Patients with angiographically documented CHD	No change in minimal luminal diameter and % stenosis as assessed by QCA	Purified fish oil versus olive oil	6 g EPA + DHA
von Schacky *et al.* (1999)	B	2	223 Men and women with angiographically documented CHD	Less progression, more regression of coronary atherosclerosis (angiography/ expert panel)	Purified fish oil versus placebo	1.65 g EPA + DHA (3.3 g during first 3 months)
GISSI-P (1999)	A	3.5	11,324 Men and women with recent acute MI	15% Reduction in death, non-fatal MI and stroke (combined)	Purified fish oil versus vitamin E versus no treatment	0.85-0.88 g EPA + DHA

Design A, randomised controlled trial, factorial design; design B, randomised controlled trial, double-blind; design C, prospective cohort study. ALA, α-linolenic acid; CHD, coronary heart disease; CVD, cardiovascular disease; MI, myocardial infarction; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GISSI-P, Gruppo Italiano per lo Studio della Sopravvivenza nell-Infarto miocardico-Prevenzione; QCA, quantitative coronary angiography.

1.3.2.4 Fibre and antioxidants

Also linked with an increased heart disease risk are a low intake of fruit and vegetables (which provide antioxidants and other phytochemicals), a low intake of sources of soluble fibre (helps to decrease blood cholesterol concentration) and a poor folate status. Folate (folic acid) is a B vitamin that is known to be associated with metabolism of certain amino acids. When supplies are inadequate, the amount of the amino acid homocysteine present in the blood rises and this has been recognised as a predictor of heart disease risk (Boushey *et al.*, 1995).

1.4 Summary of introduction and aims of this study

Cardiovascular disease involves the formation of atherosclerotic plaques. These dynamic structures include lipid, largely derived from circulating LDL, particularly following its oxidation, and inflammatory cells. These inflammatory cells can be responsible for LDL oxidation. Plaques can be destabilised, and if they rupture they can block the circulation resulting in a myocardial infarction (if in the coronary artery) or a stroke (if in the carotid artery).

Dietary fatty acids are strongly linked to the process of atherosclerosis. SFA increase the risk by increasing plasma cholesterol concentrations, and n-6 PUFA decrease risk by decreasing plasma cholesterol concentrations. However, n-6 PUFA are believed to be pro-thrombotic and pro-inflammatory and these effects might negate the decrease in plasma cholesterol. n-3 PUFA decrease risk by decreasing plasma TAG concentrations and they are anti-thrombotic and ant-inflammatory. Large studies show protection of primary and secondary cardiovascular events with increasing n-3 PUFA consumption. Both n-6 and n-3 PUFA are substrates for oxidation and therefore increasing their amounts in LDL might increase LDL oxidation and so counteract some of the benefits of increased consumption of these fatty acids. It is not clear whether these fatty acids are incorporated into active atherosclerotic plaques, and if they are, whether they act to stabilise or destabilise the plaque.

The aims of this study were to examine the relationship between fatty acids in the diet, risk factors for CVD, and atherosclerotic plaque structure and composition in

patients undergoing carotid endarerectomy. To do this the effects of moderate supplementation with n-3 and n-6 PUFA on plasma lipid concentrations, the fatty acid profile and oxidisability of plasma LDL and of lipid fractions isolated from atherosclerotic plaques, and the cell populations involved in the inflammatory activity in the carotid atherosclerotic lesions were characterised.

Chapter 2

Study design and characteristics of patients

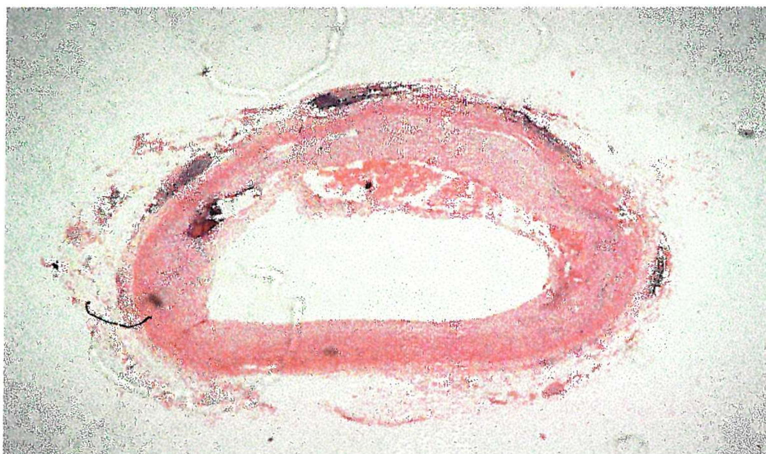
2 Study design and characteristics of patients

2.1 Introduction

2.1.1 Carotid atherosclerosis and carotid endarterectomy

Atherosclerotic lesions can be observed throughout the body but certain areas of the arterial tree are particularly prone to the development of lesions such as the aortic arch, branching sites of larger vessels (artery bifurcation) and the carotid and coronary arteries. These are all sites subject to increased haemodynamic stress, due to increased shear stresses damaging the endothelial surface and the presence of non-laminar blood flow. The human carotid bifurcation is a high-risk region for the development of atherosclerotic plaques, which may lead to complications such as thrombosis, arterio-arterial embolism and stroke (Figure 2.1, Eliasiw *et al.*, 1994).. The atherosclerotic plaques usually develop in the outer wall just opposite the carotid bifurcation (Zarins *et al.*, 1987). It has been estimated that 50-60% of all acute strokes are related to atheromatous disease of the carotid bifurcation (Pessin *et al.,* 1977; Stehbens, 1982; Bogousslavski *et al.,* 1986; Gelabert and Moore, 1991). Carotid endarterectomy was introduced in 1954 as a procedure for the prevention of ischaemic strokes caused by carotid artery stenosis. Carotid endarterectomy is particularly beneficial to certain groups of patients with ischaemic cerebral symptoms secondary to carotid artery atherosclerosis (Gelabert and Moore, 1991).

Figure 2.1 Microscopic image of a lesion from a carotid artery



2.1.2 Aims

The aims of this chapter are to describe the study design used and to describe the characteristics of the patients studied. Particular attention was paid to the habitual nutrient intakes, especially those of fat and fatty acids, and the plasma lipid concentrations.

2.2 Methods

2.2.1 Patients and study design

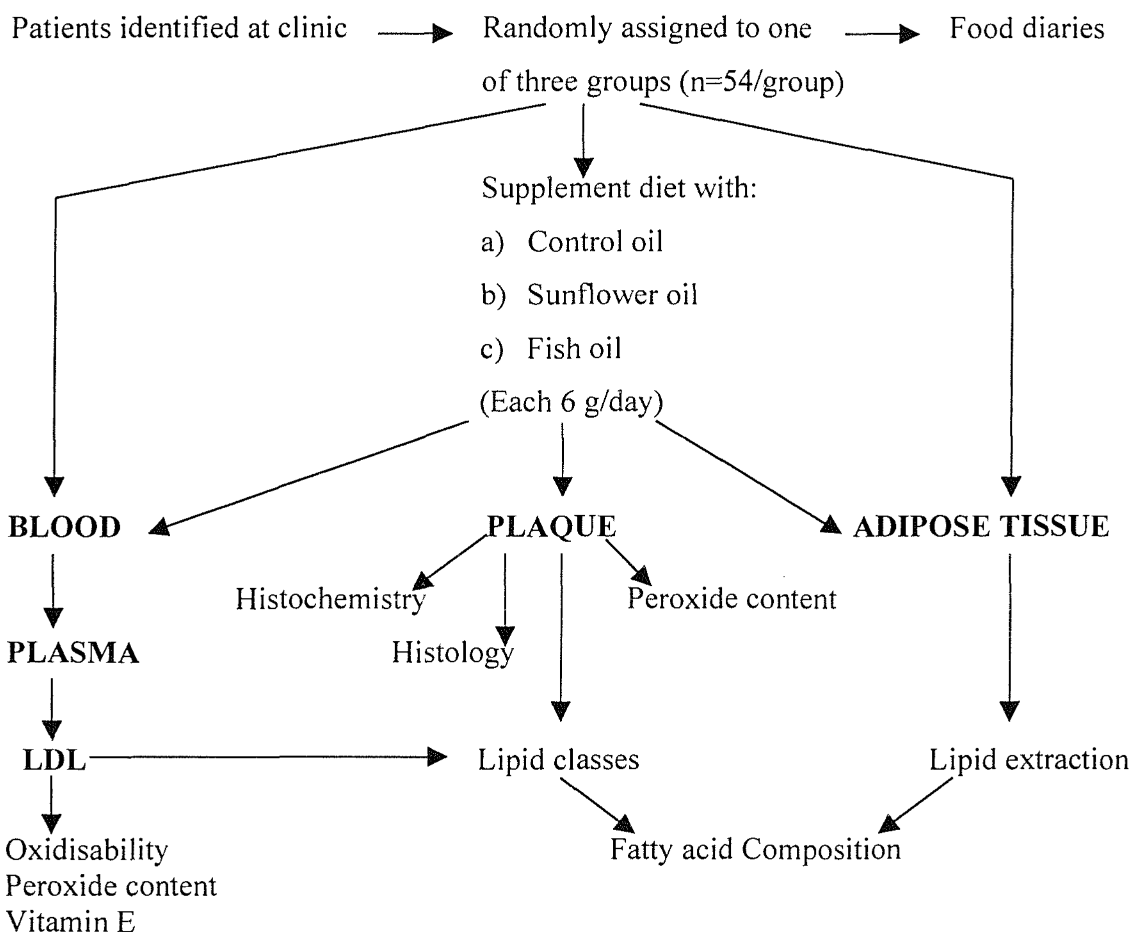
This study was approved by he Southampton and South West Hampshire Local Research Ethics Committee. Patients destined to undergo carotid endarterectomy were the patients used in this study. Patients were recruited from those attending the outpatients clinic of Mr. C.P Shearman, Consultant Vascular Surgeon, Southampton University Hospitals Trust. Patients were offered carotid endarterectomy on clinical grounds, as is the current practice in the Southampton University Hospitals Trust. If the patients agreed to participate in the study they were asked to give informed consent and were randomly assigned to one of three intervention protocols 1) habitual diet plus control oil capsules, 2) habitual diet plus sunflower oil capsules and 3) habitual diet plus fish oil capsules. Patients requiring emergency surgery were not included in the study. A total of 54 patients were assigned to each group. Randomisation was stratified for gender and this was a double blind study.

At the time of commencing the study, a fasting blood sample and a narrow gauge needle subcutaneous adipose tissue biopsy from the abdominal wall were taken. Patients were then given dietary record sheets and weighing scales and instructed in their use for completing a 7-day weighed food diary. Patients then commenced dietary supplementation with capsules until their operation (Figure 2.1). Operative removal of carotid plaques was performed at various times over a time scale ranging from two to 27 weeks. It is current clinical practice for a period of between two and 27 weeks to elapse between the time when the decision is made that a patient requires surgical removal of a carotid plaque and the time when the operation is performed. Supplementation occurred during this period, thus providing supplementation periods of various lengths. This

strategy allowed the time course of changes in the fatty acid composition of the plaque, plasma and adipose tissue to be assessed.

Immediately prior to surgery a second fasting blood and adipose tissue sample were taken and the plaque was removed (Figure 2.1) by Mr. C.P. Shearman and processed as described later (see section 4.2.1)

Figure 2.2 Study protocol



2.2.2 Composition of capsules and assessment of dietary fatty acid intake

Fatty acid intake was assessed for each patient by analysis of a seven day weighed food diary, and by measurement of the fatty acid composition of two adipose tissue biopsies, the first taken at the time that the decision was made to operate on the patient (i.e. prior to supplementation) and the second at the operation. Food diaries were analysed by staff at the Public Health Nutrition Group, University of Southampton using a non-commercial programme developed in SPSS and a nutrient composition database based upon values from McCance and Widdowson (5th edition, 1991) bought from the Royal Society of Chemistry.

Alterations in the dietary intake of n-3 and n-6 PUFA were achieved by supplementation of the patient's habitual diet with encapsulated fish oil or sunflower oil. Patients consumed capsules containing 1 g of oil at a rate of two, three times a day with food (i.e. 6 capsules/day). The placebo capsules consumed by the control group contained the same amount of fat as the test capsules and were a blend (80:20) of palm oil and soya bean oil. The fatty acid composition of this blend is very similar to that of the average UK diet, as shown in Table 2.1. Data for the fatty acid composition of the 'average' UK diet were from the British Nutrition Foundation (1992) and are from the survey of British adults in the late 1980s published by Gregory *et al.* (1990). A recent briefing paper (British Nutrition Foundation, 1999) states that these data are the most recent data of this type available. All capsules contained the same level of vitamin E (approx. 300 μg/g oil). Supplementation of the diet with 6 g sunflower oil or fish oil per day added approximately a further 1.6% and 0.8% to dietary energy in the form of n-6 and n-3 PUFA, respectively. Patients in the sunflower oil group consumed an extra 3.8 g linoleic acid per day. Patients in the fish oil group consumed an extra 0.8 g EPA and 0.5 g DHA per day. Patients were required to cease taking any dietary oil supplements prior to entry into the study. Compliance with the dietary regimens was checked verbally and from diary returns.

Table 2.1 Fatty acid composition of capsules used and the average UK diet

	Fatty acid (g/100 g total fatty acid)			
Fatty acid	**UK diet**	**Control oil**	**Sunflower oil**	**Fish oil**
12:0		0.9		
14:0		2.1	1.5	6.2
16:0	45.0*	34.9	8.6	20.4
16:1n-7		2.0	1.0	12.3
18:0		3.7	3.5	5.7
18:1n-9	33.0	33.8	18.6	10.1
18:2n-6	14.0	18.9	62.8	2.3
18:3n-3	2.0	1.8	1.4	4.6
18:3n-6				1.0
20:4n-6			1.2	1.1
22:4				1.4
20:5n-3				14.3
22:5n-3				1.5
22:6n-3				8.3

UK diet data are from the British Nutrition Foundation (1992). (*Indicates total SFA)

2.2.3 Measurement of plasma and LDL lipid concentrations

Blood was obtained from patients at the point when the decision was made that they should go to surgery and they had agreed to participate in the study and again immediately prior to operation. Blood was taken into vacutainers using EDTA (15%; 0.12 ml) as an anticoagulant. Plasma was isolated by low-speed centrifugation (2500 rpm for 10 min).

2.2.3.1 LDL Preparation

Plasma was adjusted to a density of 1.24 g/ml by the addition of 381.6 mg solid potassium bromide (KBr) per ml plasma. A two-step density gradient was formed by layering 1.7 ml of density-adjusted plasma underneath 3.3 ml of phosphate-buffered

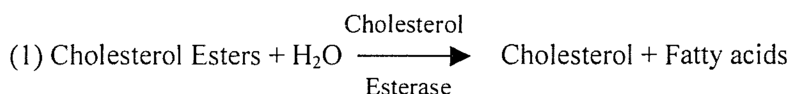
saline (d = 1.006 g/ml) in centrifuge tubes. They were then sealed and centrifuged in an Optima ultracentrifuge (TLA-100.4 rotor) at 100,000 rpm for 2 h at 15^0C to separate VLDL, LDL and HDL fractions (Sattler *et al.,* 1992). Each lipoprotein fraction was then removed separately by firstly removing the VLDL fraction from the top of the tube using a needle and syringe. The top of the tube was then cut off and the remaining VLDL, along with the LDL and HDL fractions were removed separately using pasteur pipettes. VLDL and HDL were frozen for use in other studies. Aliquots of the LDL fractions were saved for the lipid peroxide assay and conjugated diene measurements (see section 3.2.2) and the remaining LDL was frozen (-70°C) for later analysis.

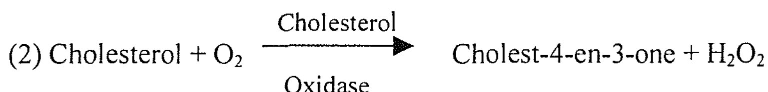
2.2.3.2 Total Cholesterol

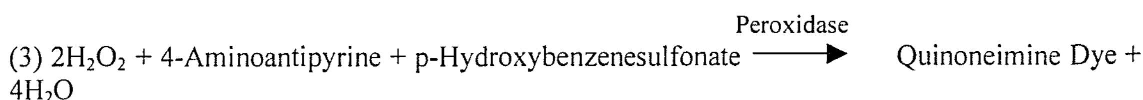
The cholesterol concentration of plasma and LDL was measured using an enzymatic assay (Sigma Diagnostics, Procedure No.352).

Principle:

In this assay cholesterol esters are first hydrolysed by cholesterol esterase to cholesterol (reaction 1). The cholesterol produced by hydrolysis is oxidised by cholesterol oxidase to cholest-4-en-3-one and hydrogen peroxide (reaction 2). The hydrogen peroxide produced is then coupled with the chromagens, 4-aminoantipyrine and p-hydroxybenzenesulfonate in the presence of peroxidase to yield a quinoneimine dye (reaction 3) which has an absorbance maximum of 500 nm. The intensity of the colour produced is directly proportional to the total cholesterol concentration in the sample.







Procedure:

1. Cholesterol reagent was prepared according to instructions.
2. The spectrophotometer wavelength was set to 500 nm, the absorbance reading was set to zero with water as reference.
3. A series of cuvettes were set up for the blank, standard and samples.
4. The reagent was warmed to assay temperature.
5. 1 ml of reagent was pipetted into each cuvette.
6. 10 μl of deionised water (blank), cholesterol standard and sample were added to the appropriate cuvettes. They were mixed by gentle inversion.
7. The cuvettes were incubated for 10 min at room temperature.
8. The absorbances were read at 500 nm.
9. The cholesterol concentration in the sample was calculated as follows:

$$\text{Cholesterol (mg/dL)} = \frac{A_{TEST} - A_{BLANK}}{A_{STANDARD} - A_{BLANK}} \times \text{Standard concentration (mg/dL)}$$

To convert to mmol/L multiply by 0.0259.

Calibration: The procedure was calibrated using a cholesterol standard also from Sigma.

Precision: To test intra-assay variation the same sample was analysed ten times and the results were as shown in Table 2.2

Table 2.2 Intra-assay variability of the total cholesterol assay

	Plasma 1	**Plasma 2**
Mean (mmol/L)	2.8	4.9
SD (mmol/L)	0.05	0.06
CV (%)	1.9	1.3
Number of Assays	10	10

2.2.3.3 HDL Total Cholesterol

The concentration of total cholesterol in HDL was calculated by using the Friedewald's formula (Friedewald *et al.*, 1972).

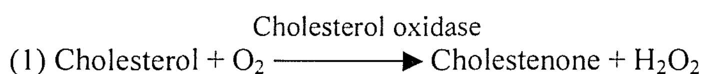
$$\text{HDL cholesterol} = \text{Total cholesterol} - \text{LDL cholesterol} - \left(\frac{\text{TAG}}{2.2}\right)$$

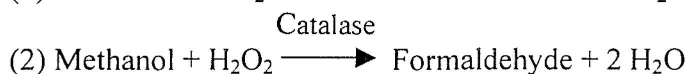
2.2.3.4 Free Cholesterol

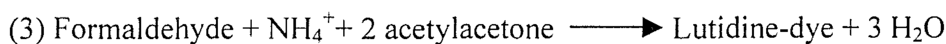
The concentration of free cholesterol in plasma and LDL was measured using an enzyme assay kit (Boehringer Mannheim Cat. No. 139 050). The assay was modified for reading on a microtitre plate reader.

Principle:

Cholesterol is oxidised by cholesterol oxidase to cholestenone (reaction 1). In the presence of catalase, the hydrogen peroxide produced in this reaction oxidises methanol to formaldehyde (reaction 2). The latter reacts with acetylacetone forming a yellow lutidine-dye in the presence of NH_4^+ ions (reaction 3). The concentration of the lutidine-dye (3,5-diacetyl-1,4-dihydro-lutidine) formed is stoichiometric to the amount of cholesterol and is measured by the increase of light absorbance in the visible range at 405 nm.







Procedure:

1. Sample (40 μl) and 0.5 ml of cholesterol reagent mix were mixed thoroughly in glass tubes. These are the sample blanks.
2. Cholesterol-oxidase (2 μl) was placed in a second set of glass tubes. To this 250 μl of the mixture from the first set of tubes was added.
3. The tubes were mixed, covered and incubated at 37°C for 60 min.
4. The tubes were cooled to room temperature.
5. 200 μl of each sample was transferred to a flat bottomed 96 well microtitre plate and the absorbance read on a plate reader at 405 nm.
7. The amount of free cholesterol in the sample was calculated as follows:

$$\text{Free cholesterol (g/l)} = \frac{V \times MW \times diln \times A}{\varepsilon \times d \times v \times 1000}$$

V = final assay volume (ml)

v = sample volume (ml)

MW = molecular weight of cholesterol (g/mol)

d = light path (cm)

ε = extinction coefficient of the lutidine-dye at 405 nm = 7.4 ($l \times mmol^{-1} \times cm^{-1}$)

diln. = 1.008 (0.252/0.25)

A = (absorbance of sample) - (absorbance of sample blank)

To convert to mg/dL multiply by 100, and multiply by 0.0259 to convert to mmol/L.

Calibration: The procedure was calibrated using a cholesterol standard also from Boehringer Mannheim.

Precision: Intra-assay precision was checked by analysing the same samples ten times and the results were as shown in Table 2.3

Table 2.3 Intra-assay variability of the free cholesterol assay

	Plasma 1	Plasma 2
Mean (mmol/L)	0.91	1.58
SD (mmol/L)	0.02	0.04
CV (%)	2.2	2.5
Number of Assays	10	10

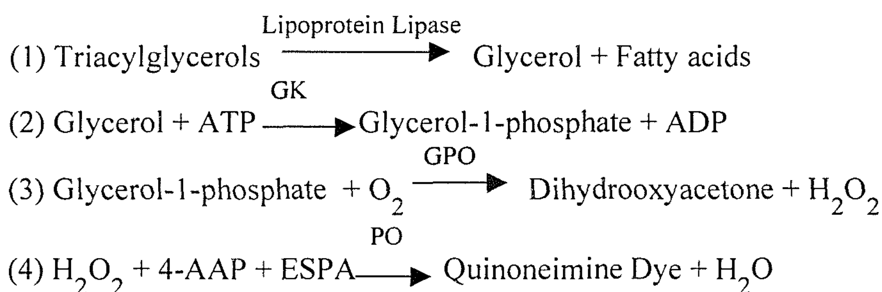
2.2.3.5 Esterified Cholesterol

The concentration of esterified cholesterol in plasma and LDL was calculated by subtracting the concentration of free cholesterol from the concentration of total cholesterol.

2.2.3.6 Triacylglycerols

The concentration of triacylglycerols in plasma and LDL was measured using an enzyme assay kit (Sigma Diagnostics, Procedure No. 337).

Principle:



Triacylglycerols are first hydrolysed by lipoprotein lipase to glycerol and three free fatty acids (reaction 1). Glycerol is then phosphorylated by adenosine triphosphate (ATP) forming glycerol-1-phosphate and adenosine-5- diphosphate (ADP) in the reaction catalysed by glycerol kinase (GK) (reaction 2). Glycerol-1-phosphate is then oxidised by glycerol phosphate oxidase (GPO) to dihydroxyacetone phosphate and hydrogen peroxide (H_2O_2) (reaction 3). A quinoneimine dye is produced by the peroxidase (PO) catalysed coupling of 4-aminoantipyrine (4-AAP) and sodium N-ethyl-N-(3-sulfopropyl)m-

anisidine (ESPA) with H_2O_2 (reaction 4) which shows an absorbance maximum at 540 nm. The increase in absorbance at 540 nm is directly proportional to the concentration of triacylglycerols in the sample.

Procedure:

1. Triglyceride Reagent A and Reagent B were prepared according to the instructions.
2. The spectrophotometer was set to 540 nm and the absorbance reading was set to zero with water as reference.
3. The reagents were warmed to room temperature.
4. A series of cuvettes for blank, standard and sample were set up.
5. Reagent A (0.8 ml) was pipetted into each cuvette.
6. Deionised water, standard and sample (10 μl) was added to the cuvettes. These were then mixed by gentle inversion.
7. Cuvettes were incubated for 5 min at room temperature.
8. The initial absorbance (IA) of blank, standard and sample cuvettes was read at 540 nm vs. water as reference.
9. Then reagent B (0.2 ml) was added to each cuvette, and the contents were mixed and incubated for a further 15 min at room temperature.
10. The final absorbance (FA) of blank, standard and sample cuvettes was read.
11. The concentration of triglycerides in the sample was calculated as follows:

$$\frac{FA_{SAMPLE} - (IA_{SAMPLE} \times F)}{FA_{STANDARD} - (IA_{BLANK} \times F)} \text{ X Concentration of Standard}$$

Where F (dilution factor) = 0.81/1.01 = 0.80

To convert to mmol/L divide by 92.

Calibration: Sigma diagnostics glycerol standard was used for calibration of the assay.

Precision: To test intra-assay variation the same sample was analysed ten times and the results were as shown in Table 2.4

Table 2.4: Intra-assay variability of the triglyceride assay

	Plasma 1	Plasma 2
Mean (mmol/L)	1.74	1.96
SD (mmol/L)	0.02	0.03
CV (%)	1.6	1.9
Number of Assays	10	10

2.2.4 Adipose tissue fatty acid composition

At the time of commencing the study and at surgery a narrow gauge needle subcutaneous adipose tissue biopsy was taken from the abdominal wall. The adipose tissue samples were then frozen at –70°C until the time of analysis.

Total lipid was extracted from adipose tissue using chloroform/methanol/water (2:1:0.8 vol/vol/vol). The homogenate of sample and solvent was closed under nitrogen and then vortexed vigorously for 1 min and chloroform and KCl (0.88%) were added. Again the sample was closed under nitrogen, vortexed and the lower phase was then collected after centrifugation (10 min, 2500rpm). The procedure was repeated from the point of addition of chloroform and KCl (Bligh and Dyer, 1957).

Fatty acid methyl esters were formed by reaction of the total lipid extract with hot methanolic boron trifluoride for one hour at 80°C. The fatty acid methyl esters were then purified using hexane/butylated hydroxytoluene and saturated aqueous sodium chloride solution. The methyl esters were then separated by gas chromatography (GC) in a Hewlett-Packard 6890 gas chromatograph (Hewlett-Packard Ltd., Bracknell, Bucks., U.K.) fitted with a 25 m x 0.32 mm SGE BPX70 capillary column with a film thickness of 0.25 μm. Helium at 1 ml/min was used as the carrier gas, and the split/splitless injector was used in the split mode with a split ratio of 20:1. Injector and detector temperatures were 280°C and 275°C, respectively, and the column oven temperature was held at 170°C for 12 min and then programmed to increase by 5°C/min until it reached 200°C. The detector was a flame ionising detector (FID). Fatty acid methyl esters were identified by comparison with standards run previously.

2.2.5 Statistical analysis

All values are presented as means ± SEM. Comparison of treatment group means was performed using one-way ANOVA followed by the least-significant difference post hoc test. Comparison of means before and after supplementation for each treatment group was performed using paired t-tests. Correlations were determined as Pearson correlation co-efficients. In all cases a statistical significance was assumed if $p<0.05$.

2.3 Results

2.3.1 Patient characteristics

The characteristics of patients by dietary treatment group are shown in Table 2.5. There were more males than females in each of the dietary groups, but the numbers of each were evenly distributed among the three groups. The age of the patients ranged from 38-85 years; mean ages were the same among groups and were the same for males and females. Patient's height and weight were measured and used to calculate body mass index (BMI). BMI for this group of patients ranged from 18.7 to 42.4; mean BMI values were the same among groups and they were not different between males and females. BMI distributions for each group are shown in Table 2.6 and were not different among groups.

Table 2.5 Characteristics of patients by treatment group

	GENDER		AGE (years)		BMI (kg/m^2)	
		N	range	(mean)	range	(mean)
Fish Oil	M	35	52-84	(68.7)	18.7-35.8	(25.9)
	F	20	56-84	(70.0)	20.2- 42.4	(26.4)
Sunflower Oil	M	34	44-81	(68.1)	20.1-34.1	(26.1)
	F	21	57-83	(71.3)	20.7-33.6	(25.5)
Control	M	37	38-85	(70.4)	20.2-37.1	(26.8)
	F	21	57-78	(70.0)	19.1-35.7	(26.4)

(M = male and F = female)

Table 2.6 BMI distributions by treatment group

	BMI (% patients)		
Diet	**<25**	**25-30**	**>30**
Fish Oil	47%	42%	11%
Sunflower oil	42%	47%	11%
Control	35%	49%	16%

2.3.2 Nutrient intakes of patients

The intakes of energy, of macronutrients and of selected micronutrients for patients by treatment group are shown in Table 2.7. Not all patients completed the weighed diaries and therefore the data presented are for about 68% of patients in each dietary group. Mean energy intake across all groups was 1916 kcal/d and did not differ among groups. Mean intake of fat was on average 60 g/day and represented about 28% of energy. Mean carbohydrate intake was 244 g/day and represented 51% of energy and mean protein intake was 75.5 g/day and represented 16% of energy. Mean intakes of nutrients were generally similar between groups, although total carbohydrate intake in the fish oil group was higher than in the control group ($p = 0.03$), dietary fat as a percent of energy in the control group was higher than in the fish oil group ($p = 0.04$) and vitamin E intake in the sunflower oil group was higher than in the fish oil group ($p = 0.05$).

The intakes of individual fatty acids in the different treatment groups are shown in Table 2.8 (as g/day) and 2.9 (as % energy). Of the 28% of energy consumed as fat, 9% was as cis-MUFA, 10.8% as SFA and 4.8% as cis-PUFA. Total n-3 fatty acid intake in this group of patients was 1.3 g/day, while total n-6 intake was 9.0 g/day.

The intakes of individual fatty acids were generally similar between groups, but mean intake of lauric acid (12:0) as a percent of energy in the control group was higher than in the fish oil group ($p = 0.05$) and mean intake of γ–linolenic acid (18:3n-6) as a percent of energy in the control group was significantly higher than the fish oil group ($p = 0.03$). Mean intakes of linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3) were 8.6

g/day and 0.9 g/day, respectively and were not different among treatment groups. Mean intakes of EPA (20:5n-3), DPA (22:5n-6) and DHA (22:6n-3) were 0.1, 0.03 and 0.13 g/day, respectively and were not different among treatment groups.

Table 2.7 Daily energy and nutrient intakes of patients by treatment group

Nutrient	Fish oil (n= 35)	Sunflower oil (n= 39)	Control (n=41)
Energy (kcals)	2003.8 ± 74.4	1911.2 ± 79.8	1833.4 ± 59.2
Fat (g)	60.0 ± 3.3	59.7 ± 3.8	60.3 ± 2.8
(% en)	26.8 ± 1.0[b]	27.7 ± 1.0	29.5 ± 0.8[a]
CHO (g)	262.0 ± 11.7[a]	239.2 ± 10.3	230.2 ± 8.66[b]
(% en)	52.1 ± 1.1	50.2 ± 0.9	50.1 ± 1.0
Protein (g)	78.2 ± 2.9	75.2 ± 2.9	73.1 ± 2.6
(% en)	15.9 ± 0.5	16.1 ± 0.4	16.2 ± 0.5
Alcohol (g)	5.7 ± 1.9	8.4 ± 1.9	5.3 ± 1.3
(% en)	1.95 ± 0.54	2.76 ± 0.59	1.84 ± 0.43
β−carotene (mg)	1.96 ± 0.23	1.88 ± 0.20	2.04 ± 0.19
Vitamin C (mg)	80.8 ± 8.0	73.0 ± 7.4	70.0 ± 6.2
Vitamin E (μg)	5.8 ± 0.6[b]	7.6 ± 0.7[a]	6.3 ± 0.6
Copper (mg)	1.28 ± 0.11	1.10 ± 0.07	1.12 ± 0.09
Zinc (mg)	9.4 ± 0.4	8.7 ± 0.4	8.3 ± 0.4
Iron (mg)	12.9 ± 0.9	11.1 ± 0.6	11.2 ± 0.6
Selenium (μg)	56.3 ± 3.9	61.4 ± 3.6	59.6 ± 3.8

Data are presented as mean ± SEM. CHO = carbohydrate. Statistical significance (one way ANOVA): groups with different letters are significantly different.

Table 2.8: Fatty acid intakes of patients by treatment group (g/day)

Fatty acid	Fish oil (n= 35)	Sunflower oil (n=39)	Control (n=41)
SFA (g)	22.88 ± 1.50	23.30 ± 2.01	23.26 ± 1.24
MUFA (g)	19.92 ± 1.23	18.79 ± 1.13	19.58 ± 1.00
PUFA (g)	9.60 ± 0.80	10.56 ± 0.77	10.07 ± 0.54
n-3 PUFA (g)	1.30 ± 0.11	1.20 ± 0.09	1.33 ± 0.11
n-6 PUFA (g)	8.51 ± 0.73	9.56 ± 0.78	8.91 ± 0.53
12:0 (g)	0.79 ± 0.08	0.88 ± 0.10	0.91 ± 0.08
14:0 (g)	2.22 ± 0.21	2.37 ± 0.28	2.33 ± 0.17
16:0 (g)	12.2 ± 0.70	12.4 ± 0.98	12.1 ± 0.64
18:0 (g)	5.02 ± 0.32	5.04 ± 0.42	5.20 ± 0.29
18:1n-9 (g)	16.6 ± 0.93	15.7 ± 1.0	15.9 ± 0.8
18:2n-6 (g)	8.14 ± 0.70	9.21 ± 0.79	8.50 ± 0.53
18:3n-3 (g)	0.98 ± 0.08	0.82 ± 0.05	0.91 ± 0.08
18:3n-6 (g)	0.02 ± 0.01	0.03 ± 0.01	0.04 ± 0.02
20:3n-6 (g)	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
20:4n-6 (g)	0.05 ± 0.00	0.05 ± 0.00	0.06 ± 0.00
20:5n-3 (g)	0.08 ± 0.01	0.11 ± 0.01	0.11 ± 0.02
22:5n-3 (g)	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
22:6n-3 (g)	0.10 ± 0.02	0.13 ± 0.02	0.15 ± 0.03

Data are presented as mean ± SEM. SFA = saturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids.

Table 2.9: Fatty acid intakes of patients by treatment group (% energy)

Fatty acid	Fish oil (n=35)	Sunflower oil (n=39)	Control (n=41)
SFA (% en)	10.28 ± 0.54	10.63 ± 0.61	11.36 ± 0.42
MUFA (% en)	8.88 ± 0.37	8.73 ± 0.30	9.54 ± 0.32
PUFA (% en)	4.28 ± 0.29	5.06 ± 0.33	4.96 ± 0.22
n-3 PUFA (% en)	0.58 ± 0.04	0.59 ± 0.04	0.65 ± 0.05
n-6 PUFA (% en)	3.80 ± 0.28	4.56 ± 0.34	4.40 ± 0.23
12:0 (% en)	0.36 ± 0.03[b]	0.40 ± 0.03	0.44 ± 0.03[a]
14:0 (% en)	1.00 ± 0.09	1.08 ± 0.10	1.14 ± 0.07
16:0 (% en)	5.51 ± 0.24	5.69 ± 0.28	5.90 ± 0.21
18:0 (% en)	2.25 ± 0.11	2.30 ± 0.12	2.53 ± 0.10
18:1n-9 (% en)	7.40 ± 0.28	7.24 ± 0.26	7.76 ± 0.28
18:2n-6 (% en)	3.64 ± 0.27	4.41 ± 0.36	4.20 ± 0.23
18:3n-3 (% en)	0.43 ± 0.03	0.40 ± 0.02	0.44 ± 0.03
18:3n-6 (% en)	0.01 ± 0.00[b]	0.01 ± 0.00	0.02 ± 0.01[a]
20:3n-6 (% en)	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
20:4n-6 (% en)	0.02 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
20:5n-3 (% en)	0.03 ± 0.00	0.05 ± 0.01	0.06 ± 0.01
22:5n-3 (% en)	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
22:6n-3 (% en)	0.04 ± 0.00	0.07 ± 0.01	0.07 ± 0.01

Data are presented as mean ± SEM. SFA = saturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids. Statistical significance (one way ANOVA): groups with different letters are significantly different.

2.3.2.1 Adipose tissue fatty acid composition

Table 2.10 shows the fatty acid composition of adipose tissue samples before supplementation. There were no differences in fatty acid composition of adipose tissue between groups. The largest contributor to adipose tissue fatty acid composition was MUFA, of which the major component was oleic acid (18:1n-9). The second largest contributor was SFA, mainly from palmitic acid (16:0).

The fatty acid composition of adipose tissue before supplementation was compared to data from the food diaries to assess the relationship between habitual fatty acid intake (in g/day) and adipose tissue fatty acid composition.

Table 2.10 Fatty acid composition of adipose tissue from patients in the different dietary groups before supplementation

Fatty acid	Fish oil (n = 48)	Sunflower oil (n = 51)	Control (n = 55)
14:0	4.00 ± 0.18	4.1 ± 0.16	4.00 ± 0.16
16:0	25.25 ± 0.40	25.73 ± 0.61	24.95 ± 0.46
16:1n-7	6.04 ± 0.32	6.38 ± 0.39	6.60 ± 0.36
18:0	3.78 ± 0.20	3.76 ± 0.14	3.61 ± 0.18
18:1n-9	43.85 ± 0.50	43.46 ± 0.55	43.55 ± 0.52
18:2n-6	13.63 ± 0.51	13.53 ± 0.54	14.21 ± 0.55
α18:3n-3	1.01 ± 0.05	1.08 ± 0.10	1.09 ± 0.07
20:1n-9	0.69 ± 0.08	0.86 ± 0.15	0.62 ± 0.07
20:4n-6	0.42 ± 0.05	0.41 ± 0.03	0.36 ± 0.02
20:5n-3	0.08 ± 0.03	0.06 ± 0.01	0.09 ± 0.02
22:6n-3	0.20 ± 0.02	0.24 ± 0.05	0.20 ± 0.02

Data are the mean ± SEM g/100g total fatty acid.

2.3.2.1.1 Adipose tissue fatty acid composition and SFA intake

There were significant positive correlations between the intakes of 16:0 ($r = 0.20$, $p<0.04$) and 18:0 ($r = 0.18$, $p<0.05$) and total SFA ($r = 0.21$, $p<0.03$) and the proportion of 16:0 in adipose tissue. However, no correlation was seen between intakes of 16:0, 18:0 and SFA and the proportion of 18:0 and SFA within adipose tissue. Significant negative correlations were found between 16:0 ($r = -0.24$, $p<0.01$), 18:0 ($r = -0.28$, $p<0.01$) and SFA ($r = -0.34$, $p<0.001$) in the diet and the proportion of 18:2n-6 in adipose tissue.

2.3.2.1.2 Adipose tissue fatty acid composition and MUFA intake

There was a significant negative correlation between intake of palmitoleic acid (16:1n-7) and the proportion of 18:2n-6 in adipose tissue ($r = -0.22$, $p<0.02$). A significant positive correlation was found between total MUFA intake and the proportion of arachidonic acid (20:4n-6) in adipose tissue ($r = 0.25$, $p<0.03$). However, no correlation was seen between intake of 18:1n-9 and the proportion of 18:1n-9 in adipose tissue.

2.3.2.1.3 Adipose tissue fatty acid composition and n-6 PUFA intake

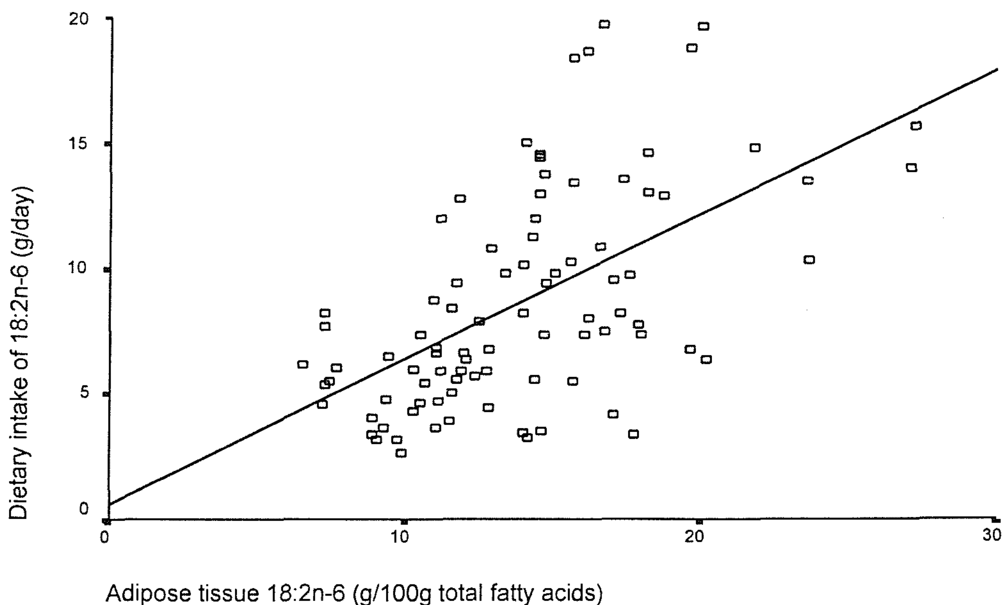
Table 2.11 shows the relationship between adipose tissue fatty acid composition and n-6 PUFA intake. There were highly significant positive correlations between 18:2n-6, total n-6 PUFA and total PUFA in the diet and the proportion of 18:2n-6 and total n-6 PUFA in adipose tissue. Figure 2.2 shows the relationship between dietary intake of 18:2n-6 and the proportion of 18:2n-6 in adipose tissue. In addition there was a significant positive correlation between dietary 18:2n-6 intake and the proportion of 20:4n-6 in adipose tissue (Table 2.11). A negative correlation was found between total dietary n-6 and total PUFA intake, and the proportion of 16:1n-7, 18:1n-9 and total MUFA in adipose tissue.

Table 2.11 Relationship between adipose tissue fatty acid composition, n-6 PUFA and total PUFA intake

Adipose fatty acid		Dietary fatty acid 18:2n-6	20:4n-6	n-6 PUFA	PUFA
16:1n-7	r	-0.053	-0.056	0.166	-0.176
	p	NS	NS	NS	0.048
18:1n-9	r	0.074	0.031	-0.189	-0.147
	p	NS	NS	0.037	NS
18:2n-6	r	0.589	0.278	0.562	0.541
	p	<0.0001	NS	<0.0001	<0.0001
20:4n-6	r	0.254	0.184	0.012	0.064
	p	0.041	NS	NS	NS
MUFA	r	-0.245	-0.044	-0.263	0.227
	p	0.01	NS	0.006	0.016
PUFA	r	0.421	-0.214	0.414	0.327
	p	0.002	NS	0.002	0.013

r = Pearson correlation coefficient; NS = not significant

Figure 2.3 Relationship between intake of 18:2n-6 and the proportion of 18:2n-6 in adipose tissue.



2.3.2.1.4 Adipose tissue fatty acid composition and n-3 PUFA intake

A negative correlation was found between 18:3n-3 intake and the proportion of 18:2n-6 in adipose tissue ($p<0.02$). A positive correlation was found between total n-3 PUFA intake and the proportion of total PUFA in adipose tissue ($p<0.04$). In addition, intake of DHA was positively correlated to the proportion of DHA in adipose tissue ($p<0.05$). However, no correlation was seen between intake of 18:3n-3 and the proportion of 18:3n-3 in adipose tissue.

2.3.3 Plasma, LDL and HDL lipid concentrations

2.3.3.1 Plasma lipid concentrations

Plasma total, free and esterified cholesterol and triacyglycerol (TAG) concentrations for the different treatment groups are shown in Table 2.12. Plasma total cholesterol concentrations ranged from 2.96-10.95 mmol/L and plasma TAG concentrations ranged from 0.40-9.98 mmol/L. Seventy per cent of patients had plasma total cholesterol concentrations below 5.2 mmol/L and 66% of patients had plasma TAG concentrations below 2.0 mmol/L. There were no significant differences between dietary groups for any of the plasma lipid concentrations.

Table 2.12: Plasma lipid concentrations in the different treatment groups before supplementation

	Fish oil (n = 53)		Sunflower oil (n = 55)		Control (n = 57)	
	Range	Mean ±SEM	Range	Mean ±SEM	Range	Mean ±SEM
TC	2.96-8.25	4.76 ± 0.15	2.97-7.39	4.72 ± 0.13	3.26-10.95	4.84 ± 0.17
FC	0.33-1.70	0.70 ± 0.04	0.41-1.51	0.71 ± 0.02	0.32-1.53	0.71 ± 0.03
CE	2.45-7.47	4.08 ± 0.14	2.47-6.55	4.00 ± 0.11	2.68-9.99	4.16 ± 0.16
TAG	0.40-9.98	1.70 ± 0.18	0.63-5.20	1.75 ± 0.15	0.56-4.26	1.67 ± 0.11

TC = Total cholesterol, FC = Free cholesterol, CE = Cholesterol ester, TAG = Triacyglycerol. All values are mmol/L.

Plasma lipid concentrations were analysed according to whether patients were being treated with statins or not (Table 2.13). 56 patients were being treated with statins; 19 (12%) in the control group, 19 (11%) in the fish oil group and 18 (11%) in the sunflower oil group. When all patients were analysed as one group at baseline plasma free, total and esterified cholesterol concentrations were all lower in patients being treated with statins compared to those not on statins. Plasma total, esterified and free cholesterol all tended to be lower in patients treated with statins in all three treatment groups. However the only significant difference was free cholesterol, which was lower in the fish oil and sunflower oil groups if patients were treated with statins.

Table 2.13 Plasma lipid concentrations in the different treatment groups according to statin treatment before supplementation

Treatment	Statin	N	FC	CE	TC	TAG
FO	Y	19	0.62 ± 0.03	3.85 ± 0.19	4.43 ± 0.20	1.57 ± 0.13
	N	34	0.75 ± 0.05*	4.20 ± 0.19	4.94 ± 0.20	1.76 ± 0.28
SO	Y	18	0.61 ± 0.03	3.79 ± 0.27	4.42 ± 0.27	1.83 ± 0.25
	N	37	0.76 ± 0.05*	4.09 ± 0.12	4.87 ± 0.13	1.70 ± 0.19
C	Y	19	0.70 ± 0.07	3.88 ± 0.38	4.57 ± 0.39	1.69 ± 0.19
	N	38	0.72 ± 0.03	4.30 ± 0.15	4.99 ± 0.16	1.66 ± 0.14
All patients	Y	56	0.65 ± 0.03	3.84 ± 0.17	4.48 ± 0.18	1.64 ± 0.10
	N	109	0.74 ± 0.03*	4.20 ± 0.09*	4.93 ± 0.09*	1.70 ± 0.12

FO = fish oil, SO = sunflower oil, C = control oil. TC = Total cholesterol, FC = Free cholesterol, CE = Cholesterol ester, TAG = Triacyglycerol. All values are mmol/L. Data are mean ± SEM, statistical significance (t-test): *denotes significantly different from statin treatment.

2.3.3.2 LDL and HDL lipid concentrations

LDL total, free and esterified cholesterol and TAG concentrations and HDL total cholesterol concentration for the different treatment groups are shown in Table 2.14. Ninety four per cent of patients had LDL total cholesterol concentrations below 3.5 mmol/L. There were no differences between treatment groups in LDL lipid concentrations or in HDL total cholesterol concentration.

Table 2.14: LDL and HDL lipid concentrations in the different treatment groups before supplementation

	Fish oil (n= 53)		**Sunflower oil (n=48)**		**Control (n=53)**	
	Range	**Mean ±SEM**	**Range**	**Mean ±SEM**	**Range**	**Mean ±SEM**
LDL TC	0.90-4.01	1.99 ± 0.09	0.80-3.69	1.93 ± 0.10	0.58-6.53	1.96 ± 0.14
LDL FC	0.14-0.51	0.27 ± 0.01	0.11-0.48	0.25 ± 0.01	0.08-0.60	0.27 ± 0.02
LDL CE	0.73-3.50	1.77 ± 0.09	0.68-3.25	1.73 ± 0.11	0.50-5.93	1.83 ± 0.17
LDL TAG	0.08-0.48	0.21 ± 0.01	0.10-0.56	0.21 ± 0.01	0.10-0.77	0.21 ± 0.01
HDL TC	0.04-6.53	1.95 ± 0.13	0.14-3.51	1.98 ± 0.09	0.22-3.86	2.19 ± 0.10

TC = Total cholesterol, FC = Free cholesterol, CE = Cholesterol ester, TAG = Triacyglycerol.
All values are mmol/L.

LDL and HDL lipid concentrations were analysed according to whether patients were being treated with statins or not (Table 2.15). When all patients were analysed as one group at baseline there were no significant differences in LDL or HDL lipid concentrations between patients being treated with statins and those who were not. All values tended to be lower in the statin treated group. However, the only significant difference was found in HDL total cholesterol in the sunflower oil group. Patients taking statins had lower HDL total cholesterol than those not taking statins in the sunflower oil group.

Table 2.15 LDL and HDL lipid concentrations in the different treatment groups according to statin treatment before supplementation

Treatment	Statin	N	LDL FC	LDL CE	LDL TC	LDL TAG	HDL TC
FO	Y	20	0.25 ± 0.02	1.62 ± 0.16	1.77 ± 0.15	0.21 ± 0.02	1.97 ± 0.11
	N	33	0.28 ± 0.02	1.87 ± 0.10	2.11 ± 0.11	0.22 ± 0.01	1.97 ± 0.20
SO	Y	16	0.23 ± 0.03	1.75 ± 0.27	1.85 ± 0.21	0.23 ± 0.03	1.73 ± 0.12
	N	32	0.26 ± 0.02	1.74 ± 0.12	1.97 ± 0.11	0.20 ± 0.01	2.10 ± 0.12*
C	Y	17	0.24 ± 0.04	1.78 ± 0.48	1.90 ± 0.33	0.22 ± 0.04	2.06 ± 0.16
	N	36	0.28 ± 0.02	1.86 ± 0.16	2.04 ± 0.14	0.20 ± 0.01	2.25 ± 0.13
All Patients	Y	53	0.24 ± 0.02	1.70 ± 0.17	1.84 ± 0.13	0.22 ± 0.02	1.92 ± 0.08
	N	101	0.27 ± 0.01	1.82 ± 0.01	2.04 ± 0.01	0.20 ± 0.00	2.11 ± 0.09

FO = fish oil, SO = sunflower oil, C = control oil. TC = Total cholesterol, FC = Free cholesterol, CE = Cholesterol ester, TAG = Triacyglycerol. All values are mmol/L. Data are mean ± SEM, statistical significance (t-test): *denotes significantly different from statin treatment.

2.4 Discussion

The aims of this chapter were firstly to describe the study design used, secondly to assess the characteristics of dietary intake, especially fatty acid intake, of patients with substantial atherosclerotic plaques, and thirdly to measure plasma lipid concentrations in these patients before dietary supplementation.

In this study patients were randomised to one of three dietary groups. Therefore, it was important to determine that there were no major differences in patient characteristics between groups. Indeed there were no major differences found between groups in terms of age, gender distribution, BMI, intakes of macro or micronutrients, blood lipid concentrations, adipose tissue fatty acid composition or proportion of patients being treated with statins.

In adults a useful surrogate for direct measurement of body fatness is the body mass index (BMI), which is weight (kg) divided by height $(m)^2$. Categories of BMI suggested for the UK are: under 20: underweight; 20-25: normal (desirable) range; 25-30: overweight; above 30: obese. It was found that values for this group of patients ranged from 18.7 to 42.4. About 40% of patients had values below 25, 46% had values between 25 and 30 and 14% had values above 30. Therefore, half of this patient group would be considered to be overweight and 14% would be considered obese; this is in general agreement with published data on the British population. In England between 1980 and 1991, the proportion of adults with a BMI between 25-30 increased from 35 to 40% of men and from 24 to 26% of women. In 1986/87 in Great Britain, 12% of women and 8% of men (aged 16-64) were obese (Gregory *et al.*, 1990), compared to 8% and 6% respectively in 1980 (OPCS, 1984). By 1991 these figures had increased to 15% and 13% respectively (White *et al.*, 1993). Data from the Health Survey for England 1996 (Prescott-Clarke and Primatesta, 1998) shows that the prevalence of obesity is still increasing, they reported that 18% of women and 16% of men were obese. The prevalence of obesity has, therefore, more than doubled in only 16 years. Obesity results from chronic energy consumption in excess of expenditure. Excess fatness is associated with metabolic changes which increase the risk of cardiovascular and other diseases. These changes include increased plasma TAG and LDL cholesterol concentrations

Blood lipid concentrations measured in plasma and LDL at baseline were not different between groups. However, the concentrations of LDL cholesterol and HDL cholesterol reported in this thesis were unusual in that the concentration of LDL cholesterol was lower than expected and that of HDL cholesterol was higher than expected. Expected concentrations would be 3.5 mM for LDL cholesterol and 1.2 mM for HDL cholesterol (Harris, 1997). The reason for this discrepancy most probably relates to the methodology used. In this study total plasma cholesterol concentration was measured and LDL was purified by centrifugation and the cholesterol concentration of the LDL fraction was measured. Recovery of LDL may have been less than 100% meaning that the LDL cholesterol concentration measured was less than the true value. Because HDL cholesterol concentration was calculated by a formula using the difference between total and LDL cholesterol concentration (See section 2.2.3.3) the HDL cholesterol concentration would be overestimated. Nevertheless, the plasma TAG and total cholesterol concentrations are considered to be correct. It will be important to reanalyse these samples for LDL and HDL cholesterol concentrations using a more appropiate method such as precipitation of VLDL and LDL with magnesium chloride and measuring the residual HDL cholesterol concentration. According to the data obtained here, about 70% of these patients had plasma total cholesterol values < 5.2 mmol/L, the threshold recommended by the European Atherosclerosis Society (1987). It was also found that 66% of patients had plasma TAG values < 2.0 mmol/L, and 94% had LDL total cholesterol values < 3.5 mmol/L. These apparently 'normal' lipid concentrations may be due to the fact that many patients may have modified their diets or lifestyles since being diagnosed with CVD and a number were being treated with statins. About one third of patients were taking statins with equal numbers in each of the lipid treatment groups When plasma lipids were analysed together for all patients plasma total, esterified and free cholesterol were all significantly lower in patients on statin treatment compared to those not taking statins. However when plasma lipids were analysed according to treatment group the lipid lowering effect of statins was not as significant but this is probably due to small number of patients in each group. HDL total cholesterol was lower in patients taking statins in the sunflower oil group but no other differences were found in LDL or HDL lipid concentrations according to statin treatment.

In 1986/87 the mean serum total cholesterol concentration in Britain was 5.8 mmol/L for both sexes and increased with age: 32% of males had serum cholesterol concentrations in the desirable range of < 5.2 mmol/L compared to 36% for females (Gregory *et al.*, 1990). Amongst patients in the 1991 Health Survey for England who were aged 19-64 and who were not taking lipid lowering drugs, the mean serum cholesterol concentration was 5.7 mmol/L for both sexes: 31% of men and 30% of women had concentrations less than 5.2 mmol/L (White *et al.*, 1993).

The background diet of patients was measured by using a 7-day weighed intake. It is possible that this method of dietary analysis can lead to under-reporting of intake or changes in intake. This is because subjects may be inclined to alter or mask their habitual intake of food because their diet is under scrutiny. Nevertheless it was important in this study to obtain a measure of nutrient intake and all available methods are subject to errors. Mean energy intake was 1916 kcal/d, compared with the estimated average requirement for this age group of 2100 kcal/d (Department of Health, 1991). The energy intake which is only 95% of the estimated average requirement may indicate under-reporting to a certain extent. Intake of fat averaged 60 g/day and represented 28% of energy, 9% was as cis-MUFA, 10.8% as SFA and 4.8% as cis-PUFA. It is recommended that no more than 35% of total dietary energy including alcohol should come from fat. SFA should provide no more than 11% of total dietary energy, cis-MUFA should provide 13% and cis-PUFA should provide 7.5% of total dietary energy and should be derived from a mixture of n-6 and n-3 PUFA (Department of Health, 1991). Therefore in this group of patients intakes of PUFA and MUFA are lower than they should be, but this is due to the lower than recommended total fat intake.

Total n-3 PUFA intake in this group of patients was 1.3 g/day with 0.9 g/day coming from α-linolenic acid, n-6 PUFA intake was 9.0 g/day with 8.6 g/day coming from linoleic acid. Intakes of EPA, DPA and DHA were 0.1, 0.03 and 0.13 g/day respectively. Current recommendations for dietary PUFA are as follows: linoleic acid should supply 15 g/day, α-linolenic acid 3 g/day, long chain n-3 PUFA 1 g/day and total PUFA intake should be 19 g/day (British Nutrition Foundation, 1999). Total PUFA intake in this group of patients was 10.3 g/day or 4.8 % of energy which is lower than the

current recommendation of 19 g/day or 7.5 % of energy, but again this largely reflects the low total fat intake among these patients.

The average intake of fat in Western countries is 75 to 150 g each day and fat generally contributes 35 to 45% of dietary energy. Intake of fat was considerably lower in this study, it averaged 60 g/day and represented 28 % of energy. This apparently lower intake of fat in these patients may be due to under-reporting and/or due to changes in dietary habits. The major fatty acids in the diet of adults in the United Kingdom are medium and long chain SFA, especially myristic, palmitic and stearic, the MUFA oleic and the PUFA linoleic and α-linolenic (see Table 1.2). Since 1970 the absolute consumption of SFA in the United Kingdom has declined by 40%, while the consumption of MUFA has declined by 30%. The consumption of PUFA increased by 25% over this period of time (Department of Health, 1991). This was largely due to increased consumption of linoleic acid which became generally available in margarines and cooking oils. According to the Adult Survey (Gregory *et al.*, 1990) the daily diet of the average adult male in the United Kingdom contains 42 g SFA, 31 g MUFA and 15.8 g PUFA. In the current study intakes of all fatty acids were lower than the average adult male, the intake of SFA was 23 g/day, MUFA was 19 g/day and PUFA was 10 g/day. The main PUFA in the diet is linoleic acid, intake is approximately 13.8 g/day for adult males, and α-linolenic acid contributes about 2 g/day. 0.7% of dietary energy in the diet of the UK population is n-3 PUFA and 6% is n-6 PUFA. However in the current study intake of linoleic acid was 8.6 g/day and α-linolenic acid was 0.9 g/day, total PUFA intake was 4.8% of energy again lower than average intakes. Total PUFA intake increased from 5% of dietary energy in 1980 to 7% in 1992 (Department of Health, 1994) and the long-term safety of diets rich in n-6 PUFA has been questioned (Ulbricht and Southgate, 1991). An estimate of the change in intake of n-3 fatty acids for the population as a whole over the past few years is available from the Total Diet Surveys for 1991 and 1995 (Ministry of Agriculture Fisheries and Food, 1997a). In 1991, total n-3 fatty acid intake was 1.61 g per day. By 1995, this had risen to 1.8 g per day. During this same period, total intake of n-6 fatty acids had fallen from 10.66 g per day to 10.2 g per day.

Adipose tissue fatty acid composition was compared to dietary intake data as it has been suggested that the fatty acid composition of adipose tissue is a good marker of

long term dietary intake. Circulating TAG, which originates partly from digestion of dietary TAG, is a major source of fatty acids for adipose tissue. Once incorporated into the tissue the fatty acids are known to have a relatively long half-life. For example, the half-life of linoleic acid (18:2n-6) in adipose tissue has been suggested to be approximately 2 years (Garland *et al.*, 1998). Adipose tissue fatty acid composition is therefore considered a strong indicator of long term dietary intake (Garland *et al.*, 1998). In this study there was a strong positive correlation between linoleic acid (18:2n-6) and total n-6 PUFA intake and the proportions of linoleic acid, arachidonic acid and total n-6 PUFA in adipose tissue. There was a positive correlation between n-3 PUFA intake and the proportion of total PUFA in adipose tissue and between DHA intake and its proportion in adipose tissue. These results show that there is a strong relationship between dietary PUFA intake and the fatty acid composition of adipose tissue. No significant correlation was seen between total SFA and MUFA intake, and the proportions of these fatty acids in adipose tissue. Other studies have investigated the relationship between diet and adipose tissue composition. The results of a study carried out by Garland *et al.* (1998) on the relationship between dietary intake and adipose tissue composition of selected fatty acids in women are supported by the data from this study. They did not find significant correlations between intakes of SFA and MUFA and the proportion of these fatty acids in adipose tissue However, they found significant correlations between total PUFA intake and the proportion of total PUFA in adipose tissue. Andersen *et al.* (1999) found significant positive correlations between intakes of linoleic acid, α-linolenic acid, EPA and DHA and the proportion of these fatty acids in adipose tissue. Leaf *et al.* (1995) found that plasma concentrations of n-3 PUFA (EPA, DPA, DHA and total n-3) significantly correlated with corresponding proportions of fatty acids in adipose tissue. The findings of this study support the results of these earlier studies. Therefore, it can be concluded that the PUFA composition of adipose tissue is a good marker of dietary PUFA intake.

The data from this study would suggest that intakes of fat and fatty acids for this group of patients are lower than intakes for the UK population which may seem surprising given that they have advanced atherosclerosis. The average diet of the patients within this study could be considered 'healthy' and not likely to promote atherosclerosis

and the onset of cardiovascular disease. However it must also be considered that these patients already have severe atherosclerosis and have been treated for this for a long period of time. Therefore, it is likely that these patients were given dietary advice about healthy eating and have modified their diets and other lifestyle factors accordingly in preparation for surgery.

Chapter 3

LDL oxidisability and fatty acid composition

3 LDL oxidisability and fatty acid composition

3.1 Introduction

3.1.1 LDL oxidation

There is evidence that oxidative modification increases the atherogenicity of LDL (Steinberg *et al.*, 1989; Salonen *et al.*, 1992). The presence of products of LDL oxidation in atherosclerotic lesions of rabbits and humans (Palinski *et al.*, 1989) is supportive of a role for oxidised LDL in the atherogenic process. The role of dietary fat and fatty acids in modifying the fatty acid composition of LDL, and thus its susceptibility to oxidation are of growing interest. It has been clearly shown that the oxidation of LDL is a lipid peroxidation process (Steinbrecher *et al.* 1984, Esterbauer *et al.* 1987, Steinbrecher, 1987) in which the PUFA of LDL are successively degraded to a variety of products. These lipid peroxidation products and their interactions with apoB are likely the cause for the altered functional properties of Ox-LDL (Esterbauer *et al.* 1987, Steinbrecher, 1987, Haberland and Fogelman, 1987, Haberland, 1988).

3.1.2 Lipid peroxidation

Lipid oxidation is a free radical-initiated process leading to oxidative deterioration of PUFA. Peroxidation involves direct attack on the unsaturated fatty acids in lipid molecules by oxygen. The reaction may be chemical, catalysed by metal ions (autoxidation), or enzymic, catalysed by the enzyme lipoxygenase. Lipid peroxidation is initiated when a hydrogen atom is removed from a methylene ($-CH_2-$) group in the hydrocarbon chain of a lipid molecule (Stage 1 in Figure 3.1). The molecular species that is formed is chemically reactive and is known as a free radical. There are many initiators of lipid peroxidation. One is 'singlet' oxygen, which itself may be formed from the normal form (ground state) of oxygen by 'sensitisers' such as chlorophylls, bilirubin, porphyrins and haem compounds, in the presence of light. Another common initiating

species is the hydroxyl radical ($OH^{\bullet}$), generated from superoxide anions (O_2^{-}) by ferric ion catalysis. Sensitisers and ferric ions are common in foods and biological tissues.

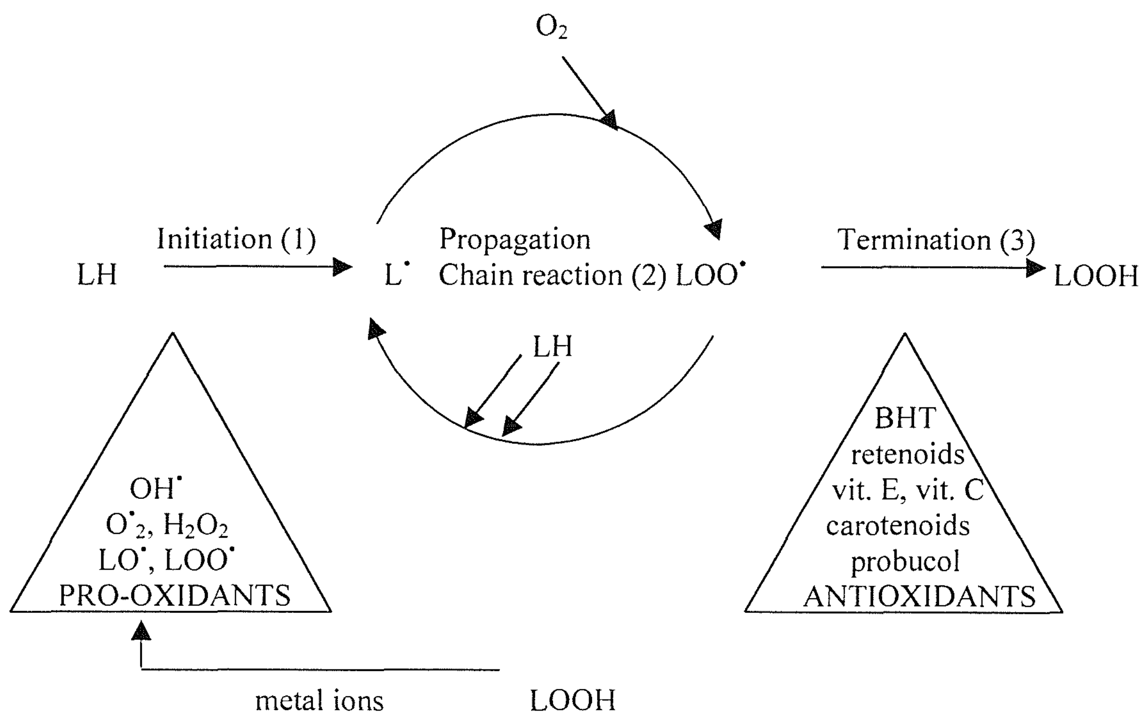
After initiation, the process of propagation gives rise to chemical rearrangements of the lipid molecules and further reaction with oxygen to form fatty acid peroxy- and hydroperoxy-radicals (Stage 2 in Figure 3.1). Once the process has started, it continues by a chain reaction of propagation steps which can be terminated (Stage 3 in Figure 3.1) in two ways: two lipid radicals can combine to form polymeric products, or fatty acid peroxyradicals can undergo cyclisation and the resulting cyclic endoperoxide breaks down into a range of end-products characteristic of lipid peroxidation, including malondialdehyde, other medium chain aldehydes, oxyacids and hydrocarbons.

One important factor in ensuring protection against uncontrolled lipid peroxidation is a well-organised membrane structure, especially in organelles involved in oxidative metabolism such as the mitochondria. Any tendency for membrane structure to break down will increase the chances of peroxidative damage. A second important protective factor is the presence of adequate antioxidant capacity. Antioxidants can be either preventative antioxidants or chain-breaking antioxidants.

Preventative antioxidants reduce the rate of chain-reaction initiation by converting peroxide radicals into inactive non-radical species: the enzymes catalase (which degrades hydrogen peroxide) and glutathione peroxidase are examples. Chain-breaking antioxidants interfere with chain propagation by trapping chain propagating radicals, for example $LOO^{\bullet}$ or $L^{\bullet}$ (Figure 3.1). Examples are the enzyme superoxide dismutase (which degrades superoxide) and vitamin E. Vitamin E is the major lipid soluble, chain-breaking antioxidant in human tissues. Vitamin C and the carotenoids are involved in the reconstitution of reduced vitamin E.

Figure 3.1 The process of lipid peroxidation

(L indicates a fatty acid chain)



3.1.3 Markers of lipid peroxidation

The extent of lipid peroxidation *in vivo, in vitro* or in model systems can be followed by the appearance of marker molecules (Halliwell, 1999; Esterbauer, 1996; Moore and Roberts, 1998). Most frequently oxidative damage to lipids is determined by measuring thiobarbituric acid-reactive substances (TBARS), an assay to determine malondialdehyde, a water-soluble product of lipid peroxidation. Another non-specific method is based on the measurement of conjugated dienes which are intermediates of lipid peroxidation (Esterbauer *et al.*, 1989). A number of different methods are available to determine lipid hydroperoxides. Recently the analysis of isoprostanes has been introduced as a measure of lipid peroxidation (Morrow and Roberts, 1997). It has been demonstrated the urinary excretion of F_2-isoprostanes increases in situations of oxidative stress (Proudfoot *et al.*, 1999). In the process of lipid peroxidation volatile products such

as ethane and pentane are also formed: they are exhaled with the breath and can be measured as a marker of lipid peroxidation (Kohlmuller and Kochen, 1993).

3.1.4 PUFA and LDL oxidation

All LDL is subject to peroxidation and the most common method used to measure this is copper- or iron-induced oxidation *ex vivo* which gives rise initially to conjugated dienes. The conjugated diene method has become one of the most frequently used assays to assess resistance of LDL to oxidation. The time course of oxidation forming conjugated dienes shows three consecutive phases: a lag-phase during which the diene formation increases only slowly, a propagation phase with a rapid increase in the diene formation and finally a decomposition phase. The increase in conjugated diene concentration is highly correlated with the increase in production of malondialdehyde (MDA) and lipid hydroperoxides. The susceptibility of LDL to oxidation is influenced by its PUFA content (i.e. the amount of substrate available to oxidise) and its antioxidant content (resists oxidation). Under *in vivo* or in vitro conditions, competition exists between oxidative and protective processes that depend on PUFA composition and on antioxidant levels. Therefore, for a given antioxidant content (especially vitamin E) increasing PUFA content should increase the susceptibility of LDL to oxidation, so decreasing lag time and increasing the amount of total oxidation products formed. However, for a given PUFA content increasing antioxidant content should increase lag time and so make LDL less susceptible to oxidation. The generation of oxidised LDL leads to enhanced uptake by macrophages via the scavenger receptor, stimulation of macrophage acyl-CoA: cholesterol acyltransferase (ACAT) activity and foam cell formation in arterial walls (section 1.2.2). The susceptibility of LDL to oxidation has been investigated in human studies, and effects of MUFA, n-6 PUFA and n-3 PUFA have been studied.

Several studies have demonstrated that the fatty acid composition of LDL is influenced by diets rich in oleic acid (Parthasarathy *et al.*, 1990; Reaven *et al.*, 1991; Berry *et al.*, 1992) and by supplementation of the diet with n-3 PUFA (Tripodi *et al.*, 1991; Nenseter *et al.*, 1992; Harats *et al.*, 1991). Oleate-rich LDL particles from patients consuming diets rich in oleic acid were reported to be highly resistant to oxidation, based

on formation of TBARS and conjugated dienes (Parthasarathy *et al.,* 1990; Reaven *et al.*, 1991; Berry *et al.*, 1992). In a study by Nenseter *et al.* (1991), the effect of dietary supplementation with fish oil on the susceptibility of LDL to oxidative modification was investigated. The patients received supplements of six capsules daily for four months; each capsule contained 1 g of n-3 PUFA (85% EPA plus DHA) or corn oil (56% linoleic acid and 26% oleic acid). LDL from both the fish oil and corn oil supplemented groups showed similar susceptibility to copper-catalysed lipid peroxidation. This was indicated by the amount of lipid peroxides formed during the oxidation process and by degradation of oxidatively modified LDL by J774 macrophages as a function of copper oxidation time. Frankel *et al.* (1994) looked at the effect of n-3 PUFA supplementation on copper-catalysed oxidation of LDL. Nine hypertriglyceridemic patients consumed 5.1 g of fish oil for 6 weeks. The volatile oxidation products from LDL were measured. Propanal and hexanal were the major volatile products formed in the oxidation of n-3 and n-6 PUFA respectively. Fish oil supplementation resulted in a significant increase in propanal formation and a small decrease in pentanal formation. However, total volatiles remained unchanged. This was interpreted as indicating that fish oil intake did not alter the overall oxidative susceptibility of human LDL. However, in other studies n-3 PUFA have been shown to enhance the susceptibility of LDL to oxidative modification. In a randomised double-blind cross-over study of fish oil and corn oil consumption by hypertensive patients, Suzukawa *et al.* (1995) examined changes in blood lipids, in lipoprotein composition and size and susceptibility of LDL to copper-induced and macrophage-mediated oxidation. They found that fish oil significantly decreased lag time before onset of copper-induced LDL oxidation and significantly increased production of TBARS during oxidation compared with corn oil. Corn oil had no significant effect on lag time or production of TBARS. Fish oil increased macrophage uptake of copper-oxidised LDL and of macrophage modified-LDL; corn oil was without effect. Therefore, the effects of dietary n-3 PUFA on the susceptibility of LDL to oxidation are contradictory.

Diets enriched with linoleic acid increase the linoleic acid content of LDL and, in some studies, increase LDL susceptibility to oxidation. In the studies referred to above in which corn oil was used as a placebo to study the effects of fish oil there was no effect of corn oil on LDL oxidisability (Nenseter *et al.,* 1991, Suzukawa *et al.*, 1995). Abbey *et al.*

(1993) fed 12 men a daily diet supplement containing 35 g linoleic acid-rich oil in one period and 35 g oleic rich-oil in the other period (2 x 3 wk crossover). The formation of conjugated dienes was measured. They found that LDL oxidised faster after linoleic acid supplementation than after oleic acid and produced more conjugated dienes in proportion to the increase in LDL linoleic acid content. However lag time was not altered by linoleic acid supplementation. Louheranta *et al.* (1996) studied the associations between the intake of dietary fatty acids and the susceptibility of plasma VLDL and LDL to oxidation, induced with either copper or hemin and hydrogen peroxide. The lipoproteins were from a population-based sample of middle aged men with "usual" fatty acid intakes and with no antioxidant supplementation. Dietary linoleic acid was the most important determinant of the maximal oxidation velocity for hemin-induced oxidation. For copper-induced oxidation the strongest determinant of maximal oxidation velocity was serum TAG and dietary linoleic acid was the second strongest determinant. Therefore, a high linoleic acid intake may be associated with increased susceptibility of atherogenic lipoproteins to oxidation.

3.1.5 Aims

The aims of this chapter were firstly to measure the fatty acid composition and oxidisability of plasma LDL in patients with advanced carotid atherosclerosis and secondly to determine whether plasma LDL fatty acid composition and oxidisability can be changed by short term increases in n-3 and n-6 PUFA intakes in these patients and to measure the time course for these changes.

3.2 Methods

3.2.1 Measurement of plasma and LDL lipid concentrations

LDL was prepared and LDL and plasma total cholesterol, free cholesterol and triacylglycerol concentrations were measured using the methods described in Section 2.2.3.

3.2.2 LDL Oxidation

3.2.2.1 Lipid Peroxides

Principle:

The method of El-Saadani *et al.* (1989) was used to measure the concentration of lipid peroxides in freshly-prepared LDL. The quantitation of lipid peroxides serves as a direct index of the oxidative status of tissues or membranes. This method provides a direct measure of lipid peroxides and it makes use of the oxidative capacity of lipid peroxides to convert iodide to iodine, which can be measured spectrophotometrically at 365nm.

Procedure:

LDL concentration was determined as mg protein/ml using the Lowry method. Bovine serum albumin was used as the standard. LDL was diluted with phosphate buffered saline (PBS) to 0.5 mg protein/ml. To an eppendorf, 20 µl EDTA (10 µM final concentration), 20 µl butylated hydroxytoluene (BHT) (10 µM final concentration), 100 µl LDL and 1 ml colour reagent (0.12 M potassium iodide, 0.15 mM sodium azide, 2 g/L Triton X-100, 0.1 g/L benzalkonium chloride, 10 µM ammonium molybate in 0.2 M potassium phosphate, 0.2 M sodium hydroxide, pH 6.2) were added. The tube was vortexed and the contents transferred to a cuvette and incubated for two hours in the dark at room temperature to allow the oxidation of iodide to iodine to be completed before the absorbance was measured at 365 nm against the colour reagent only as the blank.
The concentration of lipid peroxides in the samples was calculated using the molar absorptivity of I_3 measured at 365nm ($\varepsilon = 2.46 \times 10^4$ $M^{-1}. cm^{-1}$).

$A = \varepsilon C$ (where A = absorbance, ε = molar absorptivity of I_3 and C = concentration of lipid peroxides)

$$C = \frac{A}{\varepsilon} \times \frac{\text{Volume in cuvette}}{\text{Volume of LDL}}$$

Therefore

$$C = \frac{A}{2.46 \times 10^4} \times \frac{1140}{100} \quad \text{mol/L}$$

The concentration of lipid peroxides (mol/L) was then converted to nmol/L. To express the result as nmol/mg LDL protein, the concentration of lipid peroxides in nmol/L was divided by the protein concentration of LDL used (normally 0.1 or 0.5 mg/ml).

3.2.2.2 Conjugated Diene Formation

Principle:

The primary products of lipid peroxidation are lipid hydroperoxides of the general structure -CH=CH-CH=CH-CHOOH- with an absorption maximum around 234 nm. Since Ox-LDL is, like native LDL, fully soluble in buffer, the generation of such conjugated lipid hydroperoxides can be directly measured by recording the UV spectrum of the aqueous LDL solution.

Conjugated dienes in LDL were measured using the method of Princen *et al.* (1998) which is an adaptation of the method described previously by Esterbauer *et al.* (1989). This is used as a measure of the lag time for oxidation of LDL, which is considered to be a good indicator of the antioxidant status of an individual and of the susceptibility of LDL towards oxidative conditions. The kinetics of the oxidation of human LDL can be measured by continuously monitoring the change of absorbance at 234 nm. The kinetics of the diene formation i.e. the change in absorbance vs. time can be clearly divided into three phases. A first phase during which the dienes do not or only very slowly increase, a second phase during which the dienes very rapidly increase to a maximum value and a third phase during which the dienes strongly decrease again. The first two phases are termed the lag-phase and the propagation phase (Figure 3.2). During the lag phase the lipophilic antioxidants protect the PUFA against oxidation and thus

prevent the lipid peroxidation process entering into the propagating chain reaction. As shown in Figure 3.3 the protective action of the antioxidants progressively decreases since they are inactivated and consumed if they scavenge free radicals.

Figure 3.2 Kinetics of oxidation of LDL measured by continuous monitoring of the absorbance at 234nm. Three phases of oxidation are evident lag, propagation and decomposition.

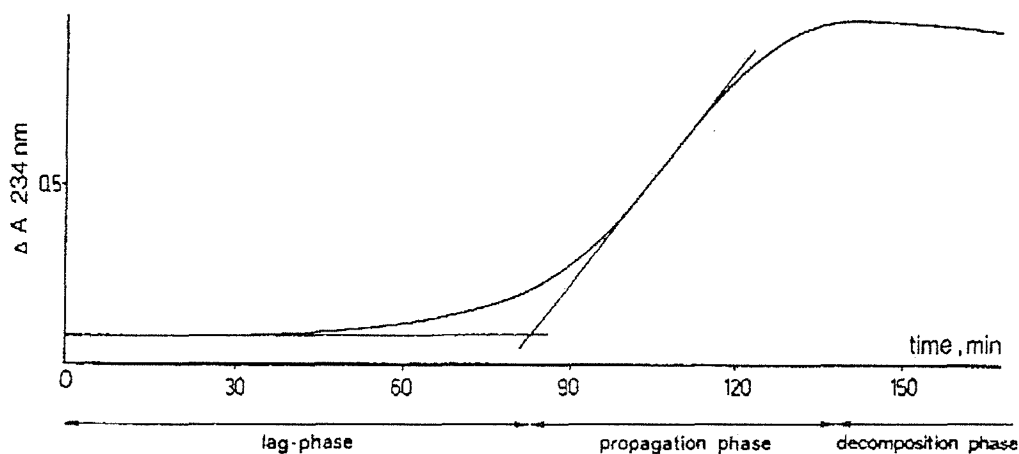
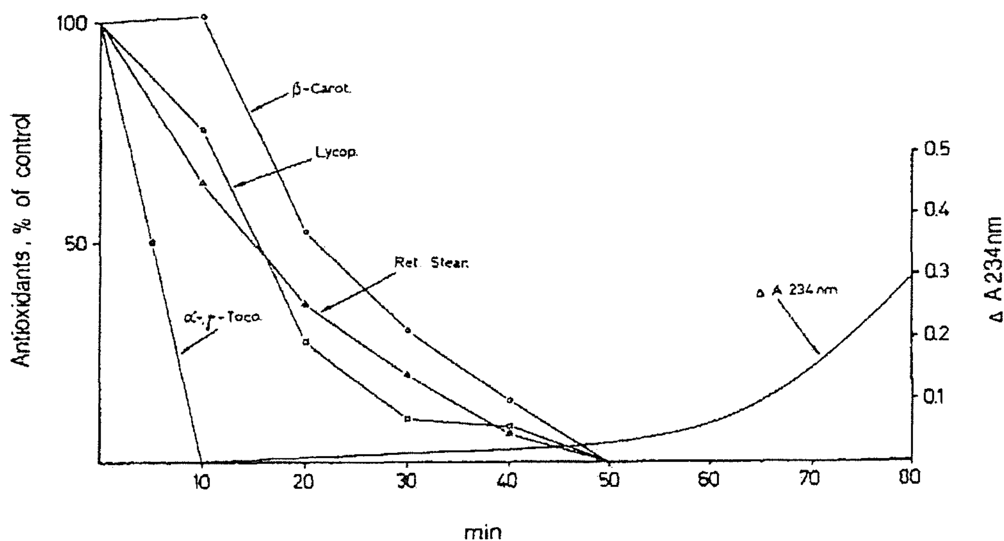


Figure 3.3 Time-course of the consumption of antioxidants and formation of conjugated dienes during oxidation of LDL (from Esterbauer *et al.*, 1989)



Preliminary experiments:

A series of preliminary experiments were performed to identify the optimal concentrations of copper and LDL protein to use in this protocol. Concentrations of copper used were 5 μM, 25 μM, 50 μM, 100 μM and 50 μM was found to be optimal. Concentrations of LDL protein used were 0.05 mg, 0.1 mg 0.25 mg, 0.5 mg and 0.1 mg was found to be optimal.

Procedure:

LDL was diluted with PBS to 0.1 mg protein/ml, and $CuSO_4$ was added to give a final copper concentration of 50 μM; this initiated lipid peroxidation. The absorbance of the sample at 234 nm was followed continuously for almost 3 hours to record the formation of conjugated dienes. These data were used to calculate the lag time, the time taken to reach half the maximum (T½), the maximum rate of oxidation and the maximum amount of conjugated dienes formed in the LDL. Absorbance curves of LDL preparations obtained from patients before and at the end of the intervention period were determined in parallel to avoid inter-assay variability. To test intra-assay variability the same LDL sample was oxidised in six consecutive oxidation runs on the same day. The results are presented in Table 3.1

Table 3.1: Intra-assay variability of Cu-induced oxidation of LDL

	Mean ± SD	**CV**
Oxidation rate (nmol diene/min/mgLDLprotein)	6.45 ± 0.38	5.9%
Maximum diene formation (nmol diene/mg LDL protein)	578 ± 20	3.4%
Lag time (min)	80.1 ± 6.8	8.4%
T ½ (min)	107.6 ± 5.4	5%

3.2.2.2.1 Role of vitamin E in preventing the oxidation of LDL

LDL was isolated from plasma (from a patient before and after fish oil supplementation) as previously described (section 2.2.3.1). LDL was diluted to 0.1 mg protein/ml. LDL was then enriched with α-tocopherol by incubating 1 ml of LDL with 5 nmol α-tocopherol for several hours: the α-tocopherol was dissolved in ethanol and 10 μl containing the desired amount were added to 1 ml LDL solution. A control sample of LDL with just ethanol added was oxidised at the same time. The LDL was then oxidised and the formation of conjugated dienes was measured as previously described (section 3.2.2.2). The lag time of oxidation was then calculated.

3.2.3 LDL fatty acid composition

Total lipid was extracted from LDL using chloroform/methanol/water as described earlier for adipose tissue (Section 2.2.4). Each lipid extract was then separated into cholesteryl ester (CE), triacylglycerol (TAG), and phospholipid (PL) by thin-layer chromatography (TLC) using hexane/ether/acetic acid (90:30:1 vol/vol/vol) as the elution phase. Fatty acid methyl esters from each lipid fraction were formed by reaction with hot methanolic boron trifluoride. The fatty acid methyl esters from each fraction were then purified using hexane/BHT and saturated aqueous NaCl. The fatty acid composition of each fraction was then determined by gas chromatography as described earlier (Section 2.2.4)

3.2.4 LDL Vitamin E content

α-Tocopherol, with tocopherol acetate added as an internal standard, was extracted from LDL with diethyl ether prior to reverse phase chromatography with UV detection at 292 nm.

Extraction of LDL

1. 100 μl of internal standard (tocopherol acetate 687.5 μM) was added to 100 μl of α-tocopherol standards (37.5 μM and 75.0 μM), sample or control.
2. 200 μl of methanol was added and the tubes were vortexed vigorously for 15 seconds.

3. 1 ml of diethyl ether was added and the tubes were then stoppered and vortexed for 1 minute and then centrifuged at 10,000 rpm for 5 minutes in a micro-centrifuge.
4. Using a glass pasteur pipette the upper diethyl ether layer was transferred into a glass extraction tube.
5. The samples were then evaporated under nitrogen at room temperature and then stored at –20°C until analysis.

The samples were analysed by HPLC by Robin West in the Department of Chemical Pathology, Southampton General Hospital.

3.2.5 Statistical analysis

All values are presented as means ± SEM. Comparison of treatment group means was performed using one-way ANOVA followed by the least-significant difference post hoc test. Comparison of means before and after supplementation for each treatment group was performed using paired t-tests. Correlations were determined as Pearson correlation co-efficients. In all cases a statistical significance was assumed if $p<0.05$.

3.3 Results

3.3.1 Treatment period

Patients received fish oil (6 g/day providing 1.7 g/day n-3 fatty acids) for 7-173 (mean 54) days depending on the time of their planned carotid endarterectomy. Over the entire study period the total amount of n-3 fatty acids provided via the capsules varied from 11.9 to 294 (mean 92) g depending on the length of fish oil treatment before surgery.

Patients received sunflower oil (6 g/day providing 3.8 g/day n-6 fatty acids) for 9-170 (mean 52) days depending on the time of their planned carotid endarterectomy. Over the entire study period the total amount of n-6 fatty acids provided via the capsules varied from 34 to 646 (mean 198) g depending on the length of sunflower oil treatment before surgery.

Patients received placebo oil (6 g/day) for 9-189 (mean 45) days depending on the time of their planned carotid endarterectomy.

3.3.2 Plasma Cholesterol and triacylglycerol concentrations

Plasma cholesterol and TAG concentrations were lowered significantly in each of the three treatment groups (Table 3.2). However, fish oil supplementation lowered plasma cholesterol and TAG concentrations to a greater extent than in the other two groups. The difference in esterified cholesterol and TAG between visits was significantly greater in the fish oil group compared to the control group. Fish oil supplementation caused a 15 % decrease in total cholesterol concentration, a 17% decrease in esterified cholesterol concentration and a 29 % decrease in TAG concentration. The TAG lowering effect of FO was not different between males and females (Table 3.3). Sunflower oil supplementation caused a 13 % decrease in total cholesterol concentration, a 13% decrease in esterified cholesterol concentration and a 12 % decrease in TAG concentration.

Table 3.2 Plasma cholesterol and triacylglycerol concentrations of patients in the different treatment groups before and after supplementation

		Fish oil (n = 53)	Sunflower oil (n = 55)	Control (n = 57)
TC	**V1**	4.8 ± 0.1	4.7 ± 0.2	4.8 ± 0.2
	V2	4.1 ± 0.1*	4.1 ± 0.1*	4.4 ± 0.2*
	V2-V1	-0.71 ± 0.14	-0.59 ± 0.12	-0.40 ± 0.10
CE	**V1**	4.1 ± 0.1	4.0 ± 0.1	4.2 ± 0.2
	V2	3.4 ± 0.1*	3.5 ± 0.1*	3.8 ± 0.2*
	V2-V1	-0.69 ± 0.13[a]	-0.43 ± 0.11	-0.30 ± 0.10[b]
FC	**V1**	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0
	V2	0.6 ± 0.2*	0.7 ± 0.2*	0.6 ± 0.2*
	V2-V1	-0.08 ± 0.03	-0.06 ± 0.02	-0.08 ± 0.02
TAG	**V1**	1.7 ± 0.2	1.7 ± 0.1	1.7 ± 0.1
	V2	1.2 ± 0.1*	1.5 ± 0.1*	1.5 ± 0.1*
	V2-V1	-0.43 ± 0.18[a]	-0.25 ± 0.09	-0.18 ± 0.08[b]

TC = Total cholesterol, FC = Free cholesterol, CE = Cholesterol ester, TAG = Triacyglycerol. All values are mean ± SEM mmol/L before (V1) and after (V2) supplementation. Statistical significance: groups with different letters are significantly different (one way ANOVA), *significantly different from visit 1 (paired t-test).

Table 3.3 The effect of gender on plasma TAG concentrations in the fish oil group

	n		TAG (mmol/L)	V2-V1 (mmol/L)	V2-V1 (% change)
Males	35	visit 1	1.8 ± 0.27		
		visit 2	1.3 ± 0.15*	-0.5 ± 0.2	-27 ± 7.6
Females	18	visit 1	1.5 ± 0.10		
		visit 2	1.0 ± 0.07*	-0.4 ± 0.08	-31 ± 4.0

Data are mean ± SEM. Statistical significance: *significantly different from visit 1 (paired t-test).

The effect of lipid treatments upon plasma cholesterol and triacylglycerol concentrations were analysed according to whether patients were taking statins or not (Table 3.4). Free cholesterol was lower in the group taking statins after both FO and SO supplementation and total cholesterol was lower after FO supplementation in the group taking statins compared to the group not taking statins. Total and esterified cholesterol were lower in both statin groups after FO supplementation whereas only patients not taking statins had significantly lower plasma total and esterified cholesterol after SO supplementation.

Table 3.4 Plasma cholesterol and triacylglycerol concentrations in the different treatment groups according to statin treatment before and after supplementation

Treatment	Statin	n	Visit	FC	CE	TC	TAG
FO	Y	19	1	0.6 ± 0.0	3.9 ± 0.2[a]	4.4 ± 0.2[a]	1.6 ± 0.1
	N	34	1	0.8 ± 0.1*	4.2 ± 0.2[a]	4.9 ± 0.2[a]	1.8 ± 0.3
	Y	19	2	0.6 ± 0.0	3.2 ± 0.1[b]	3.8 ± 0.2[b]	1.2 ± 0.1
	N	34	2	0.7 ± 0.0*	3.5 ± 0.2[b]	4.2 ± 0.2*[b]	1.2 ± 0.2
SO	Y	18	1	0.6 ± 0.0	3.8 ± 0.3	4.4 ± 0.3	1.8 ± 0.3
	N	37	1	0.8 ± 0.1*	4.1 ± 0.1[a]	4.9 ± 0.1[a]	1.7 ± 0.2
	Y	18	2	0.5 ± 0.0	3.1 ± 0.3	3.7 ± 0.3	1.4 ± 0.1
	N	37	2	0.7 ± 0.0*	3.6 ± 0.1[b]	4.3 ± 0.2[b]	1.5 ± 0.2
C	Y	19	1	0.7 ± 0.1	3.9 ± 0.4	4.6 ± 0.4	1.7 ± 0.2
	N	38	1	0.7 ± 0.0	4.3 ± 0.2	5.0 ± 0.2	1.7 ± 0.1
	Y	19	2	0.6 ± 0.0	3.5 ± 0.4	4.1 ± 0.4	1.5 ± 0.1
	N	38	2	0.7 ± 0.0	4.0 ± 0.2	4.6 ± 0.2	1.5 ± 0.2

FO = fish oil, SO = sunflower oil, C = control oil. TC = Total cholesterol, FC = Free cholesterol, CE = Cholesterol ester, TAG = Triacyglycerol. All values are mean ± SEM mmol/L. Visit 1= before supplementation, visit 2 = after supplementation. Statistical significance (t-test): *significantly different between statin treatments, groups with different letters are significantly different between visits.

3.3.3 LDL and HDL cholesterol and triacylglycerol concentrations

No significant changes were observed in LDL TAG or cholesterol concentrations in patients receiving control capsules (Table 3.5). Supplementation with fish oil resulted in a significant decrease in LDL esterified cholesterol (-11%) and HDL total cholesterol (-15%) concentration; no significant changes were seen in LDL TAG concentrations. Sunflower oil supplementation caused a significant decrease in LDL total cholesterol concentration (-5%).

Table 3.5 LDL and HDL cholesterol and triacylglycerol concentrations of patients in the different treatment groups before and after supplementation

		Fish oil (n = 53)	Sunflower oil (n = 48)	Control (n = 53)
LDL TC	**V1**	2.0 ± 0.1	1.9 ± 0.1	2.0 ± 0.1
	V2	1.8 ± 0.1	1.8 ± 0.1*	1.8 ± 0.1
	V2-V1	-0.16 ± 0.09	-0.17 ± 0.01	-0.16 ± 0.10
LDL CE	**V1**	1.8 ± 0.1	1.7 ± 0.1	1.8 ± 0.2
	V2	1.6 ± 0.1*	1.5 ± 0.1	1.5 ± 0.1
	V2-V1	-0.22 ± 0.11	-0.16 ± 0.01	-0.15 ± 0.13
LDL FC	**V1**	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
	V2	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0
	V2-V1	-0.00 ± 0.01	-0.01 ± 0.01	0.02 ± 0.01
LDL TAG	**V1**	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
	V2	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
	V2-V1	0.01 ± 0.01	0.01 ± 0.01	0.00 ± 0.01
HDL TC	**V1**	2.0 ± 0.1	2.0 ± 0.1	2.2 ± 0.1
	V2	1.7 ± 0.1*	1.7 ± 0.1	2.0 ± 0.1
	V2-V1	-0.36 ± 0.15	-0.24 ± 0.13	-0.23 ± 0.13

TC = Total cholesterol, FC = Free cholesterol, CE = Cholesterol ester, TAG = Triacyglycerol. All values are mean ± SEM mmol/L before (V1) and after (V2) supplementation. Statistical significance: groups with different letters are significantly different (one way ANOVA), * significantly different from visit 1 (paired t-test)

The effect of lipid treatments upon LDL and HDL lipid concentrations were analysed according to whether patients were taking statins or not (Table 3.6). LDL total and esterified cholesterol concentrations were lower in patients taking statins compared to those not on statins after FO and SO supplementation.

Table 3.6 LDL and HDL cholesterol and TAG concentrations in the different treatment groups according to statin treatment before and after supplementation

Treat-ment	Statin	n	Visit	LDL FC	LDL CE	LDL TC	LDL TAG	HDL TC
FO	Y	20	1	0.2 ± 0.0	1.6 ± 0.2	1.8 ± 0.1	0.2 ± 0.0	2.0 ± 0.1
	N	33	1	0.3 ± 0.0	1.9 ± 0.1	2.1 ± 0.1	0.2 ± 0.0	2.0 ± 0.2
FO	Y	20	2	0.2 ± 0.0	1.3 ± 0.1	1.5 ± 0.1	0.2 ± 0.0	1.7 ± 0.1
	N	33	2	0.3 ± 0.0	1.8 ± 0.1*	2.0 ± 0.1*	0.2 ± 0.0	1.7 ± 0.1
SO	Y	16	1	0.2 ± 0.0	1.7 ± 0.3	1.8 ± 0.2	0.2 ± 0.0	1.7 ± 0.1
	N	32	1	0.3 ± 0.0	1.7 ± 0.1	2.0 ± 0.1	0.2 ± 0.0	2.1 ± 0.1*
SO	Y	16	2	0.2 ± 0.0	1.3 ± 0.1	1.5 ± 0.1	0.2 ± 0.0	1.7 ± 0.2
	N	32	2	0.2 ± 0.0	1.7 ± 0.1*	1.9 ± 0.1*	0.2 ± 0.0	1.8 ± 0.1
C	Y	17	1	0.2 ± 0.0	1.8 ± 0.5	1.9 ± 0.3	0.2 ± 0.0	2.1 ± 0.2
	N	36	1	0.3 ± 0.0	1.9 ± 0.2	2.0 ± 0.1	0.2 ± 0.0	2.2 ± 0.1
C	Y	17	2	0.2 ± 0.0	1.4 ± 0.3	1.7 ± 0.4	0.2 ± 0.0	1.9 ± 0.1
	N	36	2	0.3 ± 0.0	1.6 ± 0.1	1.9 ± 0.1	0.2 ± 0.1	2.0 ± 0.1

TC = Total cholesterol, FC = Free cholesterol, CE = Cholesterol ester, TAG = Triacyglycerol. All values are mean ± SEM mmol/L. Visit 1= before supplementation, visit 2 = after supplementation. Statistical significance (t-test): * significantly different between statin treatment, groups with different letters are significantly different between visits.

3.3.4 Fatty acid composition of LDL

The fatty acid composition of PL, TAG, and CE of purified LDL were determined (Tables 3.7, 3.8, 3.9). Not all samples were analysed because of constraints on time and also it was not necessary to analyse all samples to observe the significant affect of treatment (Tables 3.7, 3.8, 3.9). Fewer samples of TAG and CE than of PL were analysed. The details of the patients whose LDL fatty acid composition were analysed are given in Table 3.6a. By comparing this information with that for the treatment group as a whole (Table 2.5; section 2.3.1) it is apparent that the patients whose fatty acid composition was determined are representative of the treatment group.

Table 3.6a Characteristics of patients whose LDL fatty acid composition was determined

Fraction	Treatment	N	Male (N)	Female (N)	Age (years)	BMI (kg/m^2)	Statin (% patients)
PL	FO	42	29	14	69.1	26.5	13
	SO	40	28	12	69.6	26.1	9
	C	44	26	18	70.8	26.0	10
TAG	FO	34	22	12	69.4	26.3	11
	SO	36	23	13	70.6	25.6	8
	C	36	22	14	72.1	25.9	9
CE	FO	32	21	11	69.3	26.2	12
	SO	35	23	12	70.4	25.6	10
	C	34	21	13	70.9	25.9	10

Data for age, BMI and statin are presented as mean.

Some changes were observed in LDL fatty acid composition in the control group. The proportions of 16:0 in LDL PL and 18:2n-6 in LDL TAG increased after supplementation (Tables 3.7 and 3.8). SO supplementation significantly enriched LDL CE in 18:2n-6 (Table 3.9). The proportion of 18:2 n-6 in LDL PL and CE after SO supplementation was significantly higher than in the FO group (Table 3.7 and 3.9). In contrast, FO supplementation significantly enriched LDL CE, PL and TAG in long-chain n-3 PUFA (i.e. EPA and DHA) (Tables 3.7, 3.8 and 3.9). The proportion of EPA was 3-times higher and the proportion of DHA was twice as high in the LDL fractions after fish oil supplementation. The proportion of docosapentaenoic acid (DPA) was also higher in LDL PL and TAG after FO supplementation (Table 3.7 and 3.8). The proportions of n-6 fatty acids and 18:1n-9 decreased in all LDL lipid fractions after fish oil supplementation.

The length of FO supplementation was significantly correlated with the proportion of EPA ($r = 0.54$, $p = 0.002$, Figure 3.4) and DPA ($r = 0.53$, $p = 0.003$, Figure 3.5) in LDL PL. The duration of FO supplementation did not appear to be related to the accumulation of DHA in LDL PL ($r = 0.20$, $p = 0.32$, Figure 3.6). The length of FO supplementation was not significantly correlated with the accumulation of EPA or DHA in LDL TAG or CE (Figures 3.7, 3.8 ,3.9, 3.10).

Table 3.7 Fatty acid composition of LDL phospholipids of patients in the different treatment groups before and after supplementation

Fatty acid	Visit	Fish oil (n = 43)	Sunflower oil (n = 42)	Control (n = 45)
16:0	1	30.0 ± 0.4	30.5 ± 0.4	30.1 ± 0.3
	2	31.6 ± 0.3*	30.9 ± 0.5	31.4 ± 0.3*
16:1n-7	1	0.8 ± 0.1	0.7 ± 0.1	0.8 ± 0.1
	2	0.7 ± 0.0	0.7 ± 0.1	0.7 ± 0.0
18:0	1	13.3 ± 0.1	13.3 ± 0.2	13.6 ± 0.2
	2	13.6 ± 0.2	13.2 ± 0.2	13.5 ± 0.2
18:1n-9	1	11.7 ± 0.3	11.6 ± 0.3	11.4 ± 0.3
	2	10.6 ± 0.2*	11.0 ± 0.3*	11.2 ± 0.2
18:2n-6	1	20.4 ± 0.4	19.9 ± 0.4	19.5 ± 0.5
	2	17.7 ± 0.4[a]*	20.8 ± 0.5[b]	20.1 ± 0.4[b]
18:3n-3	1	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0
	2	0.3 ± 0.1	0.4 ± 0.1	0.2 ± 0.0
20:3n-6	1	3.4 ± 0.1	3.2 ± 0.1	3.4 ± 0.1
	2	2.3 ± 0.1[a]*	3.3 ± 0.1[b]	3.4 ± 0.1[b]
20:4n-6	1	8.7 ± 0.3	8.3 ± 0.3	8.8 ± 0.3
	2	7.8 ± 0.2[a]*	8.6 ± 0.3[b]	8.7 ± 0.3[b]
20:5n-3	1	1.3 ± 0.2	1.3 ± 0.1	1.0 ± 0.1
	2	3.7 ± 0.2[a]*	1.1 ± 0.1[b]	1.0 ± 0.1[b]
22:5n-3	1	1.9 ± 0.1	2.0 ± 0.2	1.8 ± 0.1
	2	2.1 ± 0.1[a]	1.7 ± 0.1[b]*	1.7 ± 0.1[b]
22:6n-3	1	3.4 ± 0.2	3.7 ± 0.2	3.3 ± 0.2
	2	6.0 ± 0.2[a]*	3.7 ± 0.2[b]	3.6 ± 0.2[b]

Data are the mean ± SEM g/100 g total fatty acid before (visit 1) and after (visit 2) supplementation. Statistical significance: groups with different letters are significantly different (one way ANOVA), *significantly different from visit 1 (paired t-test).

Figure 3.4 Effect of length of supplementation with FO on EPA accumulation in LDL PL

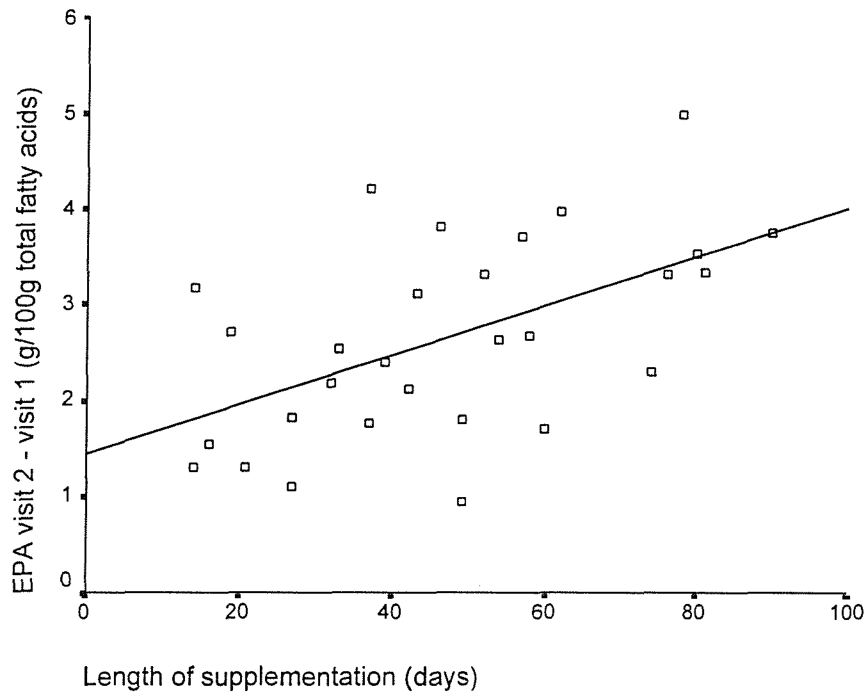


Figure 3.5 Effect of length of supplementation with FO on DPA accumulation in LDL PL

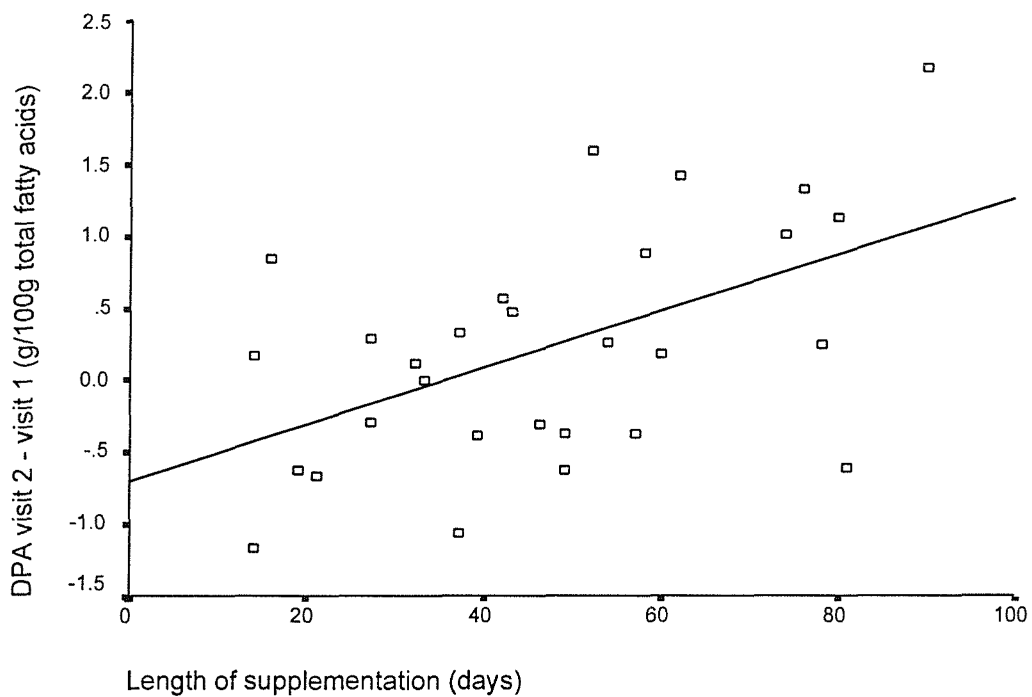


Figure 3.6 Effect of length of supplementation with FO on DHA accumulation in LDL PL

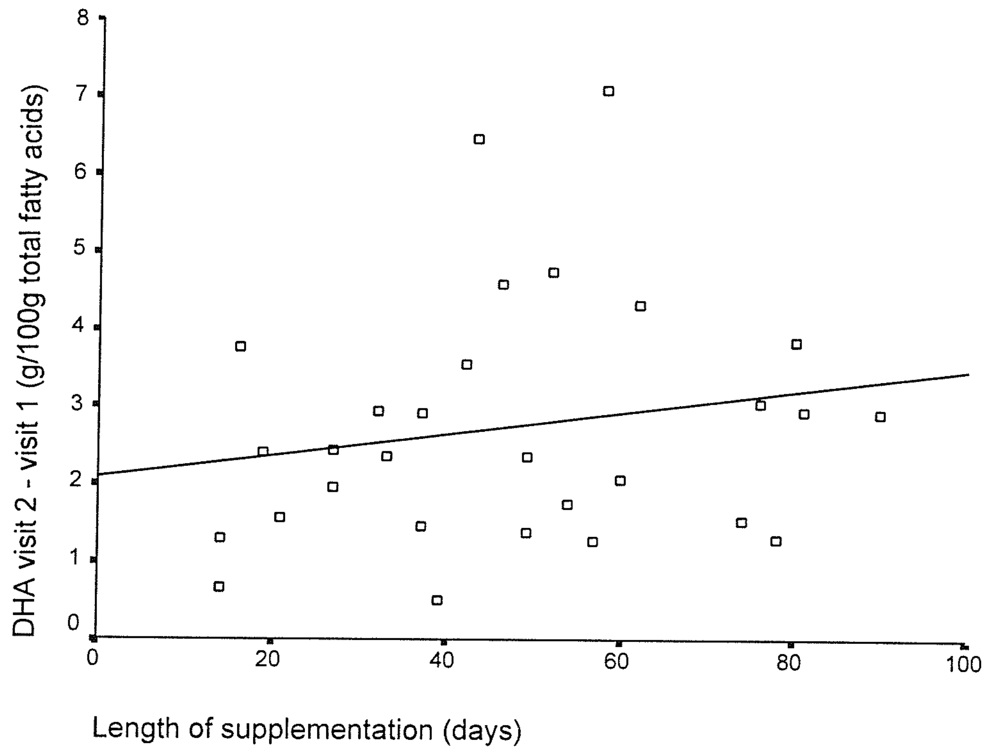


Table 3.8 Fatty acid composition of LDL TAG of patients in the different treatment groups before and after supplementation.

Fatty acid	Visit	Fish oil (n = 34)	Sunflower oil (n = 36)	Control (n = 36)
16:0	1	26.4 ± 0.5	27.3 ± 0.7	26.7 ± 0.5
	2	27.0 ± 0.6	27.0 ± 0.6	27.3 ± 0.5
16:1n-7	1	3.8 ± 0.2	4.4 ± 0.3	4.2 ± 0.2
	2	3.9 ± 0.2	3.8 ± 0.2*	4.1 ± 0.2
18:0	1	4.1 ± 0.1	4.5 ± 0.2	4.1 ± 0.1
	2	4.2 ± 0.1	5.0 ± 1.0	4.1 ± 0.1
18:1n-9	1	41.4 ± 0.5	41.6 ± 0.7	41.4 ± 0.5
	2	37.8 ± 0.6[a]*	39.4 ± 1.0*	40.7 ± 0.6[b]
18:2n-6	1	15.4 ± 0.6	14.7 ± 0.9	15.0 ± 0.7
	2	15.0 ± 0.7	16.5 ± 0.9	16.2 ± 0.7*
18:3n-3	1	0.9 ± 0.1	0.8 ± 0.1	0.9 ± 0.1
	2	1.1 ± 0.1*	0.8 ± 0.1	0.9 ± 0.1
20:3n-6	1	0.4 ± 0.0	0.4 ± 0.1	0.4 ± 0.0
	2	0.6 ± 0.4	0.4 ± 0.1	0.4 ± 0.1
20:4n-6	1	1.7 ± 0.1	1.6 ± 0.1	1.6 ± 0.1
	2	1.6 ± 0.1	1.8 ± 0.1*	1.7 ± 0.1*
20:5n-3	1	0.5 ± 0.0	0.5 ± 0.1	0.5 ± 0.1
	2	1.7 ± 0.2[a]*	0.6 ± 0.2[b]	0.4 ± 0.1[b]
22:5n-3	1	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.1
	2	1.3 ± 0.3[a]*	0.6 ± 0.1[b]	0.5 ± 0.1[b]
22:6n-3	1	0.9 ± 0.1	1.0 ± 0.1	0.9 ± 0.1
	2	2.2 ± 0.2[a]*	1.1 ± 0.2[b]	1.0 ± 0.1[b]

Data are the mean ± SEM g/100 g total fatty acid before (visit 1) and after (visit 2) supplementation. Statistical significance (one way ANOVA): groups with different letters are significantly different, *denotes significantly different from visit 1 (paired t-test).

Figure 3.7 Effect of length of supplementation with FO on EPA accumulation in LDL TAG

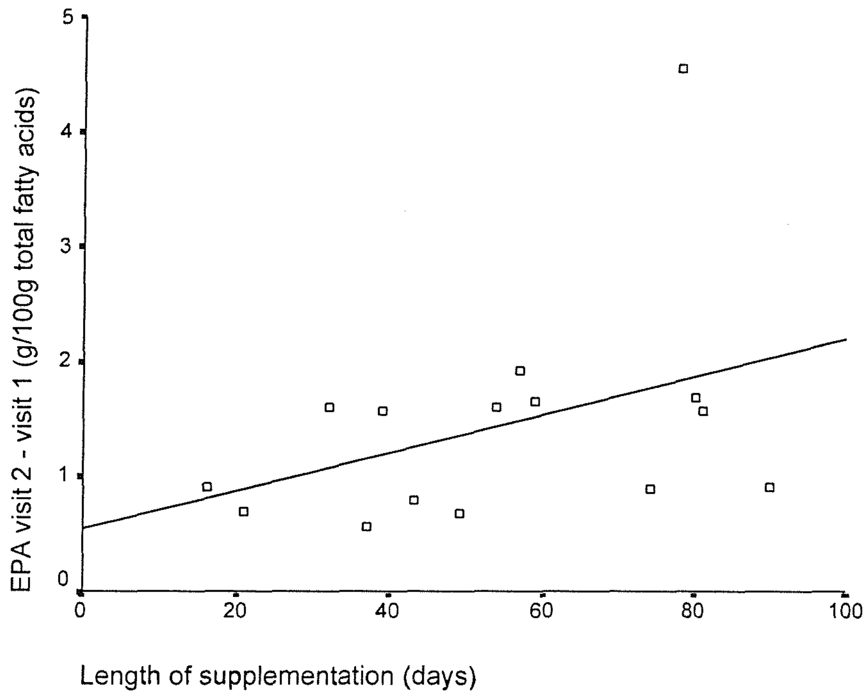


Figure 3.8 Effect of length of supplementation with FO on DHA accumulation in LDL TAG

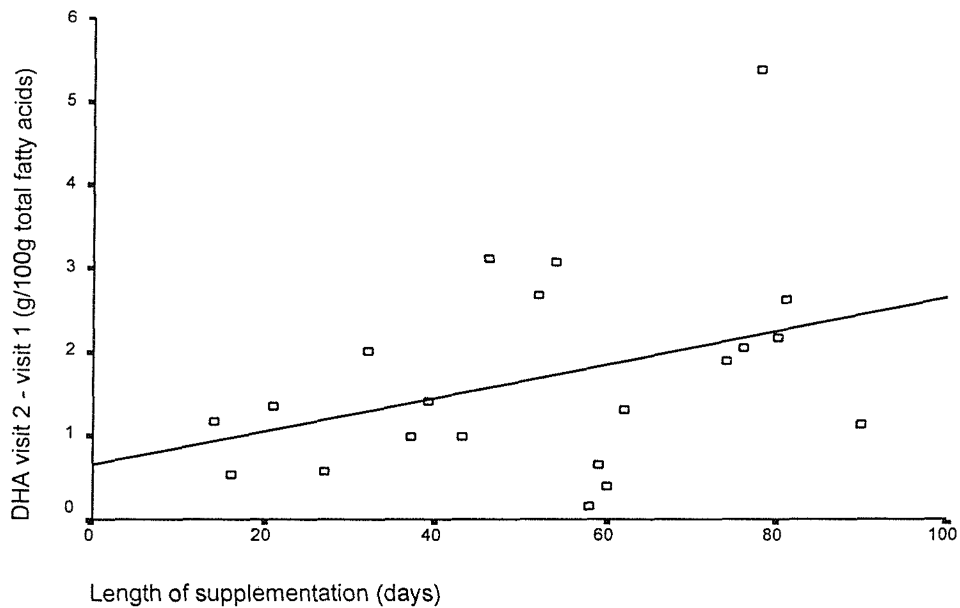


Table 3.9 Fatty acid composition of LDL CE of patients in the different treatment groups before and after supplementation

Fatty acid	Visit	Fish oil (n = 32)	Sunflower oil (n = 35)	Control (n = 34)
16:0	1	12.5 ± 0.3	12.8 ± 0.2	12.7 ± 0.3
	2	13.7 ± 0.3*	13.0 ± 0.2	12.8 ± 0.2
16:1n-7	1	3.6 ± 0.2	4.1 ± 0.3	3.9 ± 0.3
	2	3.7 ± 0.3	3.8 ± 0.3	4.0 ± 0.3
18:0	1	0.7 ± 0.1	0.7 ± 0.0	1.3 ± 0.6
	2	1.2 ± 0.5	0.8 ± 0.0	0.8 ± 0.1
18:1n-9	1	19.2 ± 0.4	19.4 ± 0.4	19.4 ± 0.8
	2	20.0 ± 1.2	18.0 ± 0.5*	18.6 ± 0.4*
18:2n-6	1	49.4 ± 0.7	48.3 ± 0.9	49.2 ± 1.5
	2	46.7 ± 0.8[a]*	51.3 ± 0.9[b]*	48.7 ± 1.3
18:3n-6	1	0.8 ± 0.0	0.8 ± 0.1	0.8 ± 0.1
	2	0.6 ± 0.1[a]	0.9 ± 0.1[b]	1.0 ± 0.1[b]
18:3n-3	1	0.6 ± 0.0	0.6 ± 0.0	0.7 ± 0.0
	2	0.6 ± 0.0	0.5 ± 0.0	0.6 ± 0.0
20:3n-6	1	0.7 ± 0.0	0.8 ± 0.0	1.0 ± 0.2
	2	0.7 ± 0.0	0.8 ± 0.0	0.8 ± 0.0
20:4n-6	1	6.7 ± 0.3	7.0 ± 0.3	6.1 ± 0.2
	2	5.9 ± 0.3*	6.6 ± 0.3	6.2 ± 0.2
20:5n-3	1	1.2 ± 0.1	1.3 ± 0.1	1.1 ± 0.1
	2	4.2 ± 0.3[a]*	1.2 ± 0.1[b]	1.0 ± 0.1[b]
22:6n-3	1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
	2	2.1 ± 0.2[a]*	1.2 ± 0.1[b]	1.2 ± 0.1[b]

Data are the mean ± SEM g/100 g total fatty acid before (visit 1) and after (visit 2) supplementation. Statistical significance (one way ANOVA): groups with different letters are significantly different, *denotes significantly different from visit 1 (paired t-test).

Figure 3.9 Effect of length of supplementation with FO on EPA accumulation in LDL CE

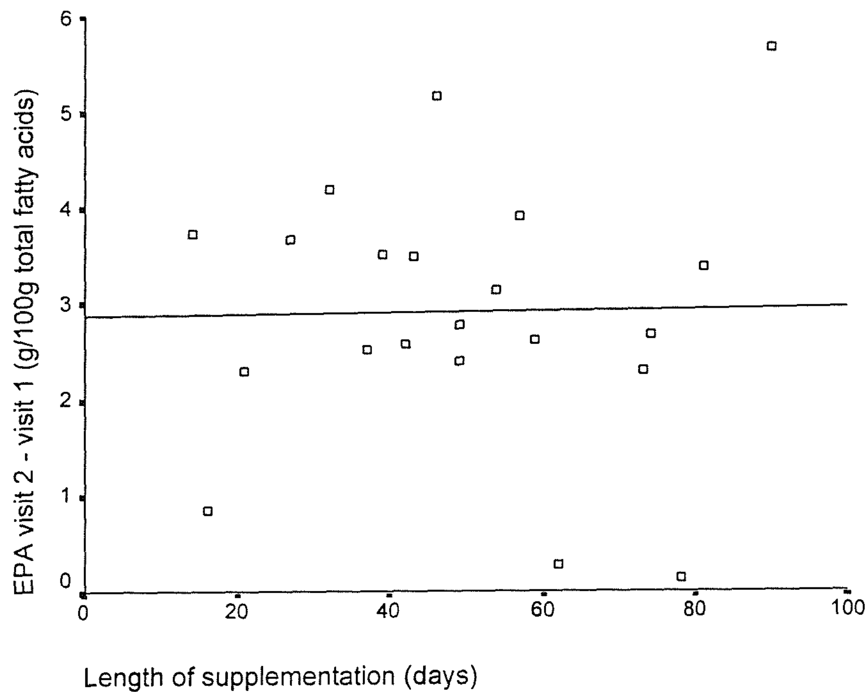
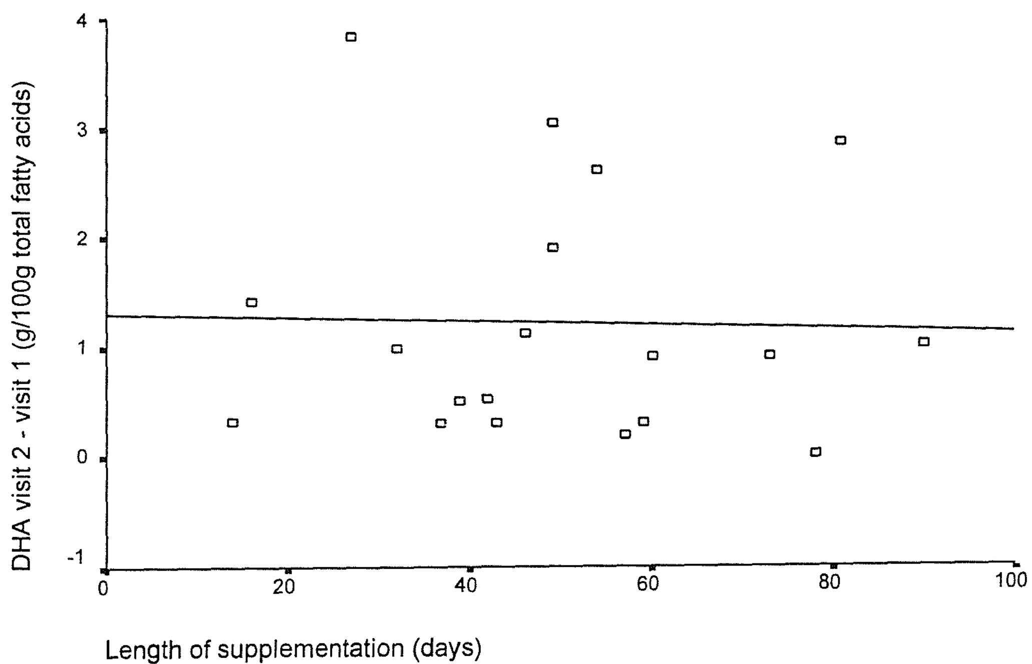


Figure 3.10 Effect of length of supplementation with FO on DHA accumulation in LDL CE



3.3.5 LDL Oxidation

3.3.5.1 Conjugated Diene Formation

The lag time of copper-induced LDL oxidation and T½ were significantly decreased in patients who received fish oil (-26% and -13% respectively), but the maximum rate of oxidation and the maximum amount of conjugated dienes formed were unchanged (Table 3.10). Lag time was significantly increased in patients on control capsules (17%), and T½ was significantly increased in patients who received sunflower oil (13%).

Table 3.10 Oxidisability of LDL from patients in the different treatment groups before and after supplementation

	Visit	Fish oil (n = 40)	Sunflower oil (n = 38)	Control (n = 48)
Lag Time (min)	1	94.2 ± 7.1	85.0 ± 11.0	83.7 ± 7.2
	2	69.8 ± 8.0[a]*	82.4 ± 9.3	101.0 ± 8.7[b]*
	V2 -V1	-28.4 ± 6.9[a]	-1.44 ± 8.46[b]	15.6 ± 5.9[b]
T½ (min)	1	93.5 ± 3.6	77.9 ± 4.6	85.1 ± 5.4
	2	80.9 ± 4.0*	88.2 ± 4.5*	93.7 ± 6.4
	V2 –V1	-14.3 ± 5.0[a]	11.8 ± 4.5[b]	5.9 ± 6.1[b]
Maximum Diene Production (nmol diene/mg LDL protein)	1	455.8 ± 16.0	467.4 ± 18.0	456.0 ± 14.1
	2	475.7 ± 21.0	470.8 ± 20.8	461.2 ± 19.5
	V2 –V1	36.6 ± 20.8	-5.1 ± 16.5	1.0 ± 16.5
Oxidation rate (nmol diene/min/mg LDL protein)	1	7.27 ± 0.59	8.22 ± 0.37	8.08 ± 0.54
	2	6.64 ± 0.48	8.28 ± 0.56	7.96 ± 0.70
	V2 –V1	0.26 ± 0.40	0.00 ± 0.50	0.17 ± 0.62

Data are presented as mean ± SEM before (visit 1) and after (visit 2) supplementation. Statistical significance: groups with different letters are significantly different (one way ANOVA), * significantly different from visit 1 (paired t-test).

3.3.5.1.1 Role of Vitamin E in preventing the oxidation of LDL

The lag time of copper induced LDL oxidation and was decreased by 27% in this patient who received fish oil (Table 3.11). However, when the LDL was enriched *ex vivo* with vitamin E lag time was increased by 6% for the sample collected before fish oil supplementation and by 20% for the sample collected after fish oil supplementation. Therefore, the reduction in lag time after fish oil supplementation was partly abolished by enriching the LDL with vitamin E *ex vivo*. Lag time was decreased by 17% compared with visit 1 as opposed to 27%.

Table 3.11 Lag time of copper-induced LDL oxidation with and without addition of α-tocopherol

Sample	**α-tocopherol added**	**Lag time** (mins)
LDL (visit 1)	None	126.9
LDL (visit 1)	5 nmol	134.4
LDL (visit 2)	None	92.4
LDL (visit 2)	5 nmol	110.8

3.3.5.2 Lipid Peroxides

No significant changes were observed in peroxide levels in freshly isolated LDL after any of the treatments (Table 3.12).

Table 3.12 Lipid peroxide concentrations in LDL from patients in the different treatment groups before and after supplementation

Group	Visit	n	**Peroxides** (nmol/mg LDL protein)
Fish oil	1	33	37.35 ± 5.00
	2	33	35.05 ± 5.70
	V2-V1		-2.30 ± 3.30
Sunflower oil	1	31	32.59 ± 5.49
	2	31	33.07 ± 5.30
	V2-V1		0.90 ± 3.70
Control	1	33	40.78 ± 5.67
	2	33	40.10 ± 5.66
	V2-V1		-0.67 ± 4.11

Data are presented as mean ± SEM

3.3.5.3 LDL Vitamin E Concentrations

LDL vitamin E was significantly higher in patients after sunflower oil supplementation but did not change in the control or fish oil groups (Table 3.13).There was no relationship between LDL vitamin E concentrations and lag time of copper-induced LDL oxidation.

Table 3.13 Vitamin E concentrations in LDL from patients in the different treatment groups before and after supplementation

Group	Visit	n	Vitamin E (nmol/mg LDL protein)
Fish oil	1	37	14.2 ± 1.2
	2	37	4.1 ± 1.1
	V2-V1		1.01 ± 0.96
Sunflower oil	1	37	12.8 ± 1.2
	2	37	15.1 ± 1.3*
	V2-V1		3.17 ± 1.14 [a]
Control	1	37	13.4 ± 0.6
	2	37	12.1 ± 1.1
	V2-V1		-0.90 ± 1.04 [b]

Data are presented as mean ± SEM before and after supplementation. Statistical: groups with different letters are significantly different significance (one way ANOVA), *significantly different from visit 1 (paired t-test).

Total PUFA content of LDL CE and PL did not differ in the different treatment groups, but FO increased the number of PUFA double bonds in LDL CE and PL (Table 3.14 and 3.15). LDL vitamin E was expressed in relation to total PUFA content and the number of PUFA double bonds in LDL CE and PL (Table 3.14 and 3.15). No significant differences were found in LDL vitamin E content between treatment groups before or after supplementation or within treatment groups between visits. However LDL vitamin E expressed in relation to total PUFA content and the number of PUFA double bonds tended to decrease in LDL CE but not PL after fish oil supplementation.

Table 3.14 LDL CE PUFA content and Vitamin E concentrations from patients in the different treatment groups before and after supplementation

Group	Visit	n	Total PUFA	Vit E/ PUFA	PUFA*DB	Vit E/ PUFA*DB
FO	1	15	60.39 ± 0.84	0.23 ± 0.02	143.31 ± 1.81	0.10 ± 0.01
	2	10	60.18 ± 0.97	0.16 ± 0.02	154.50 ± 1.86[a]*	0.06 ± 0.01
SO	1	18	59.88 ± 0.96	0.22 ± 0.03	143.62 ± 2.24	0.09 ± 0.01
	2	18	61.81 ± 1.18	0.20 ± 0.02	146.52 ± 2.42[b]	0.08 ± 0.01
C	1	19	60.76 ± 0.91	0.22 ± 0.02	142.96 ± 1.95	0.09 ± 0.01
	2	14	59.95 ± 1.07	0.18 ± 0.03	141.79 ± 2.32[b]	0.08 ± 0.01

Data are presented as mean ± SEM before and after supplementation. PUFA = Total PUFA in CE (g/100 g total fatty acids); VitE/PUFA = nmol vit E / mg LDL protein/ PUFA; PUFA*DB = PUFA * number of double bonds. Statistical significance: groups with different letters are significantly different (one way ANOVA), * significantly different from visit 1 (paired t-test).

Table 3.15 LDL PL PUFA content and Vitamin E concentrations from patients in the different treatment groups before and after supplementation

Group	Visit	n	Total PUFA	Vit E/ PUFA	PUFA*DB	Vit E/ PUFA*DB
FO	1	12	37.51 ± 0.87	0.28 ± 0.06	116.24± 2.55	0.09 ± 0.02
	2	13	40.22 ± 0.97	0.32 ± 0.03	141.80 ± 3.29[a] *	0.09 ± 0.01
SO	1	13	37.86 ± 0.79	0.25 ± 0.04	117.88 ± 3.79	0.08 ± 0.01
	2	8	39.65 ± 1.27	0.31 ± 0.06	124.86± 4.40[b]	0.10 ± 0.02
C	1	15	37.72 ± 0.80	0.35 ± 0.04	115.48 ± 2.59	0.11 ± 0.01
	2	10	37.43 ± 0.90	0.30 ± 0.08	113.37 ± 3.48 [b]	0.10 ± 0.03

Data are presented as mean ± SEM before and after supplementation. PUFA = Total PUFA in PL (g/100 g total fatty acids); VitE/PUFA = nmol vit E / mg LDL protein/ PUFA; PUFA*DB = PUFA * number of double bonds. Statistical significance: groups with different letters are significantly different (one way ANOVA), *significantly different from visit 1 (paired t-test).

3.4 Discussion

The aims of this chapter were firstly to measure the fatty acid composition and oxidisability of plasma LDL in patients with advanced carotid atherosclerosis and secondly to determine whether plasma LDL fatty acid composition and oxidisability can be changed by short term changes in n-3 and n-6 PUFA intakes in these patients and to measure the time course for these changes. It was found that supplementation with fish oil lowered plasma cholesterol and triacyglycerol concentrations, altered the fatty acid composition of plasma LDL lipid fractions (all fractions were altered in the same way) and decreased the lag time of copper-induced oxidation of LDL. Supplementation with sunflower oil lowered plasma cholesterol concentration but had few other effects.

3.4.1 Plasma and LDL cholesterol and TAG

It has generally been assumed that n-6 PUFA (and 18:2n-6 in particular) have a favourable effect on CHD risk by means of a decrease in blood cholesterol concentrations (European Atherosclerosis Society, 1987). Burr *et al.* (1989) showed a 2% decrease, Woodhill *et al.* (1978) an 11% decrease and Leren (1970) an 18% decrease in cholesterol concentrations after increasing linoleic acid intake. In the current study a 13% decrease in plasma total and esterified cholesterol and a 5% decrease in LDL total cholesterol concentration occurred after supplementation with sunflower oil rich in linoleic acid. Thus, the magnitude of the effect observed in the current study is consistent with that reported in the literature. The mechanism of the linoleic acid-induced decrease in plasma cholesterol concentration is thought to be an increased rate of removal of LDL by the hepatic receptor–dependent pathway when linoleic acid replaces saturated fatty acids (Spady and Dietschy, 1989). It was found that plasma free cholesterol and LDL total and esterified cholesterol were all lower after SO supplementation in patients taking statins compared to those not taking statins. However only patients not taking statins had lower plasma total and esterified cholesterol after SO supplementation but this may be due to the small number of patients taking statins.

There have been numerous studies investigating the effects of fish oil or its constituent n-3 fatty acids on serum or plasma lipid concentrations. It seems that a

reduction in total cholesterol concentration occurs only if large doses (4.5-6 g EPA or 6.5-10 g total n-3 fatty acids per day) are given (Davidson *et al.*, 1987). Smaller amounts of n-3 fatty acids appear to have little effect, or even to cause a slight increase in total cholesterol concentration (Burr *et al.*, 1989). However in the current study supplementation with 1.7 g n-3 fatty acids per day (or 860 mg EPA) resulted in a 15% decrease in plasma total cholesterol a 17% decrease in plasma esterified cholesterol, an 11% decrease in LDL esterified cholesterol and a 15% decrease in HDL total cholesterol concentrations. Plasma and LDL total cholesterol, plasma free cholesterol and LDL esterified cholesterol concentrations were all lower in patients taking statins after fish oil supplementation. However plasma total and esterified cholesterol were lower after FO supplementation in all patients whetheror not they were taking statins.

Epidemiological studies have demonstrated that the incidence of CHD is inversely associated with consumption of n-3 PUFA (Bang and Dyerberg, 1972). The DART and GISSI trials provide evidence that relatively low doses of n-3 PUFA reduce the risk of secondary coronary events (Burr *et al.*, 1989; GISSI-P, 1999). Plasma TAG concentrations are significantly decreased by n-3 PUFA, in a dose-dependent fashion and this may partly explain the cardio-protective effect of n-3 PUFA. In a review of 72 placebo-controlled human studies which supplemented a range of 1.0 to 7.0 g EPA plus DHA daily for at least two weeks, fasting plasma TAG concentrations were consistently decreased by 25-30% (Harris, 1996). In the current study a moderate supplementation with fish oil has been used, approximately 1.7 g/d of n-3 PUFA, and plasma TAG concentration was reduced by 28%. This was equivalent to a mean decrease of approximately 0.47 mmol/L. Males and femaleswere equally sensitive to the TAG lowering effect of FO. This shows that a moderate intake of n-3 PUFA, which could be achieved by dietary intervention or as a supplement, has a positive effect on whole body TAG metabolism in this group of patients. The mechanism of decreased TAG concentrations caused by n-3 PUFA is believed to be due to decreased hepatic TAG synthesis (Sanders *et al.*, 1985; Harris *et al.*, 1990.).

Traditionally, elevated plasma triacylglycerol (TAG) concentrations have been associated with an increased risk of CHD, but their role as an independent risk factor was unclear (Roche and Gibney, 1999). A meta-analysis of seventeen population-based

prospective studies showed that an increase in plasma TAG concentration was associated with a significant increase in risk of CHD (Hokanson and Austin, 1996). The analysis of this large cohort (n = 57,277) showed that plasma TAG concentration was an independent risk factor for CHD, particularly in women: a one mmol/L increase in plasma TAG concentration was associated with an increased cardiovascular risk of 32% in men and 76% in women.

3.4.2 LDL fatty acid composition

It has previously been found that when fish oil is incorporated into the diet plasma concentrations of EPA and DHA increase several-fold within a few weeks (Harris and Connor, 1980). In the current study it was found that supplementation with fish oil caused a significant enrichment in long chain n-3 PUFA in all LDL lipid classes. LDL has been shown to be quantitatively the most important source of lipid for atherosclerotic plaques. Therefore, if a moderate supplementation of fish oil as used in this study (1.7 g n-3 fatty acids/day) affects LDL composition it may also affect atherosclerotic plaque composition. Rapp *et al.* (1991) investigated the incorporation of n-3 PUFA into obstructive atherosclerotic plaques. N-3 PUFA consumption ranged from 16.0-21.3 g/day. There was significant enrichment of n-3 fatty acids in all plasma lipid classes (CE, PL, and TAG) after supplementation, although they did not look at LDL lipid classes. The n-3 PUFA content of atherosclerotic plaques was also greater when compared with that of plaques removed from 18 non-fish oil-supplemented controls. In the study by Rapp *et al.* (1991) dietary n-3 fatty acids were shown to be incorporated into the lipid classes of advanced atherosclerotic lesions and changes in the fatty acid compositions of plasma lipids were reflected in changes in plaque fatty acid composition. However, fish oil was provided at a very high dose and it is not currently known whether more moderate alterations in the intake of n-3 PUFA of a magnitude likely to be achievable in free living patients will alter plaque fatty acid composition and the speed at which such changes will occur. This will be investigated in chapter four.

3.4.3 LDL oxidation

There is increasing evidence that oxidatively modified LDL is an important mediator in the pathogenesis of atherosclerosis (Steinberg *et al.*, 1989; Salonen *et al.*, 1992). The conjugated diene method has become one of the most frequently used assays to assess resistance of LDL to oxidation. The resistance of LDL to oxidation depends on intrinsic properties of the lipoprotein and on the experimental conditions (Gieseg and Esterbauer, 1994). PUFA in LDL lipids are protected from peroxidation so long as endogenous antioxidants are present (Esterbauer *et al.*, 1987). Subsequently, PUFA are oxidised producing a number of breakdown products such as aldehydes and conjugated dienes. Some of the reduction in heart disease risk because of increased dietary intake of PUFA may be negated by increasing the availability of unsaturated bonds to oxidative processes.

The effects of dietary n-3 PUFA on the susceptibility of LDL to oxidation are contradictory (see Section 3.1.4). In a study by Nenseter *et al.*, (1991), the effect of dietary supplementation with n-3 PUFA on the susceptibility of LDL to oxidative modification was investigated. LDL from both fish oil and corn oil supplemented groups showed similar susceptibility to copper-catalysed lipid peroxidation (see Section 3.1.4). Frankel *et al.* (1994) looked at the effect of n-3 PUFA supplementation on copper-catalysed oxidation of LDL. The volatile oxidation products from LDL, i.e. propanal and hexanal, were measured. Total volatiles remained unchanged thus indicating that fish oil intake did not alter the oxidative susceptibility of human LDL. However, in other studies n-3 PUFA have been shown to enhance the susceptibility of LDL to oxidative modification. Suzukawa *et al.* (1995) found that fish oil consumption (3.4 g n-3 fatty acids /day for 6 weeks) decreased lag time before onset of copper-induced LDL oxidation and significantly increased production of TBARS during oxidation compared with corn oil (see Section 3.1.4). Under *in vivo* conditions, competition exists between oxidative and protective processes that depend on PUFA composition and on antioxidant levels. In the current study it was found that supplementation with 1.7 g n-3 fatty acids/ day caused an increase in the number of PUFA double bonds in LDL CE and PL, therefore making more substrate available to oxidise, and led to a 26% decrease in lag time. Therefore, fish oil supplementation may adversely affect the susceptibility of LDL to oxidation. LDL

vitamin E concentration was not changed after fish oil supplementation despite the fact that all capsules contained vitamin E. However when LDL from a patient who received fish oil was enriched with vitamin E *ex vivo*, the reduction in lag time after fish oil supplementation was partly abolished. This confirms that by increasing antioxidant content the lag time of LDL oxidation can be increased and so make LDL less susceptible to oxidation and suggests that the effect of fish oil is related to the changed relationship between the amount of PUFA and the amount of vitamin E in LDL.

It has been shown that diets enriched with linoleic acid increase the linoleic acid content of LDL and increase its susceptibility to oxidation (Parthasarathy *et al.*, 1990; Reaven *et al.*, 1993; Abbey *et al.*, 1993). However, in the current study supplementation with linoleic acid did not cause any changes in the susceptibility of LDL to oxidation although there was more linoleic acid in LDL CE.

In summary, it has been shown that plasma LDL fatty acid composition and oxidisability can be changed by short term changes in n-3 and n-6 PUFA intakes in patients with advanced carotid atherosclerosis.

Chapter Four

Plaque and adipose tissue fatty acid composition and measures of plaque morphology

4 Plaque and adipose tissue fatty acid composition and measures of plaque morphology

4.1 Introduction

4.1.1 Plaque lipid composition

Lipids are a major component of atheromatous plaques (see Section 1.2). The accumulation of lipid in plaques is a major determinant of disease progression, plays a major role in thrombosis and is a potent risk factor for coronary heart disease and stroke (Davies *et al.,* 1993; Department of Health, 1994). Fatty acids are transported in the blood mainly as components of lipoproteins (see Section 1.1.3). Lipoprotein composition is strongly influenced by the fatty acid composition of lipid absorbed from the diet; in the current study this was confirmed for LDL lipid fractions. Plaques acquire lipid mainly from LDL (Shaikh *et al.,* 1991). The process of lipid accumulation is accelerated and plaque growth stimulated if oxidative damage has occurred to LDL (Steinberg *et al.*, 1989). Macrophages play a major role in the increase in plaque lipid content by engulfing lipids (e.g. Ox-LDL) from the bloodstream. Lipid-laden foam cells develop. The death of foam cells releases their lipid contents thereby enlarging the plaque lipid core (Ball *et al.*, 1995). Plaque lipid composition may thus reflect the fatty acid composition of dietary fat. However, there have been few reports relating dietary fatty acid intake to plaque fatty acid composition and this is one of the topics investigated in this study.

4.1.1.1 Dietary PUFA and the composition of aortic plaques

Dietary fatty acids differ in their influence on the incidence of coronary heart disease (Wood and Oliver, 1992). Epidemiological studies show that increased consumption of PUFA is associated with a reduced incidence of coronary heart disease (Wood and Oliver, 1992; Kromhout *et al.,* 1985). It has been shown that after ingestion of fish oil, n-3 PUFA accumulate in plaques in human coronary arteries (Rapp *et al.,* 1991). How dietary PUFA influence atherogenesis is unknown, but the susceptibility of LDL to oxidative modification suggests that esterified forms may promote LDL infiltration into

the vessel wall. Oxidised LDL is readily taken up by macrophages resulting in lipid accumulation and lesion formation (Steinberg *et al.,* 1989).

Recent evidence suggests that although n-3 and n-6 PUFA decrease the risk of thrombosis and decrease plasma cholesterol concentrations, respectively, they may accelerate the atherosclerotic process (Felton *et al.,* 1994). How the long-term dietary intake of essential fatty acids affects the fatty acid content of atherosclerotic plaques is not clear. In a study by Felton *et al.* (1994) the fatty acid composition of atherosclerotic plaques was compared with that of post-mortem serum and adipose tissue to assess the extent to which plaque fatty acid content is affected by long-term dietary intake of fatty acids. They analysed the fatty acid composition of these tissues from nine white men (age 44-73) from consecutive necropsies and found positive associations between serum and plaque n-6 and n-3 PUFA and monounsaturates and between adipose tissue and plaque n-6 PUFA. No associations were found with SFA. The positive correlation found between plaque 18:2 n-6 content and serum and adipose tissue 18:2 n-6 were taken to suggest that dietary intake of this essential fatty acid directly influences plaque composition.

Alterations in the composition of lipids in plaque have structural and functional implications. An increase in unsaturation may increase peroxidative damage and plaque growth (Frankel *et al.,*1994; Suzukawa *et al.,* 1995). Inflammation plays a key role in atherogenesis (Hajjar and Pomerantz, 1992; Mitchinson, 1994). Changes in the proportions of n-3 and n-6 PUFA in the phospholipids of plaque cell membranes will change the amount and nature of eicosanoids, diacylglycerols and phosphatidic acids produced, thereby modulating inflammation (Grimble and Tappia, 1995).

The findings of Felton *et al.* (1994) and Rapp *et al.* (1991) support the hypothesis that dietary n-6 and n-3 PUFA significantly influence the composition of human atherosclerotic plaques. However, at present it is not clear whether the incorporation of PUFA into human atherosclerotic plaques is advantageous or disadvantageous. These observations raise the question of the potency, magnitude and speed of the dietary influence on the fatty acid composition of the lipids of LDL and the plaque. These will be addressed in the current study.

4.1.1.2 Dietary EPA and DHA from fish oil and their incorporation into atherosclerotic plaques

EPA and DHA are ordinarily present in only very small quantities in both plasma and atherosclerotic plaque of individuals consuming a diet low in marine products (Harris *et al.,* 1983; Rapp *et al.,* 1983). When fish oil is incorporated into the diet plasma concentrations of EPA and DHA increase several-fold within a few weeks (Harris and Connor, 1980). Therefore, substantial changes in plasma lipid fatty acid composition induced by diet could, over time, alter the fatty acid composition of the lipid classes of human atherosclerotic plaques.

Rapp *et al.* (1991) investigated the incorporation of n-3 PUFA into obstructive atherosclerotic plaques removed from eleven patients fed fish oil for 6-120 days before a planned arterial endarterectomy. The amount of fish oil was calculated so that the n-3 fatty acid content of the diet equalled 6% of total calories. Depending on height, weight, age and physical activity, fish oil consumption ranged from 48 to 64 g/day (16.0-21.3 g/day of n-3 fatty acids). Over the entire study period the total n-3 fatty acid consumption varied from 115 to 2,256 g depending on the length of the fish oil feeding period before surgery. The mean n-3 fatty acid content increased greatly in plasma, from 0.9% to 14.8% of fatty acids in CE, from 3.8% to 22.1% of fatty acids in PL and from 1.3% to 21.9% of fatty acids in TAG. The n-3 fatty acid content of atherosclerotic plaques was also greater when compared with that of plaques removed from 18 non-fish oil-fed controls. The mean n-3 fatty acid content in CE of plaques was 4.9% in the experimental group and 1.4% in control plaques, in PL it was 8.8% versus 1.8% and in TAG it was 4.7% versus 0.7%. DHA and EPA behaved differently compared with plasma: relatively more DHA than EPA was deposited into plaques. The increase in n-3 fatty acids in plasma reached a plateau three weeks after initiation of fish oil feeding. However, in plaques the accumulation of n-3 fatty acids increased with time. Mean total n-3 fatty acid accumulation in the CE fraction of plaques was 2.8% among the seven patients fed fish oil for less than 58 days and 7.3% for the four patients fed fish oil for 58 days or longer. Therefore in this study dietary n-3 fatty acids were shown to be incorporated into the lipid classes of advanced human atherosclerotic lesions. Changes in the fatty acid composition of plasma lipids were reflected in changes in plaque fatty acid composition. Because n-3

fatty acids affect many factors predisposing to both thrombosis and atherosclerosis these data may have clinical implications. Fish oil was fed at a very high dose in the study of Rapp *et al.* (1991) (6% of dietary energy) and at the moment it is not known whether more moderate alterations in the intake of n-3 PUFA of a magnitude likely to be achievable in free living patients will alter plaque fatty acid composition and the speed at which such changes will occur. This was investigated in the current study.

4.1.2 Plaque lipid composition and morphology and how it affects plaque stability

Thrombus formation following disruption of a coronary artery plaque is responsible for the majority of incidents of acute myocardial ischaemia (Davies and Thomas, 1984, Falk, 1985). Thrombus formation may occur after plaque disruption when the plaque core becomes exposed to blood from the arterial lumen. The plaque core is rich in tissue factor, collagen and lipid all of which are potent thrombogenic agents (Wilcox, 1991). The reasons why some plaques fissure and others do not are not fully understood, but the propensity to disruption depends on the balance between the forces acting on the plaque and its inherent mechanical strength (Davies *et al.*, 1993; 1994). Plaque disruption appears to result from connective tissue degradation, a process possibly influenced by intraplaque lipid content and macrophage activity (Davies *et al.*, 1994, Tracy *et al.*, 1985, Kragel *et al.*, 1991). Differences in lipid composition and distribution between stable and unstable plaques may reflect differences in lipid pools and the degree of macrophage involvement. The study of these differences and their interactions with indices of plaque stability, such as cap thickness, may help to determine whether plaque lipids influence plaque stability and may lead to strategies to render unstable plaques less vulnerable to disruption; such studies are part of the current investigation.

The chances of atherosclerotic plaques disrupting may be influenced by both their lipid content and the distribution of these lipids within the plaque. To investigate this, Felton *et al.* (1997) analysed the morphological and lipid profiles of 668 human aortic plaques from 30 men aged 44-69 years who died as a result of an acute ischemc event. Patients who were known to have a history of hypertension or diabetes mellitus were excluded because of the possibility of atypical plaques. Plaques were classified as disrupted or as intact types A or B, the latter distinction being based on the absence or

presence, respectively, of disrupted plaques within the same aorta. They found that disrupted plaques had a greater content of lipid and macrophages as well as a thinner cap than intact plaques. Lipid concentrations were positively associated with macrophage accumulation in all plaque types and were negatively associated with minimum cap thickness at the edge of disrupted plaques. Free cholesterol concentration at the centre of type B plaques only was inversely associated with minimum cap thickness. Esterified cholesterol concentrations were higher at the centre of type B plaques and those of free cholesterol were higher at the centre of disrupted plaques. The concentrations of all fatty acids were increased at the edge of disrupted plaques compared with the centre, but as a proportion of total fatty acids n-6 PUFA were lower at the edge possibly reflecting oxidation of PUFA. These data demonstrate differences in lipid composition and intraplaque lipid distribution between intact and disrupted plaques. At the edge of advanced plaques increased esterified lipid concentrations were inversely associated with cap thickness; this may reflect macrophage activity and a predisposition to rupture.

The study of Felton *et al.* (1997) shows that histological determinants of plaque stability such as cap thickness are inversely associated with increased plaque lipid concentration. The data confirm that disrupted plaques are characterised by an increased cross-sectional area occupied by lipid and macrophages when compared with intact type A and type B plaques. It has also shown that disrupted plaques have a significantly reduced minimum cap thickness compared with type A and type B plaques and also that minimum cap thickness is negatively associated with lipid area in type B and disrupted plaques.

Reduced proportions of n-6 PUFA and total PUFA at the edge of disrupted plaques compared with the centre may reflect oxidative damage. Such damage to PUFA may promote connective tissue degradation and influence the prevalence of disruption at this site. Both PUFA and sterol oxidation products have been detected in the human arterial wall (Fogelman *et al.*, 1980, Sevanian and McLeod, 1987). These are toxic to most arterial cells *in vitro* (Hodis *et al.*, 1991) and to macrophages in particular (Reid *et al.*, 1992, Clare *et al.*, 1995) and may provide a milieu for connective tissue degradation. Increased concentrations of proaggregatory SFA and oxidised derivatives of PUFA which can inhibit endothelial prostacyclin synthetase (Hu *et al.*, 1990, Moncada and Vane,

1978) at a site of disruption may influence the degree of thrombus formation.

4.1.3 Adipose tissue composition

The fatty acid composition of adipose tissue is determined to some extent by the type of dietary fat consumed (Field *et al.*, 1985). Given an approximate 600 day half-life of fatty acids in human adipose tissue (Beynen *et al.*, 1980) fatty acid analysis of biopsied adipose tissue provides an index of habitual dietary fat intake over the preceding 2-3 years (Heffernan, 1963). In the current study it was found that the PUFA composition of adipose tissue was a good marker of dietary PUFA intake, however only the proportion of 16:0 in adipose tissue correlated with SFA intake and no significant correlation was seen between total MUFA intake, and the proportion of MUFA in adipose tissue (see Section 2.3.2.1). Serum and adipose tissue fatty acid concentrations of the essential fatty acids, linoleic (18:2 n-6) and α-linolenic (18:3 n-3) acids, which cannot be synthesised by man, are largely determined by dietary intake (Heffernan, 1963; Hirsch *et al.*, 1960; Beynen *et al.*, 1980). Therefore, adipose tissue composition has served as a biological marker of chronic ingestion of many dietary PUFA, and this has been confirmed in this study. Chronic ingestion of n-9 fatty acids has also been reflected in the fatty acid composition of aspirated adipose tissue samples in some studies (Hirsch *et al.*, 1960; Beynen *et al.*, 1980), although in the current study this was not found (see Section 2.3.2.1). Quantitative data about the rate of incorporation into, and levels of n-3 fatty acids in, adipose tissue as a function of dietary intake are scarce.

A study by Leaf *et al.* (1995) looked at the effect of daily dietary supplementation with $\geq$ 10 g fish oil for >12 months. Twelve people who were patients of the Oregon Health Sciences University Lipid Disorders Clinic were the patients used in this study. Seven of these 12 patients had already received marine fish-oil supplements for the treatment of dyslipidemic disorders and were used as the study group. Five patients who had not received fish-oil supplementation served as control patients. The remaining three control patients were healthy volunteers. The patients received a minimum of 10 g fish oil/d (range 10-15 g/d). This provided a minimum of 1.8 g EPA and 1.0 g DHA, and a total of 2.8 g n-3 fatty acids/d for 1 y or longer (range 12-27 months). They found significantly greater proportions of EPA, DPA and DHA in adipose tissue of

supplemented patients compared with non-supplemented control patients. In another study by Katan *et al.* (1997), 58 men were fed 0, 3, 6 or 9 g/d fish oil for 12 months and the fatty acids in subcutaneous fat were measured during and after supplementation. EPA levels in adipose tissue rose, the change after 6 months was 67% of that after 12 months in gluteal and 75% in abdominal fat. After 12 months each gram of fish oil per day caused an 0.11 ± 0.01 mass % rise in gluteal fat for EPA, 0.53 ± 0.07 for DPA and 0.14 ± 0.03 for DHA. In a study by Sacks *et al.* (1995), patients with angiographically documented coronary heart disease and normal plasma lipid levels were randomised to receive either fish oil capsules (n = 31), containing 6 g of n-3 fatty acids, or olive oil capsules (n = 28) for an average duration of 28 months. At the end of the trial EPA in adipose tissue samples was 0.91% in the fish oil group compared with 0.20% in the control group ($p<0.0001$). These studies indicate that the long-term ingestion of large amounts of n-3 fatty acids in humans results in their incorporation into the adipose tissue fatty acids. Further studies are needed to identify the effects of lower intakes of n-3 fatty acids on adipose tissue composition, and also to clarify the time course of n-3 fatty acid incorporation into adipose tissue. These will be addressed in this chapter.

4.1.4 Aims

The aims of this chapter were firstly to measure the fatty acid composition of adipose tissue and atherosclerotic plaques removed from living patients, and secondly to determine whether adipose tissue and plaque fatty acid composition can be changed by short term changes in n-3 and n-6 PUFA intakes and to measure the time course for these changes. The third aim of this chapter was to investigate the morphology of plaques removed from patients in the different treatment groups, with the aim of identifying whether the incorporation of n-3 and n-6 PUFA into plaques alters the morphology or structure of the plaque and signs of inflammation within the plaque.

4.2 Methods

4.2.1 Collection of samples

At the time of entering the study and at surgery a narrow gauge needle subcutaneous adipose tissue biopsy was taken from the abdominal wall of each subject. The adipose tissue samples were then frozen at –70°C until the time of analysis.

At endarterectomy the atheromatous intima was removed in one piece. The overall length of the plaque was measured and serial transverse 3-4 mm sections were cut. The sections were then named alphabetically starting from the distal end of the internal carotid artery plaque and ending at the common cartid artery plaque. Alternate sections of the plaque were used for biochemical analysis (Section 4.2.2) and for immunohistochemistry (Section 4.2.3). The sections for biochemical analysis were snap frozen in liquid nitrogen and were stored at –70°C. The section closest to the bifurcation was frozen (-70°C) in OCT compound (Agar Scientific Ltd.) to be sectioned later using a cryostat and used for immunohistochemistry (Section 4.2.3)

4.2.2 Plaque fatty acid composition

Total lipid was extracted from the plaque using chloroform/methanol as described by Rapp *et al.* (1991). Plaque lipid was separated into CE, TAG and PL by thin layer chromatography as described earlier for LDL (Section 3.2.3). Fatty acid methyl esters were formed by reaction with hot methanolic boron trifluoride. The fatty acid methyl esters of each fraction were then purified using hexane/BHT and saturated aqueous sodium chloride solution. The fatty acid composition of each fraction was then determined by gas chromatography as described earlier (Section 2.2.4).

4.2.3 Immunohistochemistry on plaque sections

This is the localisation of cellular or tissue components (antigens) in situ by the use of an antibody:antigen reaction. Streptavidin horseradish peroxidase was used as the detection system. Sections of plaque where the specific primary antibody had been omitted served as negative controls. Normal human tonsillar tissue was used as a positive control. All antibodies used were titrated to determine the optimal concentration and conditions for use.

1. 4-6 μm cryostat sections of the frozen plaques were mounted on organosilan-coated microscopic slides.
2. The slides were then air dried overnight, and either stained the following day or wrapped in Parafilm-foil "face-to-face" and stored in a closed box at –70°C until they were needed.
3. If the slides were frozen they were left to reach room temperature before unwrapping.
4. Then the slides were fixed in acetone for 10min at 4°C, and air dried briefly for 2min.
5. The tissue sections were then encircled with an 'Immedge Pen' (Vector Laboratories, Cat. No. H-4000).
6. The endogenous peroxidase activity was blocked using 50 mg sodium azide + 500 μl hydrogen peroxide (30%) in 50 ml Tris buffered saline (TBS) for 20min at room temperature. The slides were then rinsed three times with TBS.
7. The sections were covered with normal goat serum (NGS) (10%) and incubated for 15min at room temperature.
8. The NGS was blotted off and the sections were incubated with the primary antibodies (diluted in TBS containing 1% BSA) for 60 min at room temperature; see Table 4.1 for antibodies and dilutions used.
9. Sections were washed in TBS, secondary antibodies were applied and sections were incubated for 30 min at room temperature. Secondary antibodies were diluted in TBS containing 10% human AB serum, see Table 4.1 for antibodies and dilutions used.
10. Sections were washed in TBS and Strepavidin/HRP reagent (DAKO, P397) was applied to the slides (diluted 1:400 in TBS + 1% BSA), and incubated for 30 min at room temperature.
11. Sections were again washed in TBS and enzyme activity was detected using 3,3-

amino-9-ethyl carbazole (AEC).

12. Peroxidase activity using AEC (Graham *et al.*, 1965)

- Stock solution: 25 mg AEC (Sigma A5754) and 25ml N,N dimethylformamide
- Mix:

H_2O	45ml
AEC stock solution	2.5ml
Acetate buffer (1.0 M, pH 4.9)	2.5ml

- Filter immediately
- 20 μl H_2O_2 (30%) added just before use.
- Sections stained for 10 min at room temperature.

13. Sections were fixed with 4% buffered formalin for 5 min, washed with running tap water for 5 min and counterstained with Harris haematoxylin for 15 sec.
14. Sections were differentiated in an acid solution (0.1% HCl) for 15 sec and washed again in running tap water for 5 min.
15. Coverslips were then placed on the sections using an aqueous mounting medium (glycerin-gelatin, DAKO).

After plaque sections were stained for the various antigens, they were then ranked and given a score of 0, 1 or 2 according to the staining intensity (0 = no staining present, 1 = moderate amount of staining and 2 = heavily stained).

Table 4.1 Description of primary and secondary antibodies used

Antigen	Primary Antibody	Source of Antibody	Dilution used	Secondary Antibody	Source (dilution)
T-cell, Leu 4 (CD3)	Mouse anti-human, monoclonal	BD (347340)	1/20	Goat anti-mouse (biotin)	DAKO (1/200)
VCAM-1 (CD106)	Goat anti-human, polyclonal	R&D Systems (BBA 19)	1/500	Swine anti-goat (biotin)	DAKO (1/100)
ICAM-1 (CD54)	Mouse anti-human, monoclonal	R&D Systems (BBA 3)	1/500	Goat anti-mouse (biotin)	DAKO (1/200)
KP1 (CD68)	Mouse anti-human, monoclonal	DAKO (M 0814)	1/100	Goat anti-mouse (biotin)	DAKO (1/200)
PGM1 (CD68)	Mouse anti-human, monoclonal	DAKO (M 0876)	1/200	Goat anti-mouse (biotin)	DAKO (1/200)

T-cells= T lymphocytes, VCAM-1= vascular cell adhesion molecule-1, ICAM-1= intercellular adhesion molecule-1, KP1= macrophage marker, PGM1= macrophage marker.

4.2.4 Adipose tissue fatty acid composition

Adipose tissue fatty acid composition was determined as described in Section 2.2.4.

4.2.5 Statistical analysis

All values are presented as means ± SEM. Comparison of treatment group means was performed using one-way ANOVA followed by the least-significant difference post hoc test. Comparison of means before and after supplementation for each treatment group was performed using paired t-tests. Correlations were determined as Pearson correlation co-efficients. Mean ranks between treatment groups for immunohistotochemical findings were compared using the Kruskal-Wallis and Jonckheere-Terpstra tests. The observed and expected frequency of ranks were compared using the Chi-Square test. In all cases a statistical significance was assumed if $p<0.05$.

4.3 Results

4.3.1 Plaque fatty acid composition

The fatty acid composition of plaque PL, TAG and CE were determined for about 45 patients (82 %) in each treatment group: some plaque samples were not analysed because of time consttraints and because it was not necessary to analyse all samples to see significant differences between groups. The details of the patients whose plaque fatty acid composition were determined are shown in Table 4.1a. By comparing this information with that for the treatment group as a whole (Table 2.5; section 2.3.1) it is apparent that the patients whose fatty acid composition was determined are representative of the treatment group.

Table 4.1a Characteristics of patients whose plaque fatty acid composition was determined

Fraction	**Treatment**	**N**	**Male** (N)	**Female** (N)	**Age** (years)	**BMI** (kg/m^2)	**Statin** (% patients)
PL	FO	45	24	21	69.9	26.4	11
	SO	45	30	15	70.0	25.3	9
	C	51	37	14	69.7	26.9	14
TAG	FO	44	24	20	69.1	26.5	10
	SO	37	24	13	70.3	25.3	9
	C	48	34	14	69.5	26.8	14
CE	FO	44	29	15	69.6	26.7	11
	SO	45	30	15	69.3	25.6	11
	C	51	28	23	69.5	26.3	12

Data for age, BMI and statin are presented as mean.

Some differences were observed in plaque fatty acid composition between the treatment groups. The proportion of 20:2n-6 in plaque CE and TAG in the control group was higher compared to the other two groups (Table 4.2 and 4.4) and the proportion of 20:3n-6 in plaque PL was also higher compared to the fish oil group (Table 4.3).

Dietary supplementation with sunflower oil caused a decrease in the proportion of 18:1n-9 in plaque CE compared to the control group. There was also an increase in the proportion of 16:1n-7 in plaque PL compared to the control group after SO supplementation. The proportion of 18:2n-6 in plaque TAG was increased after SO supplementation compared to the other two groups, however this was not significant.

Dietary supplementation with fish oil appeared to result in the incorporation of the long chain n-3 PUFA into advanced atherosclerotic plaques (Tables 4.2, 4.3, 4.4). EPA in plaque CE in the FO group was 35% higher ($p<0.01$) than in the control group and 28% higher ($p<0.03$) than in the SO group. DHA in plaque CE was 27% higher ($p<0.01$) than in the control group and 20% higher than in the SO group (Table 4.2). The total n-3 fatty acid content in CE of plaques was 4.1% in the FO, 3.4% in the SO and 3.2% in the control group (Table 4.2). The duration of feeding did not appear to affect the accumulation of DHA or EPA in plaque CE (Figures 4.1 and 4.2). The change in EPA in LDL CE (see Figure 3.10) was compared to the proportion of EPA in plaque CE (Figure 4.3). There was a highly significant positive correlation between EPA in the CE fraction of plaque and LDL ($r = 0.7$, $p = 0.004$).

EPA in plaque PL in the FO group was 85% higher ($p<0.001$) compared to the SO group and 93% higher than in the control group ($p<0.001$). DHA in plaque PL in the FO group was 22% higher than in the SO group ($p< 0.01$) and 9% higher than in the control group (Table 4.3). The total n-3 fatty acid content in PL of plaques was 7.1% in the FO group, 6.4% in the SO group and 6.7% in the control group. The length of FO supplementation was significantly correlated with the proportion of EPA ($r = 0.31$, $p = 0.04$, Figure 4.4) plaque PL). The duration of FO supplementation did not appear to be related to the accumulation of DHA in LDL PL (Figure 4.5).

EPA in plaque TAG in the FO group was 77% higher ($p<0.001$) compared to the SO group and 85% higher than in the control group ($p<0.001$). DHA in plaque TAG in the FO group was 16% higher than in the SO group (NS) and 32% higher than in the

control group ($p < 0.03$, Table 4.4). The total n-3 fatty acid content in TAG of plaques was 2.7% in the FO group, 2.2% in the SO group and 2.1% in the control group. The duration of feeding did not affect the accumulation of EPA or DHA in plaque TAG (Figures 4.6 and 4.7).

Table 4.2 Fatty acid composition of plaque CE of patients in the different treatment groups

Fatty acid	Fish oil (n = 45)	Sunflower oil (n = 45)	Control (n = 51)
14:0	0.91 ± 0.04	1.03 ± 0.07	1.00 ± 0.05
16:0	14.53 ± 0.25	15.00 ± 0.29	14.48 ± 0.25
16:1n-7	3.77 ± 0.13	4.10 ± 0.19	3.97 ± 0.19
18:0	0.74 ± 0.09	0.69 ± 0.10	0.97 ± 0.24
18:1n-9	26.58 ± 0.49	25.72 ± 0.53[b]	27.33 ± 0.60[a]
18:2n-6	39.47 ± 0.82	40.38 ± 0.82	38.61 ± 0.84
18:3n-3	0.27 ± 0.03	0.30 ± 0.03	0.28 ± 0.03
18:3n-6	0.34 ± 0.04	0.41 ± 0.05	0.65 ± 0.23
20:1n-9	0.10 ± 0.02	0.06 ± 0.02	0.10 ± 0.02
20:2n-6	0.50 ± 0.06[b]	0.49 ± 0.05[b]	0.68 ± 0.07[a]
20:3n-6	2.18 ± 0.16	2.08 ± 0.12	2.38 ± 0.15
20:4n-6	6.78 ± 0.16	6.64 ± 0.15	6.58 ± 0.19
20:5n-3	1.45 ± 0.07[a]	1.13 ± 0.14[b]	1.08 ± 0.06[b]
22:5n-3	0.44 ± 0.07	0.33 ± 0.06	0.34 ± 0.06
22:6n-3	1.95 ± 0.12[a]	1.63 ± 0.10[b]	1.54 ± 0.08[b]

Data are the mean ± SEM, g/100g total fatty acid. Statistical significance (one way ANOVA): groups with different letters are significantly different.

Figure 4.1 Effect of length of supplementation with FO on the proportion of EPA in plaque CE

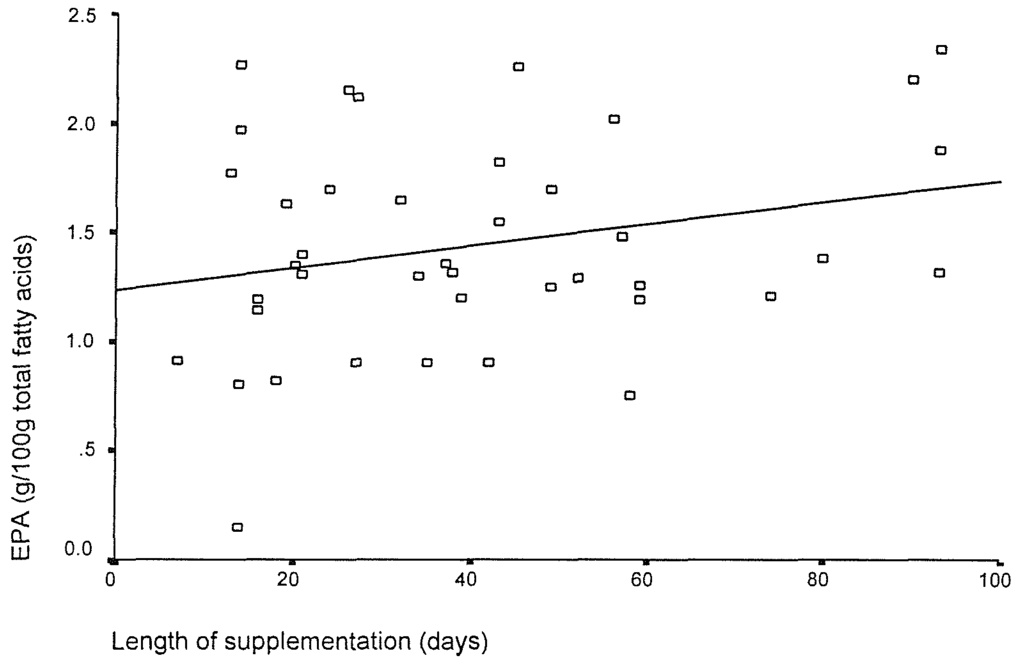


Figure 4.2 Effect of length of supplementation with FO on the proportion of DHA in plaque CE

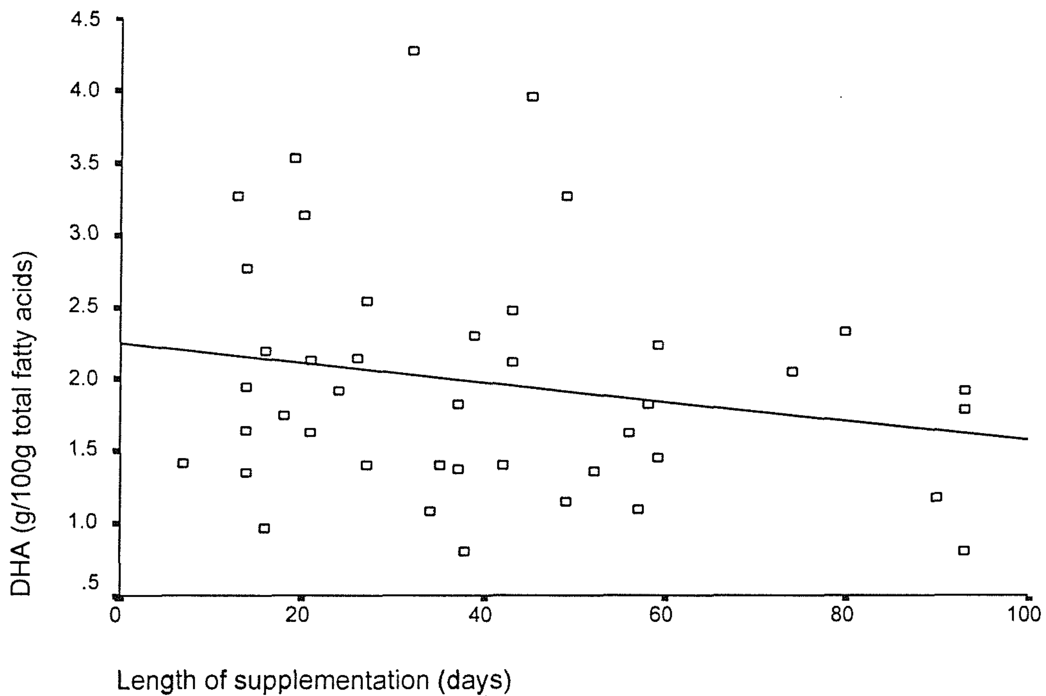


Figure 4.3 Relationship between EPA in plaque CE and LDL CE

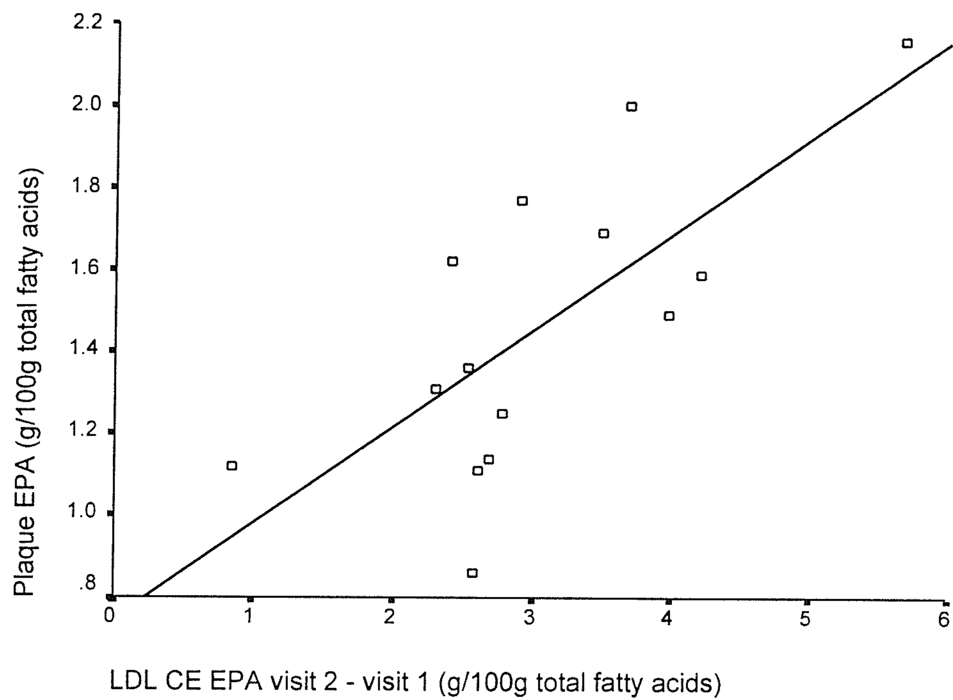


Table 4.3 Fatty acid composition of plaque PL of patients in the different treatment groups

Fatty acid	Fish oil (n = 45)	Sunflower oil (n = 45)	Control (n = 51)
14:0	1.02 ± 0.04	1.02 ± 0.05	1.03 ± 0.05
16:0	39.84 ± 0.66	39.07 ± 0.60	39.12 ± 0.49
16:1n-7	0.78 ± 0.07	1.22 ± 0.27[a]	0.74 ± 0.06[b]
18:0	14.91 ± 0.23	14.90 ± 0.31	14.78 ± 0.25
18:1n-9	14.23 ± 0.31	13.72 ± 0.25	14.07 ± 0.23
18:2n-6	9.93 ± 0.24[a]	11.20 ± 0.29[b]	10.90 ± 0.29[b]
20:2n-9	0.34 ± 0.03	0.35 ± 0.04	0.34 ± 0.04
20:3n-6	1.91 ± 0.09[a]	1.94 ± 0.07	2.14 ± 0.06[b]
20:4n-6	9.89 ± 0.30	10.06 ± 0.33	10.12 ± 0.25
20:5n-3	1.08 ± 0.08[a]	0.58 ± 0.07[b]	0.56 ± 0.06[b]
22:5n-3	2.43 ± 0.31	2.93 ± 0.34	2.84 ± 0.33
22:6n-3	3.56 ± 0.18[a]	2.92 ± 0.16[b]	3.25 ± 0.17

Data are the mean ± SEM, g/100g total fatty acid. Statistical significance (one way ANOVA): groups with different letters are significantly different.

Figure 4.4 Effect of length of supplementation with FO on the proportion of EPA in plaque PL

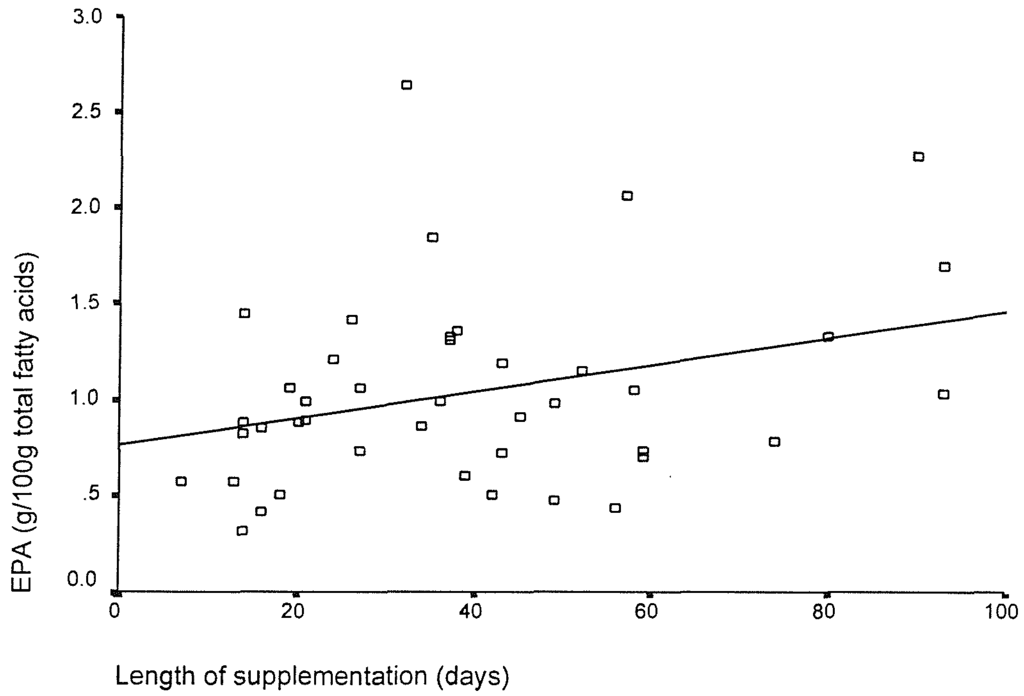


Figure 4.5 Effect of length of supplementation with FO on the proportion of DHA in plaque PL

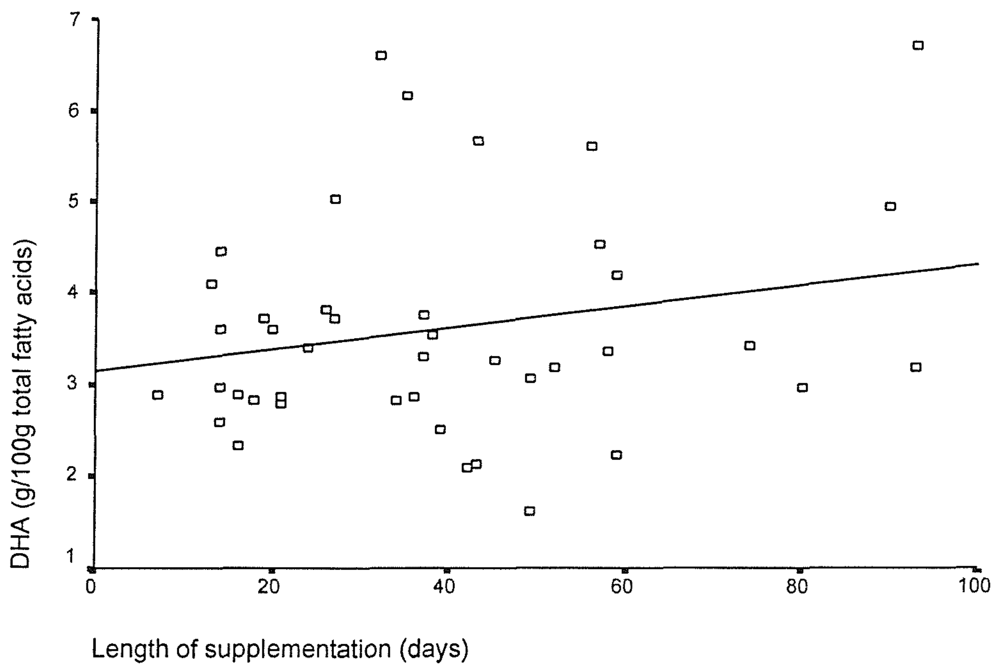


Table 4.4 Fatty acid composition of plaque TAG of patients in the different treatment groups

Fatty acid	Fish oil (n = 44)	Sunflower oil (n = 37)	Control (n = 48)
14:0	2.14 ± 0.12	2.03 ± 0.12	2.04 ± 0.16
16:0	27.44 ± 0.67	28.83 ± 0.52	28.88 ± 0.72
16:1n-7	3.78 ± 0.36	3.41 ± 0.16	3.71 ± 0.25
18:0	5.67 ± 0.17	5.78 ± 0.22	5.92 ± 0.28
18:1n-9	38.75 ± 0.64	37.55 ± 0.46	37.76 ± 0.63
18:2n-6	15.06 ± 0.44	16.39 ± 0.53	15.90 ± 0.58
18:3n-3	0.39 ± 0.05	0.34 ± 0.05	0.49 ± 0.16
20:1n-9	0.16 ± 0.04	0.15 ± 0.03	0.12 ± 0.03
20:2n-9	0.21 ± 0.04[a]	0.28 ± 0.05	0.29 ± 0.06[b]
20:3n-6	0.82 ± 0.07	1.10 ± 0.14	1.11 ± 0.11
20:4n-6	1.97 ± 0.09	2.20 ± 0.13	2.11 ± 0.13
20:5n-3	0.43 ± 0.05[a]	0.24 ± 0.04[b]	0.23 ± 0.04[b]
22:5n-3	0.63 ± 0.08	0.51 ± 0.08	0.46 ± 0.06
22:6n-3	1.24 ± 0.10[a]	1.06 ± 0.11	0.94 ± 0.08[b]

Data are the mean ± SEM, g/100g total fatty acid. Statistical significance (one way ANOVA): groups with different letters are significantly different.

Figure 4.6 Effect of length of supplementation with FO on the proportion of EPA in plaqueTAG

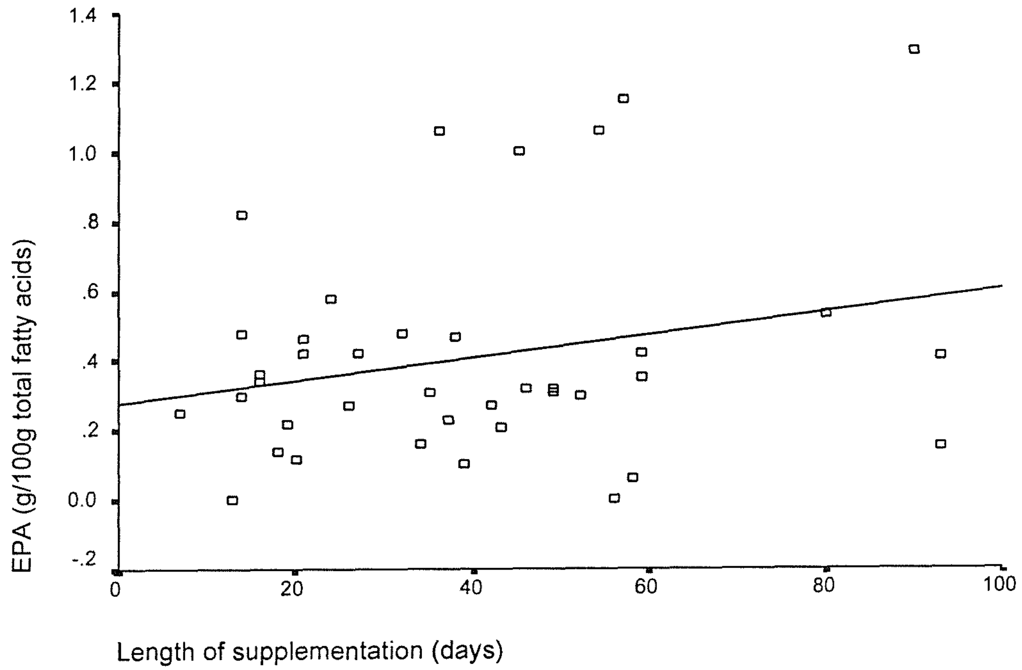
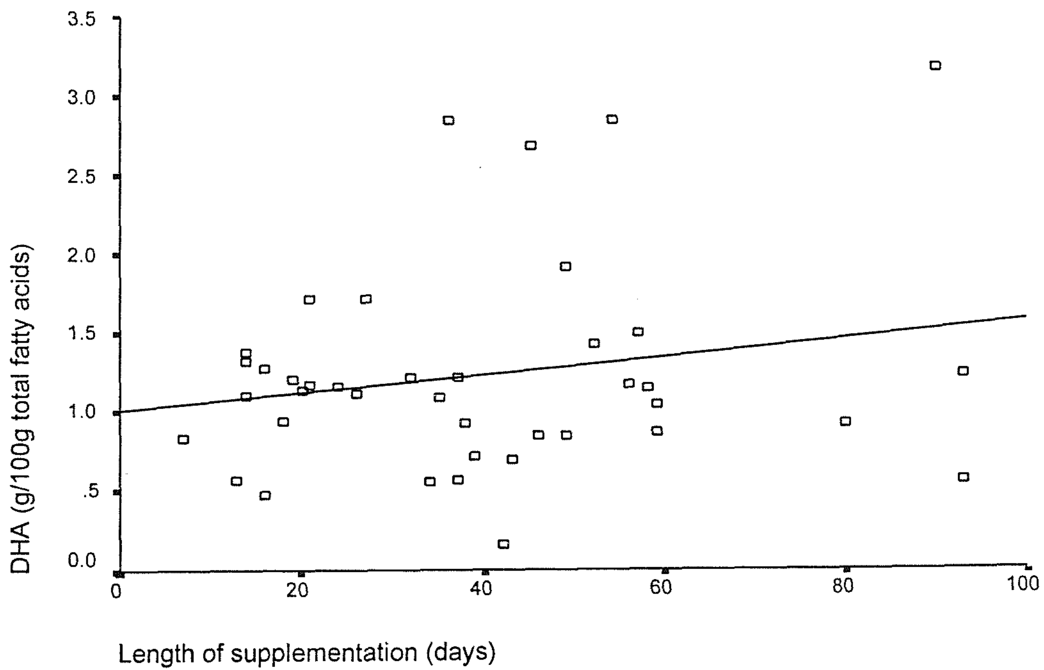


Figure 4.7 Effect of length of supplementation with FO on the proportion of DHA in plaque TAG



4.3.2 Immunohistochemistry on plaque sections

The cell populations involved in the inflammatory activity in the carotid atherosclerotic lesions were characterised with cell specific monoclonal antibodies. Plaque sections were stained for T-lymphocytes, VCAM-1, ICAM-1, and macrophages. Examples of heavily stained plaque sections for each antigen are shown in Figures 4.8 to 4.12, the brick red colour represents positive staiining and blue colour represents background staining.

Staining was performed on only a limited number of sections. This is because attempts to stain with these antibodies on paraffin sections of the first 100 plaques collected showed poor staining and so it was decided to switch to frozen sections. Unfortunately this meant that it was not possible to quantify immunological/inflammatory markers in the first 100 plaques. The details of the patients whose plaques were characterised are shown in Table 4.4a. By comparing this information with that for the treatment group as a whole (Table 2.5; section 2.3.1) it is apparent that the patients whose plaques were characterised are representative of the treatment group.

Table 4.4a Characteristics of patients whose plaque sections were stained

Treatment	**N**	**Male** (N)	**Female** (N)	**Age** (years)	**BMI** (kg/m^2)	**Statin** (% patients)
FO	25	16	9	67.8	26.2	12
SO	20	11	9	67.3	26.1	11
C	23	15	8	67.9	27.1	11

Data for age, BMI and statin are presented as mean.

The results for the five antigens investigated are shown in Table 4.5. Mean ranks for each antigen were compared between treatment groups, it was found that the mean rank for KP1, a macrophage marker, was significantly lower in the fish oil group compared to the other two groups. No other differences were found between groups, although there was a trend for the PGM1 macrophage marker to also have a lower mean rank in the fish oil group.

The frequency of ranks in each treatment group for each antigen was compared. The frequency of ranks for T-lymphocytes and PGM1 were not different between the three treatment groups but they were different from the expected frequencies with the majority of cases being heavily stained. In the sunflower oil and control groups VCAM-1 and ICAM-1 staining was quite evenly distributed between the three ranking groups, whereas after fish oil supplementation very few cases had a score of zero and the majority were heavily stained. The frequency of ranks for KP1 was similar in the sunflower oil and control groups, but was different from the expected frequency with the majority of cases being heavily stained. However, in the fish oil group less cases were heavily stained and more had a moderate amount of staining. Again this distribution of frequencies was different from expected.

Table 4.5 Immunohistochemistry scores

Antigen	Treatment	n	Rank Frequency (% cases) 0	1	2	Mean Rank
T-cell, Leu 4	FO	25	0	26.1	73.9*	33.41
(CD3)	SO	20	5.0	30.0	65.0*	30.20
	C	23	4.8	19.0	76.2*	33.69
VCAM-1	FO	25	8.3	33.3	58.3*	37.58
(CD106)	SO	20	36.8	26.3	36.8	27.18
	C	23	17.4	30.4	52.2	34.46
ICAM-1	FO	25	9.5	14.3	76.2*	32.74
(CD54)	SO	20	12.5	43.8	43.8	24.63
	C	23	21.1	21.1	51.9	27.08
KP1	FO	25	0	38.1	61.9*	51.10[a]
(CD68)	SO	20	0	19.4	80.6*	61.82[b]
	C	23	2.6	13.2	84.2*	63.54[b]
PGM1	FO	25	2.3	27.9	69.8*	58.84
(CD68)	SO	20	0	23.7	76.3*	63.17
	C	23	0	24.4	75.6*	62.74

T-cells= T lymphocytes, VCAM-1= vascular cell adhesion molecule-1, ICAM-1= intercellular adhesion molecule-1, KP1= macrophage marker, PGM1= macrophage marker. Statistical significance (Jonckheere-Terpstra test): groups with different letters are significantly different, * indicates significantly different from expected frequency (Chi-Square test).

Figure 4.8 Microscopic image of CD3 T-cell staining on a carotid plaque

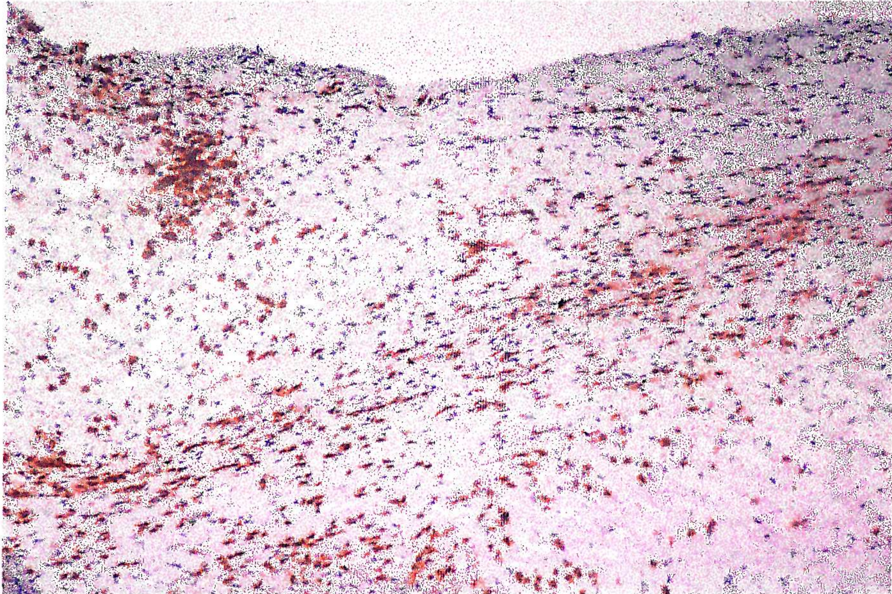


Figure 4.9 Microscopic image of VCAM-1 staining on a carotid plaque

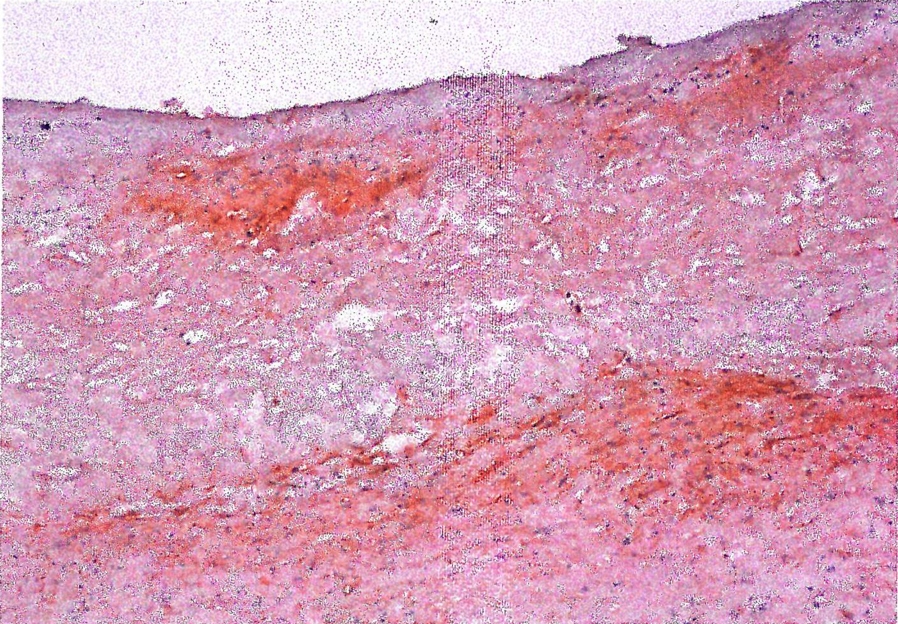


Figure 4.10 Microscopic image of ICAM-1 staining on a carotid plaque

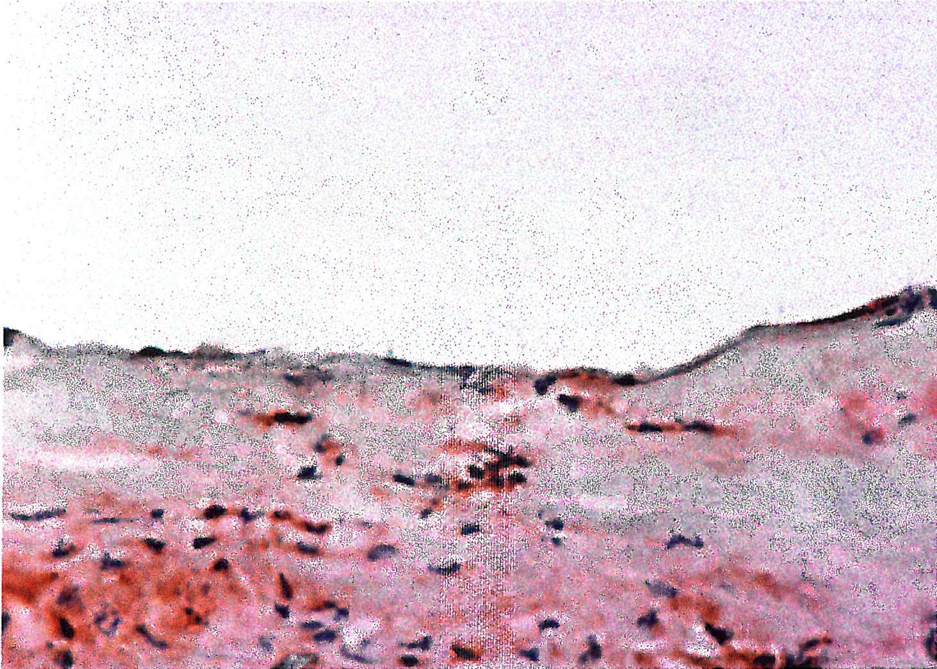


Figure 4.11 Microscopic image of KP-1 macrophage staining on a carotid plaque

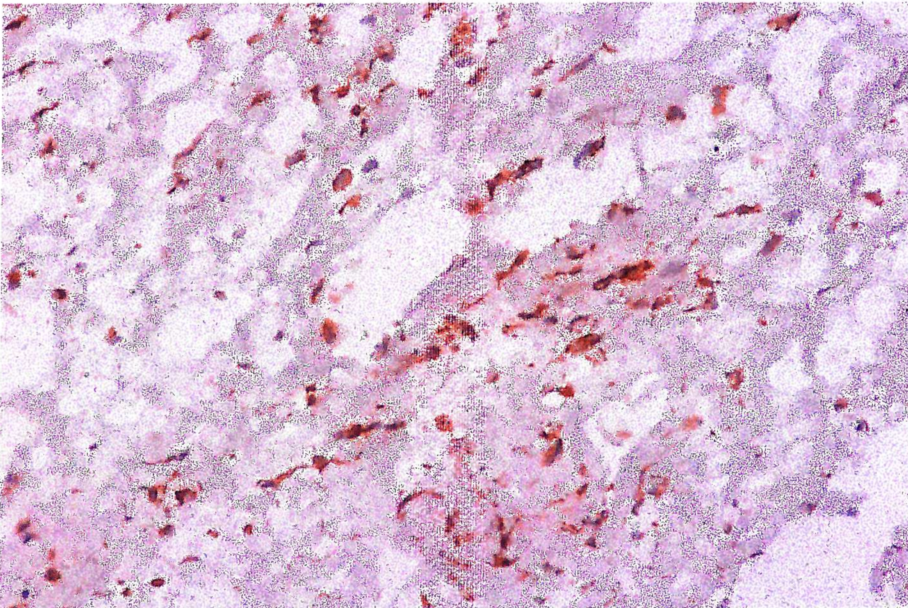
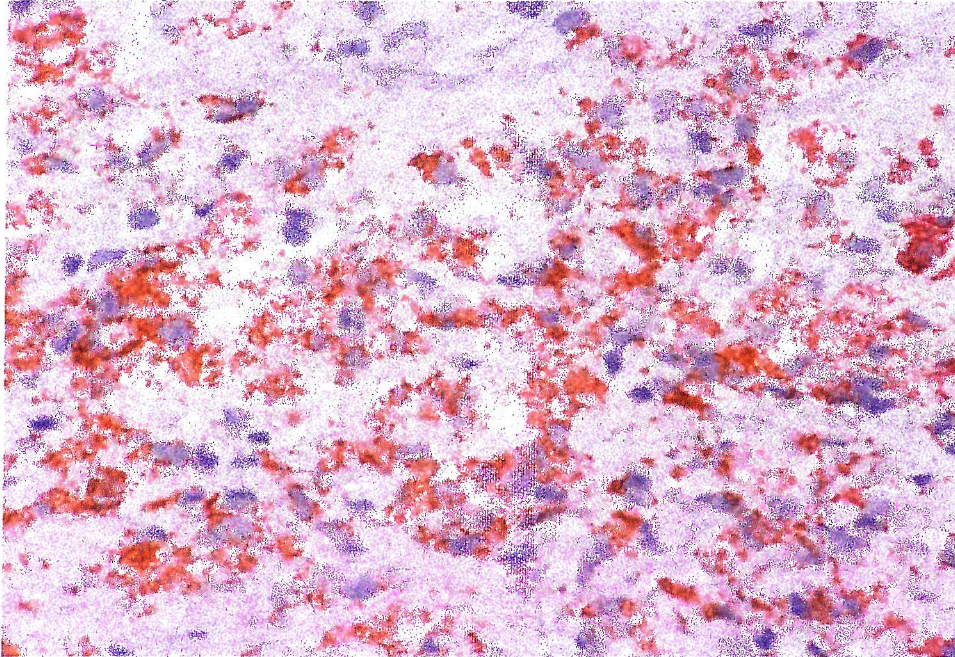


Figure 4.12 Microscopic image of PGM1 macrophage staining on a carotid plaque



4.3.3 Adipose tissue fatty acid composition

Table 4.6 summarises the differences in adipose tissue fatty acid composition between the different dietary groups before (visit 1) and after (visit 2) supplementation. There were no differences in fatty acid composition of adipose tissue between groups before supplementation (see Section 2.3.2.1). After supplementation the proportion of 18:3n-3 was higher in the SO group than in the FO group ($p<0.04$). The proportions of DHA and EPA were higher after FO supplementation than in the control group ($p<0.01$and $p<0.03$).

Dietary supplementation with fish oil resulted in the incorporation of the long chain n-3 PUFA into adipose tissue. EPA and DHA in adipose tissue in the FO group were significantly higher after supplementation. The proportions of 18:3n-3 and16:0 were significantly decreased in the FO group after supplementation. The duration of fish oil supplementation did not appear to be related to the accumulation of EPA ($r = 0.42$, $p = 0.08$; Figure 4.8) or DHA ($r = 0.36$, $p = 0.08$; Figure 4.9) in adipose tissue.

Supplementation with sunflower oil resulted in an increased proportion of 20:4n-6 and a decreased proportion of 16:1n-7. There was also a tendency for an increased proportion of 18:2n-6 after SO supplementation however this was not significant. In the control group the proportions of 16:0, 16:1n-7, and 18:3n-3 were decreased after supplementation.

Table 4.6 Fatty acid composition of adipose tissue from patients in the different treatment groups before and after supplementation

Fatty acid	Visit	Fish oil (n = 48)	Sunflower oil (n = 51)	Control (n = 55)
14:0	1	4.00 ± 0.18	4.08 ± 0.16	4.00 ± 0.16
	2	3.95 ± 0.17	3.79 ± 0.14	3.75 ± 0.15
16:0	1	25.25 ± 0.40	25.73 ± 0.61	24.94 ± 0.46
	2	24.51 ± 0.51*	24.16 ± 0.40	23.84 ± 0.34*
16:1n-7	1	6.04 ± 0.32	6.38 ± 0.39	6.60 ± 0.36
	2	5.34 ± 0.31	5.58 ± 0.27*	5.96 ± 0.36*
18:0	1	3.78 ± 0.20	3.76 ± 0.14	3.60 ± 0.18
	2	3.56 ± 0.15	3.79 ± 0.16	3.53 ± 0.17
18:1n-9	1	43.85 ± 0.50	43.46 ± 0.55	43.55 ± 0.51
	2	43.96 ± 0.38	43.87 ± 0.42	43.83 ± 0.41
18:2n-6	1	13.63 ± 0.51	13.53 ± 0.54	14.22 ± 0.55
	2	13.43 ± 0.54	14.02 ± 0.62	14.14 ± 0.62
18:3n-3	1	1.01 ± 0.05	1.08 ± 0.10	1.09 ± 0.06
	2	0.88 ± 0.04[a]*	1.01 ± 0.05[b]	0.92 ± 0.03*
20:1n-9	1	0.69 ± 0.08	0.86 ± 0.15	0.62 ± 0.07
	2	0.53 ± 0.04	0.54 ± 0.05	0.49 ± 0.04
20:4n-6	1	0.42 ± 0.05	0.41 ± 0.03	0.36 ± 0.02
	2	0.48 ± 0.05	0.45 ± 0.03*	0.44 ± 0.04
20:5n-3	1	0.08 ± 0.03	0.06 ± 0.01	0.09 ± 0.02
	2	0.14 ± 0.02[a]*	0.12 ± 0.03	0.06 ± 0.01[b]
22:6n-3	1	0.20 ± 0.02	0.24 ± 0.05	0.20 ± 0.02
	2	0.29 ± 0.03[a]*	0.24 ± 0.03	0.20 ± 0.02[b]

Data are the mean ± SEM g/100g total fatty acid before (visit 1) and after (visit 2) supplementation.. Statistical significance: groups with different letters are significantly different (one way ANOVA), *significantly different from visit 1 (paired t-test).

Figure 4.13 Effect of length of supplementation with FO on EPA accumulation in adipose tissue

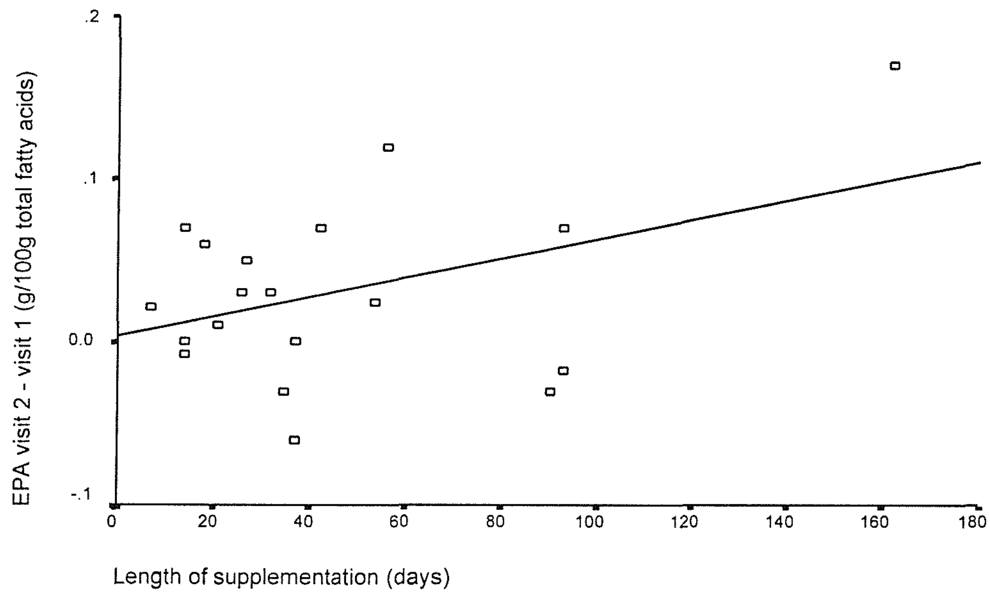
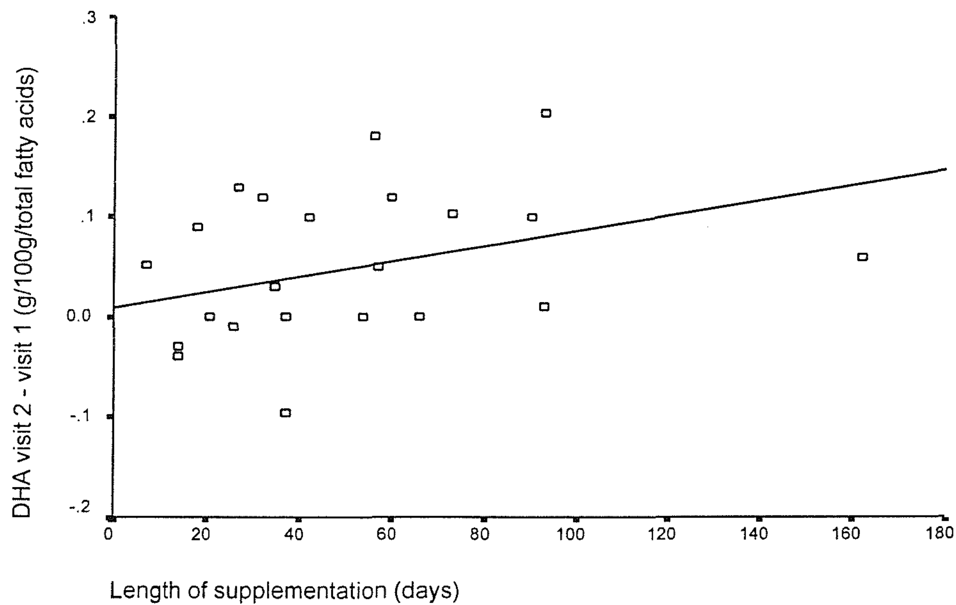


Figure 4.14 Effect of length of supplementation with FO on DHA accumulation in adipose tissue



4.4 Discussion

4.4.1 Plaque fatty acid composition

One aim of this study was to determine whether moderate supplementation with n-3 and n-6 PUFA could change plaque fatty acid composition and to measure the time course for these changes.

Cholesterol esters are the predominant lipid fraction in atherosclerotic plaques and triglycerides are the least abundant lipid (Felton *et al.*, 1997; Smith, 1960). In this study, dietary n-3 fatty acids were shown to be incorporated into the CE TAG and PL classes of advanced human carotid atherosclerotic lesions. These results agree with other published data (Rapp *et al.*, 1991), but in this study fish oil was provided at a much lower dose than in the study by Rapp *et al.* (1991). Patients in this study received 6 g fish oil providing 1.7 g n-3 fatty acids/day (0.8 g EPA plus 0.5 g DHA), whereas in the study by Rapp *et al.* (1991) fish oil was fed at a dose providing 16-21.3 g n-3 fatty acids/day, over ten times higher. Habitual intake of total n-3 fatty acids for the patients in the current study was estimated to be 1.3 g/day (see section 2.3.2). Therefore, in this study patients in the FO group more than doubled their intake of total n-3 fatty acids. The habitual intakes of EPA and DHA by patients in this study were estimated to be 0.1 g/day and 0.13 g/day respectively (see section 2.3.2). Therefore, during supplementation with FO, intakes of EPA were 8-times higher and DHA almost 4-times higher than habitual intakes. EPA in plaque CE in the FO group was 52% higher than in the control group and 24% higher than in the SO group. DHA was 30% higher compared to the other two groups. Rapp *et al.* (1991) found an increase of 79% in EPA and 69% in DHA in plaque CE. In the current study EPA in plaque PL from the FO group was 125% higher compared to the SO group and 104% higher than in the control group. DHA was also slightly increased in the FO group compared to the SO (29%) and control (11%) groups. Rapp *et al.*(1991) found an increase of 90% and 72%, respectively, for EPA and DHA in plaque PL. In this study no effect of FO feeding was seen in the plaque TAG fraction, whereas in the study by Rapp *et al.* (1991) they found a 92% increase in EPA and a 94% increase in DHA in the plaque TAG fraction. Fish oil was fed at a very high dose in the study by Rapp *et al.* (1991), whereas in this study a more moderate dose of fish oil was used. The differences

between the two studies most likely relate to the dose of fish oil used. Nevertheless, the lower dose of fish oil used here resulted in significant alterations in plaque fatty acid composition.

Patients received sunflower oil at a dose of 6 g/day. This represented 3.8 g of n-6 PUFA/day. Habitual intake of n-6 PUFA for this group of patients was estimated to be 9.0 g/day (see Section 2.4) . Therefore, patients in the SO group increased their intake of n-6 PUFA by about 42%. This is quite a small increase in comparison to the increase in n-3 fatty acid intake in the FO group, and no significant differences were seen in plaque n-6 PUFA composition between groups.

Dietary n-3 fatty acids have been previously demonstrated to associate themselves rapidly with the CE, PL and TAG moieties of the plasma lipoproteins (Bromsgust-Schonte *et al.*,1981). This was confirmed in this study (see section 3.3.3). It is known that the isotopically-labelled cholesterol of lipoproteins will also exchange with the cholesterol of atherosclerotic plaques resulting in net cholesterol accumulation in the plaque (Lin *et al.*, 1980; Jagananthan *et al.*, 1974). The information from the current study indicates that long chain n-3 fatty acids can also accumulate in plaques.

It is possible that these findings have clinical significance. N-3 fatty acids have discreet yet powerful biologic effects. They inhibit the production of thromboxane A_2 (Needleman *et al.*,1979), stimulate the production of nitric oxide (Shimokawa *et al.*,1987), decrease the endothelial cell production of a platelet-derived growth factor like protein (Fox and DiCorleto, 1988) and reduce the inflammatory response (Lee *et al.*, 1985; Fisher *et al.*, 1986). While the influx of n-3 fatty acids from plasma into the atherosclerotic arterial wall, as shown in this study, could have an ameliorating effect on the atherosclerotic process, as yet there are no data on the effect of fish oil feeding on pre-existing atherosclerotic lesions, even in experimental animals. Since lipid peroxidation has been shown to be an important stimulus for macrophage uptake of lipoproteins (Fogelman *et al.*, 1980, Henriksen *et al.*, 1981), loading the plaque with n-3 fatty acids could theoretically promote atherogenesis. In this study it was found that the lag time of copper-induced LDL oxidation decreased in patients who received fish oil (see section 3.3.4) . Therefore, fish oil supplementation may make LDL more susceptible to oxidation, and therefore more readily taken up by macrophages. However, there are studies showing

the inhibition of atherosclerosis by n-3 fatty acids in monkeys, pigs (Davis *et al.*, 1987; Weiner *et al.*, 1986) and mice (Renier *et al.*, 1993). This inhibition occurred even though the plasma cholesterol levels remained high and HDL was low. In the SCIMO and GISSI trials (see Section 1.3.2.3.2) the concentration of LDL cholesterol slightly increased in the n-3 PUFA treatment groups. Therefore n-3 PUFA seem to act through mechanism(s) other than simply an alteration of plasma lipid concentrations.

In this study it has been shown that moderate supplementation with PUFA is able to induce changes in the fatty acid composition of advanced atheromatous plaques. A recent study by Felton *et al.* (1997) has shown that disrupted plaques have a higher content of PUFA and a PUFA/SFA ratio three-fold greater than intact plaques. Felton *et al.* (1997) also suggested that increased lipid has an adverse influence on plaque stability. Therefore, the incorporation of PUFA into plaques of patients with advanced atherosclerosis may in fact make these plaques more unstable and therefore could promote an atherosclerotic event.

4.4.2 Immunohistochemistry on plaques

Atherosclerotic plaque formation results from complex cellular interactions in the intima of arteries, which take place between resident cells of the vessel wall (smooth muscle cells and endothelial cells) and cells of the immune system (leukocytes). Local flow disturbances and lipids as a driving force appear to be obligatory in this process (Ross, 1993). Once an atherosclerotic plaque has formed, it shows the highly characteristic architecture of a fibrous cap encaging a central core of extracellular lipids and debris: the atheroma. Fibrous tissue provides the structural integrity of a plaque, whereas the atheroma is soft, weak and highly thrombogenic. It is rich in extracellular lipids and is bordered by a rim of lipid-laden macrophages.

The intrinsic features that characterise a plaque as vulnerable are an increased lipid content, an increased macrophage, foam cell and T lymphocyte content, and a reduced collagen and smooth muscle content (Gertz *et al.*, 1990). Rupture tends to occur at the margins or 'shoulder region' of plaques where the overlying fibrous cap is necrotic, very thin and extensively infiltrated by macrophages and adjacent to relatively normal tissue (Constantinides, 1990). However the exact mechanisms causing plaque rupture are not yet completely known.

In this study some of the cellular and vascular components of surgically excised carotid endarterectomies were analysed. In other words, some of the cell populations involved in the inflammatory activity in atherosclerotic lesions were characterised with cell specific monoclonal antibodies. It was found that after fish oil supplementation the expression of KP1, a macrophage marker, was less intense than in the other two treatment groups and there was also a similar trend for PGM1 another macrophage marker. This may suggest that there is less macrophage activity in these plaques and thus that these plaques are more stable and less likely to rupture.

4.4.3 Adipose tissue fatty acid composition

Serum and adipose tissue fatty acid concentrations are largely determined by dietary intake, especially for the essential fatty acids, linoleic (18:2n-6) and α-linolenic (18:3n-3) acids, which cannot be synthesised by man. Adipose tissue composition has served as a biological marker of chronic ingestion of many dietary PUFA. In this study it was found that there is a strong relationship between dietary PUFA intake and fatty acid composition of adipose tissue (see Section 2.3.2.1) .

Dietary supplementation with fish oil resulted in the incorporation of the long chain n-3 PUFA into adipose tissue.. EPA and DHA in adipose tissue in the FO group were significantly higher after supplementation. Patients received fish oil at a dose of 6 g/day this represented 1.7 g n-3 fatty acids/day for 1 to 24.7 weeks. Previous studies have shown similar findings but generally with a higher dose of n-3 PUFA and for a longer supplementation period (see Section 4.1.2). These studies indicate that the long-term ingestion of large amounts of n-3 fatty acids in humans results in their incorporation into the adipose tissue fatty acids. However, the results of the current study show that lower intakes of n-3 fatty acids for a shorter period of time can still result in the incorporation of EPA and DHA into adipose tissue.

In summary, it has been shown that adipose tissue and plaque fatty acid composition can be changed by short term changes in n-3 PUFA intake in patients with advanced carotid atherosclerosis and that the incorporation of n-3 PUFA into carotid plaques may make them more stable.

Chapter 5
General Discussion

5 General Discussion

Cardiovascular disease involves the formation of atherosclerotic plaques. These dynamic structures include lipid, largely derived from circulating LDL particularly following its oxidation, and inflammatory cells. The inflammatory cells can be responsible for LDL oxidation. Plaques can be destabilised and if they rupture can block the circulation resulting in a myocardial infarction (if in the coronary artery) or a stroke (if in the carotid artery). It is not clear whether n-6 and n-3 PUFA are incorporated into active atherosclerotic plaques and, if they are, whether they act to stabilise or destabilise the plaque.

The aims of this study were to examine the relationship between fatty acids in the diet, risk factors for CVD, and atherosclerotic plaque structure and composition in patients undergoing carotid endarterectomy. To do this the effects of moderate supplementation with n-3 and n-6 PUFA on plasma lipid concentrations, the fatty acid profile and oxidisability of plasma LDL and of lipid fractions isolated from atherosclerotic plaques, and on the characteristics of atherosclerotic plaques in these patients were determined.

It was found that supplementation with fish oil containing n-3 PUFA lowered plasma cholesterol and triacyglycerol concentrations, altered the fatty acid composition of plasma LDL lipid fractions, adipose tissue and atherosclerotic plaques and decreased the lag time of copper induced oxidation of LDL. Supplementation with sunflower oil rich in n-6 PUFA lowered plasma cholesterol concentrations and resulted in increased levels of linoleic acid in LDL CE.

It has generally been assumed that n-6 PUFA (and linoleic acid in particular) have a favourable effect on CHD risk by means of a decrease in blood cholesterol concentrations (European Atherosclerosis Society, 1987). Burr *et al.* (1989) showed a 2% decrease, Woodhill *et al.,* (1978) an 11% decrease and Leren (1970) an 18% decrease in cholesterol concentrations after increasing linoleic acid intake. In the current study a 13% decrease in plasma total cholesterol concentration occurred after supplementation with sunflower oil rich in linoleic acid. There have been numerous studies investigating the effects of fish oil or its constituent n-3 fatty acids on serum or plasma lipid concentrations. It seems that a reduction in total cholesterol concentration occurs only if

large doses (4.5-6 g EPA or 6.5-10 g total n-3 fatty acids per day) are given (Davidson *et al.*, 1987). Smaller amounts of n-3 fatty acids appear to have little effect, or even to cause a slight increase in total cholesterol concentration (Burr *et al.*, 1989). However in the current study supplementation with 1.7 g n-3 fatty acids per day (including 860 mg EPA) resulted in a 15% decrease in plasma total cholesterol concentration.

Epidemiological studies have demonstrated that the incidence of CHD is inversely associated with consumption of n-3 PUFA (Bang and Dyerberg, 1972). The DART and GISSI-P trials provided evidence that relatively low doses of n-3 PUFA reduce the risk of secondary coronary events (Burr *et al.*, 1989; GISSI-P, 1999). Plasma TAG concentrations are significantly reduced by n-3 PUFA, in a dose dependent fashion and this may partly explain the cardio-protective effect of n-3 PUFA. In a review of 72 placebo-controlled human studies which supplemented a range of 1.0 to 7.0 g EPA plus DHA daily for at least two weeks, fasting plasma TAG concentrations were consistently decreased by 25-30% (Harris, 1996). In the current study a moderate supplementation with fish oil has been used, providing approximately 1.7 g/d of n-3 PUFA, and plasma TAG concentration was reduced by 29%. This shows that a moderate intake of n-3 PUFA, which could be achieved by dietary intervention or as a supplement has a positive effect on TAG metabolism in this group of patients.

It has previously been found that when fish oil is incorporated into the diet plasma concentrations of EPA and DHA increase several-fold within a few weeks (Harris and Connor, 1980). In the current study it was found that supplementation with fish oil caused a significant enrichment in long chain n-3 PUFA in all LDL lipid classes. LDL has been shown to be quantitatively the most important source of lipid for atherosclerotic plaques. Therefore, if a moderate supplementation of fish oil as used in this study affects LDL composition it may also affect atherosclerotic plaque composition.

In this study of human atherosclerotic plaques, dietary n-3 fatty acids were found to be incorporated into the CE and PL classes of advanced atherosclerotic lesions. These results agree with other published data (Rapp *et al.*, 1991) but in this study fish oil was provided at a much lower dose than in the study by Rapp *et al.* (1991). Patients in this study received 6 g fish oil providing 1.7 g n-3 fatty acids/day (0.8 g EPA and 0.5 g DHA), whereas in the study by Rapp *et al.* (1991) fish oil was fed at a dose of 16-21.3 g n-3 fatty

acids/day, over ten times higher. This explains the smaller changes seen in the current study. Nevertheless, the lower dose of fish oil used here resulted in significant alterations in plaque fatty acid composition. The influx of n-3 fatty acids from plasma into the atherosclerotic arterial wall could have an ameliorating effect on the atherosclerotic process. However, lipid peroxidation has been shown to be an important stimulus for macrophage uptake of lipoproteins (Fogelman *et al.*, 1980, Henriksen *et al.*, 1981) and therefore loading the plaque with n-3 fatty acids could theoretically promote atherogenesis. A recent study by Felton *et al.* (1997) has shown that disrupted plaques have a higher content of PUFA and a P/S ratio threefold greater than intact plaques. Felton *et al.* (1997) also suggested that increased lipid has an adverse influence on plaque stability. Therefore, the incorporation of PUFA into plaques of patients with advanced atherosclerosis may in fact make these plaques more unstable and therefore could promote thrombosis and rupture of plaques.

There is increasing evidence that oxidatively modified LDL is an important mediator in the pathogenesis of atherosclerosis (Steinberg *et al.*, 1989; Salonen *et al.*, 1992). Some of the reduction in heart disease risk because of increased dietary intake of PUFA may be negated by increasing the availability of unsaturated bonds to oxidative processes. The effects of dietary n-3 PUFA on the susceptibility of LDL to oxidation are contradictory. In a study by Nenseter *et al.* (1991), the effect of dietary supplementation with n-3 PUFA on the susceptibility of LDL to oxidative modification was investigated. LDL from both the fish oil and corn oil supplemented groups showed similar susceptibility to copper-catalysed lipid peroxidation. Frankel *et al.* (1994) looked at the effect of n-3 PUFA supplementation on copper-catalysed oxidation of LDL. The volatile oxidation products from LDL (propanal and hexanal) were measured. Total volatiles remained unchanged thus indicating that fish oil intake did not alter the oxidative susceptibility of human LDL. However, in other studies n-3 PUFA have been shown to enhance the susceptibility of LDL to oxidative modification. Suzukawa *et al.* (1995) found that fish oil decreased lag time before onset of copper-induced LDL oxidation and significantly increased production of thiobarbituric acid-reactive substances (TBARS) during oxidation compared with corn oil. In the current study it was found that supplementation with 1.7 g n-3 fatty acids/day caused a 26% decrease in lag time.

Therefore, fish oil supplementation may adversely affect the susceptibility of LDL to oxidation. It has been shown that diets enriched with linoleic acid increase the linoleic acid content of LDL and increase its susceptibility to oxidation (Parthasarathy *et al.*, 1990; Reaven *et al.*, 1993; Abbey *et al.*, 1993). However, in the current study supplementation with linoleic acid did not cause any changes in the susceptibility of LDL to oxidation.

Adipose tissue composition has served as a biological marker of chronic ingestion of many dietary PUFA. In this study a strong relationship between dietary fatty acid intake and fatty acid composition of adipose tissue was demonstrated, although this relationship was not always direct. No significant correlation was seen between total SFA and MUFA intake, and adipose tissue SFA and MUFA content respectively. PUFA intake however was much more strongly correlated with PUFA content of adipose tissue. In this study dietary supplementation with fish oil resulted in the incorporation of the long chain n-3 PUFA into adipose tissue. Previous studies have shown similar findings, but generally with a higher dose of n-3 PUFA than used here and for a longer supplementation period. A study by Leaf *et al.* (1995) looked at the effect of daily dietary supplementation with $\geq$ 10 g n-3 fatty acids from fish oil for > 12 months. They found significantly greater EPA, 22:5n-3 and DHA concentrations in adipose tissue of supplemented patients compared with non-supplemented control patients. In another study by Katan *et al.* (1997), 58 men were fed 0, 3, 6 or 9 g/d fish oil for 12 months and fatty acids monitored in subcutaneous fat during and after supplementation. They found that levels of EPA in adipose tissue increased after supplementation, in a dose dependent manner. In a study by Sacks *et al.* (1995), patients with CHD were randomised to receive either fish oil capsules, containing 6 g of n-3 fatty acids, or olive oil capsules for an average duration of 28 months. At the end of the trial EPA in adipose tissue samples was 0.91% in the fish oil group compared with 0.20% in the control group. These studies indicate that the long-term ingestion of large amounts of n-3 fatty acids in humans results in their incorporation into adipose tissue. However, the results of the current study show that lower intakes of n-3 fatty acids for a shorter period of time can still result in the incorporation of EPA and DHA into adipose tissue.

The results of this study show that a moderate supplementation of fish oil in patients with carotid atherosclerosis results in incorporation of the long chain n-3 PUFA (EPA and DHA) into LDL fractions, adipose tissue and atherosclerotic plaques. Plasma TAG and total cholesterol concentrations were decreased but LDL was more susceptible to oxidation. However the incorporation of n-3 PUFA into atherosclerotic plaques led to a lower macrophage content which may make these plaques more stable and less likely to rupture.

Atherosclerotic plaques can be destabilised, and if they rupture they can block the circulation resulting in a myocardial infarction (if in the coronary artery) or a stroke (if in the carotid artery). Previous studies have shown protection of primary and secondary cardiovascular events with increasing n-3 PUFA consumption (see Section 1.3.2.3). This study has also shown beneficial effects of increasing n-3 PUFA consumption largely through decreasing lipid levels and through the incorporation of n-3 PUFA into active atherosclerotic plaques, which may act to stabilise them. However further work is needed to look at the stability of these plaques by investigating other factors that may affect plaque stability such as matrix metalloproteinases, activated T-cells and inflammatory cytokines. Also it is not known if the effects seen in this study on carotid plaques would also occur in other atherosclerotic plaques for example in the coronary artery and this warrants further investigation. There is emerging consensus that plaque instability rather than progression should be the target for new therapies, because it is instability that underlies most clinical events. The ability to identify plaque composition and monitor its progression can therefore give valuable information regarding the development of acute coronary syndromes and target areas for intervention to reduce or prevent the socio-economic impact of cardiovascular disease.

6 REFERENCES

Abbey M., Belling G. B., Noakes M., Hirata F., Nestel P.J. (1993). Oxidation of low density lipoproteins: intraindividual variability and the effect of dietary linoleate supplementation. Am J. Clin. Nutr. **57**, 391-398.

Abbey M., Clifton P.M., McMurchie E.J., McIntosh G.H., Nestel P.J. (1990). Effect of a high fat/cholesterol diet with or without eicosapentaenoic acid on plasma lipids, lipoproteins and lipid transfer protein activity in the Marmoset. Atherosclerosis **81**, 163-174.

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

Andersen L.F., Solvoll K., Johansson L.R.K., Salminen I., Aro A., Drevon C.A. (1999). Evaluation of a food frequency questionnaire with weighed records, fatty acids and alpha-tocopherol in adipose tissue and serum. Am. J. Epidem. **150** (1), 75-87.

Badimon J.J., Fuster V., Chesebro J., Badimon L. (1993). Coronary atherosclerosis: a multifactorial disease. Circulation. **87** (2), 3-16.

Ball M. (1988) Atherosclerosis: In Lipids and Heart Disease, A Practical Approach. pp. 20-29

Ball R. Y., Stowers E. C., Burton J.H., Cary N. R., Skepper J.N. and Mitchinson M.J. (1995). Evidence that death of macrophage foam cells contribute to the lipid core of atheroma. Atherosclerosis. **114**, 45-54.

Bang H.O. and Dyerberg J. (1972). Plasma lipids and lipoproteins in Greenlandic West-coast Eskimos. Acta. Medica. Scandinavia. **192**, 85-94.

Berry E.M., Eisenberg S., Haratz D. Friendlander Y., Norman Y., Kaufmann N.A., Stein Y. (1991). Effects of diets rich in monounsaturated fatty acids on plasma lipoproteins- the Jerusalem Nutrition Study: high MUFA vs. high PUFA. Am. J. Clin. Nutr. **53** (4), 899-907.

Berry E.M., Eisenberg S., Friedlander M., Harats D., Kaufmann N.A., Norman Y., Stein Y. (1992). Effects of Diets rich in MUFA on plasma-lipoproteins-the Jerusalem Nutrition Study. 2. MUFA vs Carbohydrates. Am. J. Clin. Nutr. **56** (2), 394-403.

Beynen A.C., Hermus R.J.J., Hautvast J.G.A.J. (1980). A mathematical relationship between fatty acid composition of the diet and that of adipose tissue in man. Am. J. Clin. Nutr. **33**, 81-85.

Bini A., Fenoglio J.J., Mesa-Tejada. (1989). Identification and distribution of fibrinogen, fibrin and fibrin(ogen) degradation products in atherosclerosis. Arteriosclerosis. **9**, 109-121.

Bjorkerud B., Bjorkerud S. (1996). Contrary effects of lightly and strongly oxidised LDL with potent promotion of growth vs. apoptosis on arterial smooth muscle cells, macrophages and fibroblasts. Arterioscler. Thromb. Vasc. Biol. **16**, 416-424.

Blasi F., Ranzi M. L., Erba M., Tarsia P., Raccanelli R., Fagetti L. and Allegra L. (1996). No evidence for the presence of *Helicobacter pylori* in atherosclerotic plaques in abdominal aortic aneurysm specimens. Atherosclerosis. **126**, 339-340.

Bligh E.G. and Dyer W.J. (1959). A rapid method of total lipid extraction and purification. Can J. Biochem. Physiol. **37**, 911-917.

Bogousslavsky J., Hachinski V.C., Boughner D.R., Fox A.J., Vinuela F, Barnett H.J.M. (1986). Cardiac and arterial lesions in carotid Transient Ischemic Attacks. Arch. Neurol. **43 (3)**, 223-8.

Bonanome A., Grundy S.M. (1988). Effect of dietary stearic acid on plasma cholesterol and lipoprotein levels. N. Engl. J. Med. **318**, 1244-1248.

Bonanome A., Pagnan A., Biffanti S. Opportuno A., Sorgato F., Dorella M., Maiorino M., Ursini F. (1992). Effect of dietary monounsaturated and polyunsaturated fatty acids on the susceptibility of plasma low density lipoproteins to oxidative modification. Arterioscler. Thromb. **12** (4), 529-533.

Boushey C.J., Beresford S.A., Omenn G.S., Motulsky A.G. (1995). A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Possible benefits of increasing folic acid intakes. **274** (13), 1049-1057.

British Nutrition Foundation (1992). Unsaturated fatty acids: Nutritional and physiological significance. Task Force Report. London: Chapman and Hall.

British Nutrition Foundation (1999). Briefing Paper. N-3 fatty acids and health. London: BNF.

Brown M.S., Goldstein J.L. (1983). Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclerosis. Annu. Rev. Biochem. **52,** 223-261.

Brown M.S., Goldstein J.L. (1986). A receptor-mediated pathway for cholesterol homeostasis. Science **232**, 34. © The Nobel Foundation.

Bruckdorfer K. R. (1990). Free radicals, lipid peroxidation and atherosclerosis. Curr. Op. Lipidol. **1**, 529-535.

Bromsgust-Schonte H.C., Van Gruet C.M., Luten J.B., Ruiter A. (1981). The effect of various intakes of n-3 fatty acids on the blood lipid composition in healthy human subjects. Am. J. Clin. Nutr. **34**, 1752-1757.

Burr M. L., Gilbert J. F., Holliday R. M., Elwood P. C., Fehily A. M., Rogers S., Sweetman P. M., Deadman N. M. (1989). Effects of changes in fat, fish and fibre intake

on death and myocardial reinfarction: diet and reinfarction trial (DART). Lancet **2**, 757-761.

Calder P.C. (1996). Immunomodulatory and anti-inflammatory effects of n-3 polyunsaturated fatty acids. Proceedings of the Nutrition Society. **55**, 737-774.

Cahill P. D., Sarnis G. E., Cooper A. D., Wood P. D., Kosek J.C., Mitchell R. S., Miller D. C. (1988). Inhibition of vein graft intimal thickening by eicosapentaenoic acid. Reduced thromboxane production without change in lipoprotein levels or low density lipoprotein receptor density. J. Vasc. Surg. **7**, 108-118.

Chobanian A.V. (1992). Pathophysiology of atherosclerosis. Am. J. Cardiol. **70**, 3G-7G.

Clare K., Hardwick S.J., Carpenter K.L., Weeratunge N., Mitchinson M.J. (1995). Toxicity of oxysterols to human monocyte-macrophages. Atherosclerosis. **118**, 67-75.

Clark L.T. (1992). Atherogenesis and thrombosis: mechanisms, pathogenesis and therapeutic implications. Am. Heart J. **123**, 1106-1109.

Collins P., Fox K. (1992). The pathogenesis of atheroma and the rationale for its treatment. Eur. Heart J. **13**, 560-565.

Constantinides P. (1990). Cause of thrombosis in human atherosclerotic arteries. Am J. Cardiol. **66**, 37G-40G

Curb J. D., Reed D. M. (1985). Fish consumption and mortality from coronary heart disease. N. Engl. J. Med. **313**, 820-821.

Davidson M.H., McKenna R. Sullivan D.P. Liebson P.R. Schoenberger J.A. and Messer J.V. (1987). J. Am. Coll. Cardiol. **9**, 185A.

Davies M.J., Richardson P.D., Woolf N., Katz D.R., Mann J. (1993). Risk of thrombosis in human atherosclerotic plaques: role of extracellular lipid, macrophage and smooth muscle cell content. Br. Heart J. **69**, 377-381.

Davies M.J., Thomas A.C. (1984). Thrombosis in acute coronary artery plaques in sudden ischaemic death. N. Engl. J. Med. **310**, 1137-1140.

Davies M.J., Woolf N., Rowles P., Richardson P.D. (1994). Lipid and cellular constituents of unstable human aortic plaques. Basic Res. Cardiol. **89**, 33-39.

Daviglus M.L., Stamler J., Orencia A.J., Morris D., Shekelle R.B. (1997). Fish consumption and the 30-year risk of fatal myocardial infarction. N. Engl. J. Med. **336**, 1046-1053.

Davis H.R., Benstine R.T., Vesrelinovitch D., Weissler R.W. (1987). Fish oil inhibits development of atherosclerosis in rhesus monkeys. **7**, 441-449.

Dehmer G.J., Popma J.J., Vanderberg E.K., Eichorn E.J., Previtt J.B., Campbell W.B., Jennings L., Willerson J.T., Schmitz J.M. (1988). Reduction in the rate of early restenosis after coronary artery angioplasty by a diet supplemented with n-3 fatty acids. N. Engl. J. Med. **319**, 733-740.

de Logeril M., Renaud S., Mamelle N., Salen P., Martin J.L., Monjaud I. (1994). Mediterranean alpha-linolenic acid-rich diet in secondary prevention of coronary heart disease. Lancet **343**, 1454-1459.

de Logeril M., Salen P., Martin J.L., Monjaud I., Delaye J., Mamelle N. (1999). Mediterranean diet, traditional risk factors, and the rate of cardiovascular complications after myocardial infarction: final report of the Lyon Diet Heart Study. Circulation **99**, 779-785.

Department of Health (1991). Dietary Reference Values for Food Energy and Nutrients for the United Kingdom. Committee on Medical Aspects of Food Policy. Report on Health and Social Subjects. **41**. HMSO. London.

Department of Health. Report on Health and Social Subjects (1994). **46**. Nutritional aspects of cardiovascular disease. HMSO.

Dolecek T. A., Grandits G., Caggiula A.W., Tillotson J.(1989). Dietary omega-3 fatty acids and mortality in the multiple risk factor intervention trial. Proc. Second World Congress Prev. Cardiol., USA . Abs. **255**.

Dyerberg J., Bang H.O., Stofferson E., Moncada S., Vane J.R. (1978). Eicosapentaenoic acid and prevention of thrombosis and atherosclerosis? Lancet **ii**, 117-119.

Editorial. Fish Oil. (1988) Lancet **14** (1), 1081-1083.

El-Saadani M., Esterbauer H., El-Sayed M., Goher M., Nassar A.Y., Jurgens G. (1989). A spectrophotometric assay for lipid peroxides in serum lipoproteins using a commercially available reagent. J. Lipid Res. **30** (4), 627-630.

Eliaziw M., Streifler J.Y., Fox A.J., Hachinski V.C., Ferguson G.G., Barnett H.J.M. (1994). For the North American Symptomatic Carotid Endarterectomy Trial. Significance of plaque ulceration in symptomatic patients with high-grade carotid stenosis. Stroke **25**, 304-308.

Emeson E.E., Robertson A.L. (1988). Lymphocytes-T in aortic and coronary intimas-their potential role in atherogenesis. Am. J. Pathol. **130** (2), 369-376.

Eritsland J., Arnesen H., Berg K., Seljeflot I., Abdenoor M. (1995). Serum Lp(a) lipoprotein levels in patients with coronary artery disease and the influence of long-term n-3 fatty acid supplementation. Scan. J. Clin. Lab. Invest. **55**, 295-300.

Esterbauer H., Jurgens G., Quehenberger O., Koller E. (1987). Autoxidation of human low density lipoprotein: loss of polyunsaturated fatty acids and vitamin E and generation of aldehydes. J. Lipid Res. **28**, 495-509.

Esterbauer H., Striegel G., Puhh H., Rotheneder M. (1989). Continuous monitoring of in vitro oxidation of human low density lipoprotein. Free Rad. Res. Communs. **6** (1), 67-75.

Esterbauer H. (1996). Estimation of peroxidative damage. Path. Biol. **44**, 25-28.

European Atherosclerosis Society (1987). Strategies for the prevention of coronary heart disease: a policy statement of the European Atherosclerosis Society. Eur, Heart J. **8**, 77-88.

European carotid plaque study group (1995). Carotid artery plaque composition. Relationship to clinical presentation and ultrasound B-mode imaging. Eur. J. Endovasc. Surg. **10**, 23-40.

Fagiotto A. and Ross R. (1984). Studies of hypercholesterlemia in the nonhuman primate. 2. Fatty streak conversion to fibrous plaque. Arteriosclerosis.**4**, 341-356.

Falk E. (1983). Plaque rupture with severe pre-existing stenosis precipitating coronary thrombosis: characteristics of coronary atherosclerotic plaques underlying fatal occlusive thrombi. Br. Heart J. **50**, 127-134.

Falk E. (1985). Unstable angina with fatal outcome: dynamic coronary thrombus leading to infarction and/or sudden death. Circulation. **71**, 699-708.

Felton C.V., Crook D., Davies M.J. Oliver M.F. (1994). Dietary polyunsaturated fatty acids and composition of human aortic plaques. Lancet. **344**, 1195-1196.

Felton C.V., Crook D., Davies M.J. Oliver M.F. (1997). Relation of plaque lipid composition and morphology to the stability of human aortic plaques. Arterioscler. Thromb. Vasc. Biol. **17**, 1337-1345.

Field C.S., Angel A., Clandinin M.T. (1985). Relationship of diet to the fatty acid composition of human adipose tissue structural and stored lipids. J. Clin. Nutr. **42**, 1206-1220.

Fisher M., Upchurch K. S., Johnson M. H., Vandeil C.H., Natale A., Hoogasian J. J. (1986). Effects of dietary fish oil supplementation on polymorphonuclear leukocyte inflammatory potential. Inflammation. **10**, 387-392.

Fogelman A.M., Shechart I., Seager J., Hokom M., Child J.S., Edwards P.A. (1980). Malondialdehyde alteration of low density lipoproteins leads to cholesteryl ester

accumulation in human monocyte-macrophages. Proc. Natl. Acad. Sci. USA **77**, 2214-2218.

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

Fox P. L., DiCorleto P. E. (1988). Fish oils inhibit endothelial cell production of platelet derived growth factor-like protein. Science. **241**, 453-456.

Frankel E. N., Parks E. J., Xu R., Schneeman B. O., Davies P.A., German J.B. (1994). Effect of n-3 fatty acid rich fish oil supplements on the oxidation of low density lipoproteins. Lipids. **29**, 233-236.

Fraser I., Hughes D. and Gordon S. (1993). Divalent cation-independent macrophage adhesion inhibited by monoclonal antibody to murine scavenger receptor. Nature. **364**, 343-346.

Frayn K. (1996). Metabolic regulation: a human perspective. Portland Press, London.

Friedewald W.T., Levy R.I., Friderickson D.S. (1972). Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of preparative ultracentrifugation. Clin. Chem. **18** (6), 499-502.

Frostegard J., Wu R., Giscombe R., Holm G., Lefvert A. K., Nielson J. (1992). Induction of T-cell activation by oxidised low-density-lipoprotein. Arterioscler. Thromb. **12** (4), 461-467.

Fuster V., Badimon L., Badimon J.J., Chesebro J.H. (1992). The pathogenesis of coronary artery disease and the acute coronary syndromes: part 1. New. Engl. J. Med. **326** (4), 242-250.

Fuster V., Lewis A. (1994). Conner Memorial Lecture, mechanisms leading to myocardial infarction: insights from studies of vascular biology. Circulation. **90**, 2126-2146.

Fuster V., Fayad A.Z., Badimon J.J. (1999). Acute coronary syndromes : biology. Lancet. **353** (2), 5-9.

Garland M., Sacks F.M., Loditz G.A., Rimm E.B., Sampson L.A., Willett W.C., Hunter D.J. (1998). The relation between dietary intake and adipose tissue composition of selected fatty acids in US women. Am. J. Clin. Nutr. **67**, 25-30.

Gelabert H.A. and Moore W.S. (1991). Carotid endarterectomy: current status. Curr. Probl. Surg. **28**, 181-262.

Gertz S.D., Kragel A.H., Kalan J.M., Braunwald E., Roberts W.C.(1990). Comparison of coronary and myocardial morphologic findings in patients with and without thrombolytic therapy during fatal first acute myocardial infarction. The TIMI Investigators. A. J. Cardiol. **66**, 904-909.

Gerszten R. E., Luscinskas F.W., Ding H.T., Dichek D. A., Stoolman L.M., Gimbrone M. A. and Rosenzweig A. (1996). Adhesion of memory lymphocytes to vascular cell adhesion molecule-1 transduced human vascular endothelial cells under simulated physiological flow conditions in vitro. Circ. Res. **79**, 1205-1215.

Geng Y-J., and Hansson G.K. (1995). High endothelial cells of postcapillary venules express the scavenger receptor in human peripheral lymph nodes. Scand. J. Immunol. 42, 289-296.

Gieseg S.P. and Esterbauer H. (1994). Low density lipoprotein is saturable by prooxidant copper. FEBS Lett. **434**, 188-194.

Goldstein J.L., Brown M.S. (1977). The low-density lipoprotein pathway and its relation to atherosclerosis. Ann. Rev. Biochem. **46,** 897-930.

Graham R.C.J.R., Karnovsky M.J. (1965). The histochemical demonstration of monoamine oxidase activity by coupled peroxidase oxidation. J. Histochem. Cytochem. **13** (7), 604-605.

Gregory J., Foster K., Tyler H and Wiseman M. (1990). The Dietary and Nutritional Survey of British Adults. London: HMSO.

Griendling K.K., Alexander R.W. (1997). Oxidative stress and cardiovascular disease. Circulation. **96**, 3264-3265.

Gries A., Malle H., and Kostner G.M. (1990). Influence of dietary fish oils on plasma Lp(a) levels. Throm. Res. **58,** 667-668.

Grimble R. F., Tappia P. S. (1995). Modulatory influence of unsaturated fatty acids on the biology of tumour necrosis factor-α. Biochem. Soc. Trans. **23**, 282-287.

Grimble R.F. (1998). Dietary lipids and the inflammatory response. Proc. Nut. Soc. **57**, 535-542.

Groener J.E.M., Kostner G.M. (1987). Lipid transfer protein-catalysed exchange of cholesteryl ester between high-density lipoproteins and apo-B-containing lipoproteins. J. Lipid Res. **28** (9), 1053-1056.

Grundy S.M. (1986). Comparison of monounsaturated fatty acids and carbohydrates for lowering plasma cholesterol. N. Engl. J. Med. **314**, 745-748.

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

Guallar E., Aro A., Jimenez F.J., Martin Moreno J.M., Salminen I., van't veer P. (1999) Omega-3 fatty acids in adipose tissue and risk of myocardial infarction: the EURAMIC study. Arterioscler. Thromb. vasc. Biol. **19**, 1111-1118.

Gurr M.I. (1992). Dietary lipids and coronary heart disease, old evidence, new perspective. Prog. Lipid. Res. **31** (3), **195-243.**

Haberland M.E., Fogelman A.M. (1987). The role of altered lipoproteins in the pathogenesis of atherosclerosis. American Heart Journal. **113**, 573-577.

Haberland M E., Fong D., Cheng L. (1988). Malondialdehyde-altered protein occurs in atheroma of Watanabe heritable hyperlipidemic rabbits. Science. **241**, 215-218.

Haglund O., Mehta J.L., Saldeen T. (1994). Effects of fish oil on some parameters of fibrinolysis and lipoprotein(a) in healthy subjects. Am. J. Cardiol. **74**, 189-192.

Hajjar D. P., Fabricant C.G., Minick C.R. and Fabricant J. (1986). Virus-induced atherosclerosis. Herpes virus infection alters aortic cholesterol metabolism and accumulation. Am. J. Pathol. **122**, 62-69.

Hajjar D. P., Pomerantz K. B. (1992). Signal transduction in atherosclerosis: integration of cytokines and the eicosanoid network. FASEB. J. **6**, 2933-2941.

Halliwell B. and Gutteridge J.M.C. (1990). Role of free radicals and catalytic metal ions in human disease: an overview. Methods Enzymol. **186** (B), 1-85.

Halliwell B. (1999). Establishing the significance and optimal intake of dietary antioxidants: the biomarker concept. Nutr. Rev. **57**, 104-113.

Hamilton J.G. and Comai K. (1988). Rapid separation of neutral lipids, free fatty acids and polar lipids using prepacked silica sep-pak columns. Lipids. **23** (12), 1146-1149.

Hansson G.K. (1997). Cell mediated immunity in atherosclerosis. Current Opinion in Lipidology. **8,** 301-311.

Hansson G.K., Jonasson L., Seifert P. S., Stemme S. (1989). Immune mechanisms in atherosclerosis. Arteriosclerosis. **9**, 567-578.

Harats D., Dabach Y., Hollander G., Ben-Naim M., Schwartz R., Berry E.M., Stein O., Stein Y. (1991). Fish oil ingestion in smokers and nonsmokers enhances peroxidation of plasma lipoproteins. Atherosclerosis **90**, 127-139.

Harris W.S. (1997). N-3 fatty acids and serum lipoproteins: human studies. Am. J. Clin. Nutr. **65**, 1645S-1654S.

Harris W.S. (1996). N-3 fatty acids and lipoproteins: Comparison of results from human and animal studies. Lipids. **31** (3), 243-252.

Harris W.S., Connor W.E. (1980). The effects of salmon oil upon plasma lipids, lipoproteins and triglyceride clearance. Trans. Assoc. Am. Phys. **93**, 148-155.

Harris W. S., Connor W. E., Mc Murray M. P. (1983). The comparative reductions of the plasma lipids and lipoproteins by dietary polyunsaturated fats: Salmon oil vs. vegetable oil. Metabolism. **32**, 179-184.

Harris W.S., Connor W.E., Illingworth D.R., Rothrock D.W., Foster D.M. (1990). Effects of fish oil on VLDL triglyceride kinetics in humans. J. Lipid. Res. **31**, 1549-1558.

Hayes K.C. (1995). Saturated fats and blood lipids – new slant on an old story. Can. J. Cardiol. **11**, G39-G46.

Heffernan A. G. (1963). The fatty acid composition of human adipose tissue. Clin. Sci. **25**, 423-429.

Hendrix M.G.R., Dormans P.H.J., Kitslaar P., Bosman F. and Bruggeman C.A. (1989). The presence of cytomegalovirus nucleic acids in arterial walls of atherosclerotic and nonatherosclerotic patients. Am. J. Pathol. **134**, 1151-1157.

Henriksen T., Mahoney E.M., Steinberg D. (1981). Enhanced macrophage degradation of low density lipoprotein previously incubated with cultured endothelial cells: Recognition by receptors for acetylated low density lipoproteins. Proc. Natl. Acad. Sci. USA **78**, 6499-6503.

Hermann W., Biermann J., Lindhofer H.G. and Kostner G. (1989). Modification of the atherogenic risk factor Lp(a) by supplementary fish oil administration in patients with moderate physical training. Med. Klin. **84**, 429-433.

Hirsch J., Farquhar J.W., Ahrens E.H., Peterson M.L., Stoffel W. (1960). Studies of adipose tissue in man: a microtechnique for sampling and analysis. Am. J. Clin. Nutr. **8**, 499-512.

Hodis H.N., Crawford D.W., Sevanian A. (1991). Cholesterol feeding increases plasma and aortic tissue cholesterol oxide levels in parallel: further evidence for the role of cholesterol oxidation in atherosclerosis. Atherosclerosis. **89**, 117-126.

Hokanson J.E. and Austin M.A. (1996). Plasma triglyceride level is a risk factor for cardiovascular disease independent of high-density lipoprotein cholesterol level: a meta-analysis of population based prospective studies. J. Cardiovascular Risk. **3**, 213-219.

Holvoet P., Perez G., Zhao Z., Brouwers E., Bernar H., Collen D. (1995). Malondialdehyde-modified low density lipoprotein in patients with atherosclerotic disease. J. Clin. Invest. **95**, 2611-2619.

Hornstra G., Lussenberg R. N. (1975). Relationship between the type of dietary fatty acid and the arterial thrombus tendency in rats. Atherosclerosis. **22**, 499-516.

Hrelia S., Celadon M., Rossi C.A., Biagi P.L., Boroni A. (1990). Delta-6-desaturation of linoleic acid and alpha-linolenic acids in aged rats: a kinetic analysis. Biochemistry International. **22**, 659-667

Hu B., Peng S. K., Brubaker D., Morin R.J. (1990). Influence of cholesterol oxides and antioxidants on prostacyclin production by cultured endothelial cells. FASEB J. **4**, 3734-3738.

Jagananthan S.N., Connor W.E., Baker W.H., Bhattacharyya A.K. (1974). The turnover of cholesterol in human atherosclerotic arteries. J. Clin. Invest. **54**, 366-377.

Jen K.L.C., Alexander M., Lin P.K.H., Kasim S.E. (1989). Lipid lowering effect of omega-3 fatty acids in genetically obese Zucker rats. Nutr. Res. **9**, 1217-1228.

Jones P.J.H., Ridgen J.E., Benson A.P. (1990). Influence of dietary fatty acid composition on cholesterol synthesis and esterification in hamsters. Lipids **25**, 815-820.

Katan M.B., Deslypere J.P., van Birgelen A.P.J.M. (1997). Kinetics of the incorporation of dietary fatty acids into serum cholesteryl esters, erythrocyte membranes and adipose tissue: an 18 month controlled study. J. Lipid Res. **38** (10), 2012-2022.

Katan M. B., Van-Birgelen A., Deslypere J.P., Penders M., Van Staveren W. A. (1991). Biological markers of dietary intake, with emphasis on fatty acids. Ann. Nutr. Metab. **35**, 249-252.

Katan M. B., Zack P. L. and Mensink R.F. (1994). Effect of fats and fatty acids on blood lipids in humans: an overview. Am. J. Clin. Nutr. **60** (6 suppl), 1017S-1022S.

Keys A., Anderson J.T., Grande F. (1965). Serum cholesterol response to changes in the diet. Metabolism. **14**, 747-787.

Kiessling R., Gronberg A., Ivanyi J., Siderstrim K., Ferm M., Kleinau S., Nilsson E., Klareskog L. (1991). Role of hsp60 during autoimmune and bacterial inflammation. Immunol. Rev. **121**, 91-111.

Kinsella J.E., Lokesh B., Stone R.A. (1990). Dietary n-3 polyunsaturated fatty acids and amelioration of cardiovascular disease: possible mechanisms. Am. J. Clin. Nutr. **52**, 1-28.

Kisanuki A. et al. (1992). Contribution of the endothelium to intimal thickening in normocholesterolemic and hypercholesterolemic rabbits. Arterioscler. Thromb. **12**, 1198-1205.

Kohlmuller D., Kochen W. (1993). Is n-pentane really an index of lipid peroxidation in humans and animals? A methodological reevaluation. Anal. Biochem. **210**, 268-276.

Kragel A., Gertz S. D., Roberts W. C. (1991). Morphologic comparison of frequency and type of acute plaques in the major epicardial coronary arteries in unstable angina pectoris; sudden coronary death and acute myocardial infarction. J. Am. Coll. Cardiol. **18**, 801-808.

Kristensen S.D., Roberts K.M., Lawry J., Martin J.F. (1988). The effect of fish oil on atherogenesis and thrombopoiesis in rabbits on high cholesterol diet. Artery **15**, 250-258.

Kromhout D., Bosschieter E. B., Coulander C. L. (1985). The inverse relationship between fish consumption and 20-year mortality from coronary heart disease. N. Engl. J. Med. **312**, 1205-1209.

Kume N., Cybulsky M. I. and Gimbrone M. A. (1992). Lysophosphatidylcholine, a component of atherogenic lipoproteins, induces mononuclear leukocyte adhesion molecules in cultured human and rabbit arterial endothelial cells. J. Clin. Invest. **90**, 1138-1144.

Kuo C.C., Gown A.M., Benditt E. P. and Grayston J.T. (1993). Detection of Chlamydia pneumoniae in aortic lesions of atherosclerosis by immunocytochemical stain. Arterioscler. Thromb. **13**, 1501-1504.

Ku D.N., Giddens D.P., Zarins C.K., Glasgov S. (1985). Pulsatile flow and atherosclerosis in the human carotid bifurcation: positive correlation between plaque location and low oscillating shear stress. Arteriosclerosis. **5**, 293-302.

Lagarde M., Guichardant M., Dcehavanne M. (1981). Human platelet PGE, and dihomogammalinoleic acid. Comparison to PGE2 and arachidonic acid. Prog. Lipid Res. **20**, 439-443.

Landymore R.W., MacAulay M., Sheridan B., Cameron C. (1986). Comparison of cod-liver oil and aspirin-dipyridamole for the prevention of intimal hyperplasia in autologous vein grafts. Ann. Thorac. Surg. **41**, 54-60.

Lapidus L., Anderson H., Bengsston C., Bosaeus I. (1986). Dietary habits in relation to incidence of cardiovascular disease and death in women: a 21 year follow up of

participants in the population study of women in Gottenburg, Sweden. Am. J. Clin. Nutr. **44**, 444-448.

Leaf D.A., Connor W.E., barstad L., Sexton G. (1995). Incorporation of dietary n-3 fatty acids into the fatty acids of human adipose tissue and plasma lipid classes. Am. J. Clin. Nutr. **62**, 68-73.

Leaf A., Weber P.C. (1988). Cardiovascular effects of n-3 fatty acids. N. Engl. J. Med. **318**, 549-557.

Lee T.H., Hoover R.L., Williams J. D. (1985). Effects of dietary enrichment with eicosapentaenoic and docosahexaenoic acids on in-vitro neutrophil and monocyte leukotriene generation and neutrophil function. N. Engl. J. Med. **312**, 1217-1224.

Leren P. (1970). The Oslo diet-heart study. Eleven-year report. Circulation. **42**, 935-942.

Libby P. and Hansson G.K. (1991). Involvement of the immune system in human atherogenesis – current knowledge and unanswered questions. Lab. Invest. **64**, 5-15.

Libby P., Sukhova G., Lee R.T., Galis Z.S. (1995). Cytokines regulate vascular functions related to stability of the atherosclerotic plaque. Journal of Cardiovascular Pharmacology. **25** (S2), S9-S12.

Lin D.S., Connor W.E., Wissler R.W., Vesselinovitch D., Hughes R. (1980). A comparison of the turnover and metabolism of cholesterol in normal and atherosclerotic monkey aortas. J. Lipid Res. **21**, 192-201

Loscalzo J. (1990). Lipoprotein (a). A unique risk factor for atherothrombotic disease. Arterioscler. Thromb. **10**, 672-679.

Louheranta A.M., Porkkala-Sarataho E.K., Nyyssonen M.K., Salonen R.M., Salonen J.T. (1996). Linoleic acid intake and susceptibility of very low density and low density lipoproteins to oxidation in men. Am. J. Clin. Nutr. **63**, 698-703.

MacIntyre D. E., Hoover R.L., Smith M. *et al* (1984). Inhibition of platelet function by cis-unsaturated fatty acids. Blood. **63**, 848-857.

MAFF (Ministry of Agriculture Fisheries and Food) (1997a) Food Information. Surveillance Sheet 127. Dietary intake of iodine and fatty acids. London:MAFF.

Marckman P., Lassen A., Haraldsdothir J., Sandstrom B. (1995). Biomarkers of habitual fish intake in adipose tissue. Am. J. Clin. Nutr. **62**, 956-959.

Mensink R. P., Katan M. B. (1989). Effects of a diet enriched with monounsaturated or polyunsaturated fatty acids on levels of low density and high density lipoprotein cholesterol in healthy women and men. N. Engl. J. Med. **321**, 436-441.

Mensink R.P., Zock P.L., Katan M.B., Hornstra G. (1992). Effect of dietary cis and trans fatty acids on serum lipoprotein (a) levels in humans. J.Lipid Res. **33**, 1493-1501.

Meydani M., Natiello F., Goldin B., *et al.* (1991). Effect of long term fish oil supplementation on vitamin E status and lipid peroxidation in women. J. Nutr. **121**, 484-491.

McGregor L., Morazain R., Renaud S.A. (1980). A comparison of the effects of dietary short and long chain saturated fatty acids on platelet functions, platelet phospholipids and blood coagulation in rats. Lab. Invest. **43**, 438-442.

Miettinen T. A., Naukkaninen V., Hattinen J. K., Mathial S., Kumlin T. (1982). Fatty acid composition of plasma predicts myocardial infarction. Br. Med. J. **285**, 993-996.

Mitchinson M.J. (1994). The new face of atherosclerosis. Br. J. Clin. Pract. **48**, 149-151.

Moncada S., Vane J.R. (1978). Pharmacology and endogenous roles of prostacyclin endoperoxides, thromboxane A_2 and prostacyclin. Pharmacol. Rev. **30**, 293-331.

Montenegro M.R. (1999). Atherosclerosis morphology and pathogenesis. Ann. Rev. Biomed. Sci. **1**, 133-144.

Moore K., Roberts L.J. (1998). Measurement of lipid peroxidation. Free Radic. Res. **28**, 659-671.

Morrow J.D. Roberts L.J. (1997). The isoprostanes: unique bioactive products of lipid peroxidation. Prog. Lipid Res. **36**, 1-21.

Muhlestein J.B., Anderson J.L., Hammond E.H., Zhao L., Trehan S., Schwobe E.P., Carlquist J.F. (1998). Circulation **24**, 633-636.

Navab M., Berliner J.A., Watson A.D. (1996). The Yin and Yang of oxidation in the development of the fatty streak: a review based on the 1994 George Lyman Duff Memorial Lecture. Arterioscler. Thromb. Vasc. Biol. **16**, 831-842.

Needleman P., Raz A., Minkes M. S., Ferrendelli J. A., Sprecher H. (1979). Triene prostaglandins: Prostacyclin and thromboxane biosynthesis and unique biological properties. Proc. Natl. Acad. Sci. USA. **76**, 944-948.

Nenseter M. S., Rustan A. C., Lend-Katz S., Soyland E., Maelandsmo G., Phillips M.C., Drevon C.A. (1992). Effect of dietary supplementation with n-3 polyunsaturated fatty acids on physical properties and metabolism of low density lipoproteins in humans. Arterioscler. Thromb. **12**, 369-379.

Norell S., Ahlbom A., Feychling M., Pedersen N.L. (1986). Fish consumption and mortality from coronary heart disease. Br. Med. J. **293**, 426.

Office of Public Censuses and Surveys. (1984). The heights and weights of adults in Great Britain.London: HMSO, SS 1138.

Office of Population Censuses and Surveys. Mortality Statistics: area 1990. London: HMSO, Series DH5; no 17.

Ohlsson B.G., Englund M.C.O., Karlsson A.L.K., Knutsen E., Erixon C., Skribeck H., Liu Y., Bondjers G., Wiklund O. (1996). Oxidised low density lipoprotein inhibits lipopolysaccharide induced binding of nuclear factor-kappa B to DNA and the subsequent

expression of tumour necrosis factor-alpha and interleukin-1 beta in macrophages. J. Clin. Invest. **98**, 78-89.

Oostenberg G.S., Mensink R. P., Hornstra G. (1994). Effects of fish oil and vitamin E supplementation on copper catalysed oxidation of human low density lipoprotein in vitro. Eur. J. Clin. Nutr. 48, 895-898.

Packer L. (1999). Oxidants and antioxidants. Part B. Methods in enzymology. Vol. 300. San Diego, CA: Academic Press.

Palinski W., Horkko S., Miller E., Steinbrecher U.P., Powell H. C., Cartiss L.K., Witcham J. (1996). Cloning of monoclonal autoantibodies to epitopes of oxidised lipoproteins from apolipoprotein E-deficient mice. Demonstration of epitopes of oxidised low density lipoprotein in human plasma. J. Clin. Invest. **98**, 800-814.

Palinski W., Rosenfeld M.E., Yla0Herttuala S., *et al.* (1989). Low density lipoprotein undergoes oxidative modification in vivo. Proc. Natl. Acad. Sci. USA. **86**, 1372-1376.

Parks J.S., Kaduck-Sayer J., Bullock B. C., Rudd C.C. (1995). Effect of dietary fish oil on coronary artery and aortic atherosclerosis in African green monkeys. Arteriosclerosis. **10**, 1102-1112.

Parthasarathy S., Khoo J.C., Miller E., Barnett J., Witztum J.L., Steinberg D. (1990). Low density lipoprotein rich in oleic acid is protected against oxidative modification: implications for dietary prevention of atherosclerosis. Proc. Natl. Acad. Sci. USA. **87**, 3894-3898.

Pessin M. S. *et al.* (1977). Clinical and angiographic features of carotid TIAs. N. Engl. J. Med. **296**, 358-362.

Prescott-Clarke P, Primatesta P (1998). Health Survey for England 1996. London:HMSO.

Princen H.M.G, Duyvenvoorde W., Buytenhek R., Blonk C., Tijburg L.B.M., Langius J.A.E., Meinders A.E., Pijl H. (1998). No effect of consumption of green and black tea on

plasma lipid and antioxidant levels and on LDL oxidation in smokers. Atherioscler. Thromb. Vasc. Biol.**18**, 833-841.

Proudfoot J., Barden A., Mori T.A., Burke V., Croft K.D., Beilin L.J., Puddey I.B. (1999). Measurement of urinary F(2)-isoprostanes as markers of in vivo lipid peroxidation – a comparison of enzyme immunoassay with gas chromatography/mass spectrometry. Anal. Biochem. **272**, 209-215.

Puolakkainen M., Kuo C.C., Shor A., Wang S. P., Grayston J.T. and Campbell L. A. (1993). Serological response to Chlamydia pneumoniae in adults with coronary arterial fatty streaks and fibrolipid plaques. J. Clin. Microbiol. **31**, 2212-2214.

Quinn M. T., Partharsarathy S., Fong L. C. and Steinberg D. (1987). Oxidatively modified LDL: a potential role in recruitment and retention of monocyte/macrophage in atherogenesis. Proc. Natl. Acad. Sci. 84, 2995-2997.

Rao G.H.R., Radha E., White J.G. (1983). Effect of docosahexaenoic acid on arachidonic acid metabolism and platelet function. Biochem. Biophys. Res. Commun. **117**, 549-555.

Rapp J.H., Connor W.E., Lin D.S., Inahana T., Porter J.M. (1983). Lipids of human atherosclerotic plaques and xanthomas: Clues to the mechanism of plaque progression. J. Lipid Res. **24**, 1329-1335.

Rapp J. H., Connor W. E., Lin D. S., Porter J. M. (1991). Dietary eicosapentaenoic acid and docosahexaenoic acid from fish-oil: their incorporation into human atherosclerotic plaques. Arterioscler. Thromb. **11**, 903-911.

Reaven P., Parthasarathy S., Grasse B.J., Miller E., Almazan F., Mattson F.H., Khoo J.C., Steinberg D., Witztum J.L. (1991). Feasibility of using an oleate-rich diet to reduce the susceptibility of low density lipoprotein to oxidative modification in humans. Am. J. Clin. Nutr. **54**, 701-706.

Reaven P., Parthasarathy S., Grasse B.J., Miller E., Steinberg D., Witztum J.L. (1993). Effects of oleate rich and linoleate rich diets on the susceptibility of low density

lipoprotein to oxidative modification in mildly hypercholesterolemic subjects. J. Clin. Invest. **91**, 668-676.

Reid V.C., Brabbs C. E., Mitchinson M.J. (1992). Cellular damage in mouse peritoneal macrophages exposed to cholesteryl linoleate. Atherosclerosis. **92**, 251-260.

Renier G., Skamene E., DeSanctis J., Radzioch D. (1993). Dietary n-3 polyunsaturated fatty acids prevent the development of atherosclerotic lesions in mice. Arterioscler. Thromb. **13**, 1515-1524.

Renaud S., Morazain R., Godsey F., Thevenon C., Martin J.L., Mendy F. (1986). Nutrients, platelet function and composition in nine groups of French and British farmers. Atherosclerosis. **60**, 37-48.

Rich S., Miller J.F., Cherous R., Davis H.R., Shanks P., Glasgov S., Lends W.E.M. (1989). Development of atherosclerosis in genetically hyperlipidaemic rabbits during chronic fish oil ingestion. Arteriosclerosis. **9**, 189-194.

Riemersma R.A., Wood D. A., Butler S., et al. (1986). Linoleic acid content in adipose tissue and coronary heart disease. Br. Med. J. **292**, 1423-1427.

Rose G., Shipley M. (1986). Plasma cholesterol concentration and death from coronary heart disease: 10-year results of the Whitehall study. BMJ. **293**, 306-307.

Ross R. (1986). The pathogenesis of atherosclerosis- an update. N. Engl. J. Med. **314**, 488-500.

Ross R. (1993). The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature. **362**, 801-809.

Ross R. Agius L. (1992). The process of atherogenesis- cellular and molecular interaction: from experimental animals to humans. Diabetologia. **35**, S34-40.

Rubanyi G.M. (1993). The role of endothelium in cardiovascular hmeostasis and diseases. J. Cardiovasc. Pharmacol. **4**, S1-S14.

Sacks F. M., Stone P. H., Gibson C. M., Silverman D. I., Rosner B., Pasternak R. C. (1995). Controlled trial of fish oil for regression of human coronary atherosclerosis. HARP research group. J. Am. Coll. Cardiol. **25** (7), 1492-1498.

Sakai M., Miyazaki A., Hakamata H., Sato Y., Matsumura T., Kobori S., Shichiri M. and Horiuchi S. (1996). Lysophosphatidylcholine potentiates the mitogenic activity of modified LDL for human monocyte-derived macrophages. Arterioscler. Thromb. Vasc. Biol. **16**, 600-605.

Salonen J.T., Yia-Hertuala S., Yamamoto R., Butler S., Korpela H., Salonen R., Nyyssanen K., Palinski W., Witztum J.L. (1992). Autoantibody against oxidised LDL and progression of carotid atherosclerosis. Lancet. **339**, 883-887.

Sanders T.A.B., Hinds A., Perreira C.C. (1989). Influence of n-3 fatty acids on blood lipids in normal subjects. J. Int. Med. **225**(1), 99-104.

Sanders K., Johnson L., O' Dea K., Sinclair A.J. (1994). The effect of dietary fat level and quality on plasma lipoprotein lipids and plasma fatty acids in normocholesterolaemic subjects. Lipids. **29**, 129-138.

Sattler W., Bone P., Stocker R. (1992). Isolation of human VLDL, LDL, HDL, and two HDL subclasses in the TL-100 tabletop centrifuge using the TLA-100.4 Rotor. Ultracentrifugation Technical Information. Beckman Inst. UK LTD.

Saynor R., Gillot T. (1988). Fish oil and plasma fibrinogen. Br. Med. J. **297**, 1196.

Scott J. (1991). Lipoprotein (a). Br. Med. J. **303**, 663-664.

Sevanian A., McLeod L.L. (1987). Cholesterol autoxidation in phospholipid bilayers. Lipids. **22**, 627-631.

Shaikh M, Wootton R., Nordestgaard B. G., Baskerville P., Lumley J. S., Leville A. E., Quinley J. and Lewis B. (1991). Quantitative studies of transfer in vivo of low density Sf 12-60 and Sf 60-400 lipoproteins between plasma and arterial intima in humans. Arterioscler. Thromb. **11**, 569-577.

Shekelle R.B., Paul O., MacMillain, Shyrock A., Stamler J. (1985). Fish consumption and mortality from coronary heart disease. N. Engl. J. Med. **313**, 820.

Shepherd J., Packard C. J., Patsch J.R., Gotto A.M., Taunton O. D. (1978). Effects of dietary polyunsaturated and saturated fat on the properties of high density lipoproteins and the metabolism of apolipoprotein A-1. J. Clin. Invest. **61**, 1582-1592.

Shimokava H., Lam J.Y., Chesebro J.H., Bowie E.J.W., Vanhoutte P.M.. (1987). Effects of dietary supplementation with cod-liver oil on endothelium-dependent responses in porcine coronary arteries. Circulation. **76**, 898-905.

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

Singh R.B., Niaz M.A., Sharma J.P., Kumar R., Rastogi V., Moshiri M. (1997). Randomised double-blind, placebo-controlled trial of fish oil and mustard oil in patients with suspected acute myocardial infarction: the Indian experiment of infarct survival-4. Cardiovasc. Drugs Ther. **11**, 485-491.

Smith C., Mitchinson M.J., Aruoma O.I. and Halliwell B. (1992). Stimulation of lipid peroxidation and hydroxyl radical generation by the contents of human atherosclerotic lesions. Biochem. J. **286**, 901-905.

Spady D.K., Dietschy J.M. (1989). Interaction of aging and dietary fat in the regulation of low density lipoprotein transport in the hamster. J. Lipid. Res. **30**, 559-569.

Stary H. C. (1989). Evolution and progression of atherosclerotic lesions in coronary arteries of children and young adults.Arteriosclerosis. **9** (1), 19-32.

Stary H. C. (1989). Evolution and progression of atherosclerotic lesions in coronary arteries of children and young adults. Arterioscler. Thromb. Suppl. 1, 119-132.

Stary H.C., Chander A.B., Dinsmore R.E. (1995). The definition of advanced type of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the committee on vascular lesions of the council on arteriosclerosis. Circulation. **92**, 1355-1374.

Stehbens W. E. (1982). Hemodynamics and atherosclerosis. Biorheolgy. **19**, 95-101.

Steinberg D. (1991). Antioxidants and atherosclerosis. Circulation. **84**, 1420-1425.

Steinberg D., Parthasarathy S., Carew T. E., Khoo J. C., Witztum J. L. (1989). Beyond cholesterol: modifications of low- density lipoprotein that increase its atherogenicity. N. Engl. J. Med. **320**, 915-924.

Steinbrecher U.P., Parthasarathy S., Leake D.S., Witztum J.L., Steinberg D. (1984). Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. Proc. Natl. Acad. Sci. USA. **81** (12), 3883-3887.

Steinbrecher U P. (1987). Oxidation of human low density lipoprotein results in derivatisation of lysine residues of apolipoprotein B by lipid peroxide decomposition products. J. Biol. Chem. **262** (8), 3603-3608.

Stemme S., Faber B., Holm J., Wiklund O., Witztum J.L., Hansson G.K. (1995). T lymphocytes from human atherosclerotic plaques recognise oxidised LDL. Proc. Natl. Acad. Sci. USA. **92**, 3893-3897.

Strong J.P. (1992). Atherosclerotic lesions. Natural history, risk factors and topography. Arch. Pathol. Lab. Med. **116**, 1268-1275.

Suzukawa M., Abbey M., Howe P. R., Nestel P.J. (1995). Effects of fish oil fatty acids on low density lipoprotein size, oxidisability and uptake by macrophages. J. Lipid. Res. **36**, 473-484.

Tholstrup T., Marckmann P., Vessby., Sandstrom B. (1995). Effects of fats high in individual saturated fatty acids on plasma lipoprotein (a) levels in young healthy men. J. Lipid Res. **36**, 1447-1452.

Tracy R., Devaney K., Kissling G. (1985). Characteristics of the plaque under a coronary thrombus. Virchows Arch. A. **405**, 411-427.

Traub O., Berk B.C. (1998). Laminar shear stress: mechanisms by which endothelial cells transduce an atheroprotective force. Arterioscler. Thromb. Vasc. Biol. **18**, 677-685.

Tremoli E. et al. (1993). Increased synthesis of plasminogen activator inhibitor-1 by cultured human endothelial cells exposed to native and modified LDLs. Arterioscler. Thromb. **13**, 338-346.

Tripodi A., Loria P., Dilengite M.A., Carulli N. (1991). Effect of fish oil and coconut oil diet on the LDL receptor activity of rat liver plasma membranes.Biochim. Biophys. Acta. **1083** (3), 298-304.

Ulbricht T. L.V., Southgate D. A. T. (1991). Coronary heart disease: seven dietary factors. Lancet. **338**, 985-992.

Ulbricht T.L.V., Wheelock J.V. Dietary fat and coronary heart disease. Bradford: Horton, 1989.

Uusitupa M.I.J., Niskanen L., Luoma J., Viija P., Mercuri M., Rauramaa R., Yia-Hertuala S. (1996). Autoantibodies against oxidised LDL do not predict atherosclerotic vascular disease in non-insulin-dependent diabetes mellitus. Arterioscler. Thromb. Vasc. Biol. **16**, 1236-1242.

Vanhoutte P.M. (1989). Endothelium and control of vascular function: state of the art lecture. Hypertension. **13**, 658-667.

Van der Wal A. C. et al. (1992). Adhesion molecules on the endothelium and mononuclear cells in human atherosclerotic lesions. Am. J. Pathol. **141**, 1427-1433.

Van- Houwelingen R., Zevenbergen H., Groot P., Kester A., Hornstra G. (1990). Dietary fish effects on serum lipids and apolipoproteins, a controlled study. Am. J. Clin. Nutr. **51**, 393-398.

VanStaveren W. A., Deurenberg P., Katan M. B. et al. (1986). Validity of the fatty acid composition of subcutaneous fat tissue microbiopsies as an estimate of long term average fatty acid composition of the diet of separate individuals . Am. J. Epidemiol. **123**, 455-463.

Vollset S. E., Heach I., Bjelke E. (1985). Fish consumption and mortality from coronary heart disease. N. Engl. J. Med. **313**, 821.

Von Rokitansky C. (1852). A manual of pathological anatomy, vol. 4. Berlin: Syndherman Soc. 261.

Von Schacky C., Angerer P., Kothny W., Theisen K., Mudra H. (1999). The effect of dietary omega-3 fatty acids on coronary atherosclerosis. A randomised , double-blind, placebo-controlled trial. Ann. Intern. Med. **130**, 554-562.

Wardlaw G. M., Snook J.T. (1990). Effect of diets high in butter, corn oil, or high oleic acid sunflower oil on serum lipids and apolipoproteins in men. Am J. Clin. Nutr. **51**, 815-821.

Wick G., Schnett G., Amberger A., Kleindienst R., and Qingbo X. (1995). Is atherosclerosis an immunologically mediated disease. Immunol. Today. **16**, 27-33.

Weiner B. H., Ockene I. S., Levine P.H., Vaudreuil C., Hoogasian J.J. (1986). Inhibition of atherosclerosis by cod-liver oil in a hyperlipidaemic swine model. N. Engl. J. Med. **315**, 841-846.

White A., Nicolaas G., Foster K., Browne F., Carey S. (1993). Health Survey for England 1991: a survey carried out by the Social Survey Division of the OPCS on behalf of the Department of Health. London: HMSO,Series HS; no1.

Wilcox J. (1991). Analysis of local gene expression in human atherosclerotic plaques by in-situ hybridisation. Trends Cardiovasc. Med. **1**, 17-24.

Wood K. M., Cadogan M. D., Ramshaw A. L. and Parums D.V. (1993). The distribution of adhesion molecules in human atherosclerosis. Histopathology. **22**, 437-444.

Wood D. A., Oliver M. F. (1992). Linoleic acid, antioxidants, vitamins and coronary heart disease. In: Marmot M., Elliott P. eds. Coronary heart disease epidemiology. Oxford: Oxford Medical Publications. pp. 179-202.

Woodhill J.M., Palmer A.J., Leelarthaepin B., McGilchrist C., Balcket R.B. (1978). Low fat, low cholesterol diet in secondary prevention of coronary heart disease. Adv. Exp. Med. Biol. **109**, 317-330.

Xu Q.B., Oberhuber G., Gruschwitz M. (1990). Immunology of atherosclerosis: cellular composition and major histocompatibility complex class II antigen expression in aortic intima, fatty streaks, and atherosclerotic plaques in young and aged human specimens. Clin. Immunol. Immunopathol. **56** (3), 344-359.

Xu Q., Dietrich H., Steiner H.J., Gown A.M., Schoel B., Mikuz G., Kaufmann S. and Wick G. (1992). Induction of atherosclerosis in normocholesterolemic rabbits by immunisation with heat shock protein 65. Arterioscler. Thromb. **12**, 789-799.

Xu Q., Kleindienst R., Waitz W., Dietrich H., Wick G. (1993) Increased expression of heat shock protein 65 coincides with a population of infiltrating T lymphocytes in atherosclerotic lesions of rabbits specifically responding to heat shock protein. J. Clin. Invest. **91**, 2693-2702.

Xu Q., Willeit J., Marosi M., Kleindienst R., Oberhollenzer F., Kiechi S., Stulnig T., Luef G. and Wick G. (1993). Association of serum antibodies to heat shock protein 65 with carotid atherosclerosis. Lancet. **341**, 255-259.

Yamashiroya H.M., Ghosh L., Yang R. and Robertson A. L. (1988). Herpesviridae in the coronary arteries and aorta of young trauma victims. Am. J. Pathol. **130**, 71-79.

Zaman A.G., Helft G., Worthley S.G., Badimon J.J. (2000). The role of plaque rupture and thrombosis in coronary artery disease. Atherosclerosis. **149**, 251-266.

Zarins C.K., Zatina M.A., Giddens D.P., Ku D.N., Glagov S. (1987). Shear stress regulation of artery lumen diameter in experimental atherogenesis. J.Vasc. Surg. **5**, 413-420.

Zhou Y.F., Leon M. B., Waclawiv M. A., Popma J.J., Yu Z.X., Finkel T. and Epstein S. E. (1996). Association between prior cytomegalovirus infection and the risk of restenosis after coronary atherectomy. N. Engl. J. Med. **335**, 624-630.

7 Appendix

The results presented in this thesis formed part of a larger study (see Figure 2.2) and as is common in such studies a number of individuals made vital contributions to this work. These were as follows:

Patient recruitment and randomisation: Adhisabandh Chulakadabba (Research Fellow), Kittipan Rerkasem (Research Fellow), Jenny Williams (Research Nurse).

Initiation on seven day weighed intakes Adhisabandh Chulakadabba (Research Fellow), Jenny Williams (Research Nurse).

Analysis of Food Diaries: Rachel Thompson and staff in the Public Health Nutrition group, University of Southampton.

Interaction with patients: Jenny Williams (Research Nurse).

Collection of blood and adipose tissue: Adhisabandh Chulakadabba (Research Fellow), Kittipan Rerkasem (Research Fellow), Jenny Williams (Research Nurse).

Surgical removal of plaques: Cliff Shearman (Professor of Vascular Surgery).

Plasma lipid measurements, lipoprotein separation, and LDL oxidation: Jennifer Garry.

LDL vitamin E analysis: Jennifer Garry (extraction and preparation); Robin West (Department of Chemical Pathology; HPLC analysis).

Fatty acid composition of LDL, adipose tissue and plaque: Frank Thies, Jennifer Garry, and Parveen Yaqoob.

Plaque morphology: Frank Thies, Jennifer Garry (under supervision of Patrick Gallagher).