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NUTRITIONAL AND METABOLIC ASPECTS OF n-3 POLYUNSATURATED FATTY ACIDS IN HEALTH AND DISEASE

By

Anne-Marie Tully

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Department of Clinical Medicine
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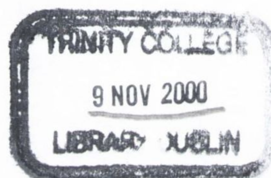
Trinity College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The health benefits of fish oils, rich in the n-3 polyunsaturated fatty acids (PUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are multi-faceted, spanning a number of human systems, including the cardiovascular, inflammatory, nervous and sensory systems. The present thesis focuses on aspects of the benefits of fish oils on two of these systems in the adult - cardiovascular and nervous. The positive effects of these oils on coronary heart disease (CHD) risk factors are well regarded and established, whereas little research has examined the effects of these fatty acids on diseases of the nervous system, particularly in relation to the older person. The effects of n-3 PUFA on these systems are not mutually exclusive. For example, inflammatory processes are involved in CHD and Alzheimer's disease (AD), and these processes can be attenuated through the use of fish oils. Fish oils have also been shown to benefit other inflammatory conditions such as Crohn's Disease, rheumatoid arthritis, asthma, and psoriasis; are beneficial in reducing hypertension and improving depression, and are necessary in the development of the brain and retina in the last trimester of pregnancy and during infancy.

Despite the extensive investigations into the benefits of fish oils on all these systems, the minimum effective dose remains elusive. This is an important point, not only with respect to CHD but also more importantly with respect to public health issues and chronic disease prevention. Current intakes of n-3 PUFA in Ireland and the U.K. fall well below recommendations, yet these fats play an extremely important therapeutic role for all ages, from the foetus to the elderly. The only sources of EPA and DHA, apart from oily fish are biosynthesis from α-linolenic acid (ALA), from fish oil supplements, or from functional foods. The rational behind the studies presented here lies in the fact that large amounts of n-3 PUFA cannot be incorporated into a functional food, be it wet or dry. Until we know the optimal minimal effective level of n-3 PUFA, functional foods containing a potent dose of n-3 PUFA cannot be developed.

Chapters 3 & 4 of this thesis examine aspects of a large multi-centre, multi-disciplinary international study funded from the EU 4th Framework Programme. "Nutrifish" or 'Nutritional studies on dried functional food ingredients containing n-3 polyunsaturated fatty acids' incorporated 2 main areas of research, involving technology and quality experts on one side, and a consortium of nutritionists on the other. The aim the Technology & Quality Groups was to develop dried microencapsulated fats and oils with particular emphasis on oils rich in n-3 PUFA. This process imparts stability to the end product. A range of uses to which the end ingredient may be put was also evaluated. The Nutrition consortium carried out a double-blind placebo-controlled study examining the effects of a range of low doses of fish oils on a wide range of coronary heart disease risk factors. The results revealed that 0.9g fish oil, an amount much lower than that recommended by Government and Scientific bodies, effectively attenuate the postprandial triacylglycerol (TAG) response and increase high density lipoprotein (HDL) cholesterol concentrations, although not significantly. These volunteers were healthy and normolipidaemic thus it is possible that hyperlipidaemic volunteers, if supplemented with the same low dose, would display an even greater response.

Previous studies have revealed a dose-response relationship between the amount of n-3 PUFA supplied in the diet and the amount incorporated into cell membranes, imparting beneficial effects on biological parameters. However, finding precisely the amount of n-3 PUFA supplied in the diet is a difficult task if done by conventional means, as traditional dietary assessment methods are fraught with errors, with underreporting being one of the main problems. The most effective way of overcoming this dilemma is by using

biomarkers. The present study examined two biomarkers for chronic fish oil intake; platelet phospholipid fatty acids and cholesteryl ester fatty acids, and also a biomarker for fat quality intake in the acute setting, TAG-rich lipoproteins (TRL) fatty acids. Their effectiveness is evaluated not only in assessing current intakes, but also in relation to predicting a particular dose of n-3 PUFA which would produce a specific level of these fatty acids in membranes, subsequently resulting in a beneficial biological response. In this case, the beneficial biological response observed with 0.9g of fish oil, was a lowering in peak TAG concentrations. A clear dose-response relationship was observed between the amount of fat supplemented and the level in each lipid fraction, in both the acute and chronic setting.

Whilst Chapter 4 examines the dose-response relationship between fish oils and incorporation into lipid fractions, this knowledge is carried through to Chapter 5 and applied to a disease of the Nervous system, Alzheimer's disease. To date there is tentative evidence for the benefits of fish oils on AD incidence and severity, but as of yet a consensus has not been made on this matter. Results from the present study revealed that patients with Alzheimer's disease have significantly lower levels of EPA and DHA, the two biologically fatty acids in fish oil, when compared to healthy elderly controls. All patients assessed in this study had mild-moderate forms of the disease. There are a number of biological theories which could explain this difference, however from these results it is not possible to determine whether the disease causes a lowering in these fatty acids or *vice versa*. It is possible that the maintenance of a particular level of n-3 PUFA in the elderly at risk of AD, or in patients with AD will dramatically improve their quality of life and thus have huge health care implications. From the work in Chapter 4 it will be possible to predict what dose of fish oil will increase CE EPA and DHA levels to a point which imparts some protection from AD.

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Table of Contents

Declaration	i
Summary	ii
Acknowledgements	iv
Table of Contents	vi
Abbreviations	xii

Chapter One	**General Introduction**	1
1.1	**Fish oils and chronic diseases**	2
1.2	**Fish oils and CHD**	6
1.2.1	Prevailing evidence	6
1.2.1 (i)	Epidemiology	6
1.2.1 (ii)	Clinical trials / intervention studies	8
1.2.2	CHD in Ireland	10
1.2.3	Current intakes and recommendations	14
1.2.4	Elongation and desaturation of ALA to EPA and DHA	19
1.3	**Postprandial lipaemia and CHD**	21
1.3.1	Relationship between postprandial lipaemia and CHD	21
1.3.2	The postprandial response	22
1.3.2 (i)	Digestion and absorption of dietary fat	22
1.3.2 (ii)	TRL formation (chylomicron and VLDL)	23
1.3.2 (iii)	TRL catabolism	24
1.3.2 (iv)	Reverse cholesterol transport	25
1.3.2 (v)	TRL clearance	26
1.3.3	Atherogenicity of remnant lipoproteins	27
1.3.4	Physiological factors affecting postprandial response	28
1.3.4 (i)	Effect of age and gender	28
1.3.4 (ii)	Effect of LPL activity	29
1.3.4 (iii)	Effect of exercise	33
1.3.4 (iv)	Effect of obesity and weight reduction	34
1.3.4 (v)	Effect of insulin and glucose metabolism	35

1.3.4 (vi)	Effect of apo E phenotype	36
1.3.5	Dietary factors affecting the postprandial response	36
Studies in the acute phase – test meal composition		37
1.3.5 (i)	Test meal fat quality composition	37
1.3.5 (ii)	Test meal fat quantity composition	39
1.3.5 (iii)	Test meal carbohydrate composition	40
1.3.5 (iv)	Test meal protein composition	41
Studies in the chronic phase – background diet composition		42
1.3.5 (v)	Manipulation of background dietary fatty acid composition	42
1.3.5 (vi)	Comparison of populations with varying habitual dietary fat intake	43
1.3.5 (vii)	Dietary carbohydrate intake	44
1.3.6	Fish oils and TAG concentrations	45
1.4	**Fish oils and Neurological disorders**	46
1.4.1	Introduction	46
1.4.2	Fish oils and Alzheimer's disease – prevailing evidence	47
1.4.2 (i)	Cross-sectional	47
1.4.2 (ii)	Prospective	48
1.4.2 (iii)	Intervention	50
1.4.3	Association between fish oils and Alzheimer's disease	51
1.4.4	Diagnosis of Alzheimer's disease	53
1.5	**Biomarkers of fat quality intake**	55
1.6	**Functional Foods**	60

Chapter Two Materials and Methods 63

2.1 Collection and storage of samples 64

2.2 TAG- rich lipoprotein (TRL) separation 64

2.3 Platelet phospholipid, cholesteryl ester and TRL fatty acid composition 65

2.3.1 Isolation of plasma and platelets 65

2.3.2 Lipid extraction 66

2.3.3 Isolation of phospholipids and cholesteryl esters (Thin Layer Chromatography) 66

2.3.4 Generation of methyl esters of fatty acids for gas liquid chromatography (Transesterification). 68

2.3.5 Determination of platelet phospholipid, cholesteryl ester and TRL fatty acid composition by Gas Liquid Chromatography (GLC) 69

2.4 Separation of plasma HDL, HDL_2 and HDL_3 70

2.5 Measurement of TAG concentration 72

2.6 Measurement of cholesterol concentration 74

2.7 Measurement of NEFA concentration 75

2.8 Measurement of glucose concentration 76

2.9 Measurement of insulin concentration 77

2.10 Apo B-48 preservative preparation 78

2.11 Measurement of apo B-48 concentration 78

2.12 Statistical analysis 83

Chapter Three: The Effect of Low Doses of Fish Oils on Postprandial Lipaemia. 84

3.1	**Introduction**	85
3.2	**Methods**	88
3.2.1	Study Design	88
3.2.2	Collection and Storage of Samples	91
3.2.3	Biochemical Parameters Investigated	92
3.2.4	Statistical Analysis	93
3.3	**Results**	94
3.3.1	Subject information	94
3.3.2	Fish oil consumption	95
3.3.3	Plasma glucose, insulin and haemoglobin concentrations	96
3.3.4	Plasma triacylglycerol concentrations	97
3.3.5	Postprandial cholesterol response	100
3.3.6	Postprandial NEFA response	102
3.3.7	Postprandial apo B-48 concentrations	105
3.3.8 a	Plasma lipoprotein concentrations – TRL and TPL	107
3.3.8 b	Plasma lipoprotein concentrations – HDL	113
3.4	**Discussion**	115

Chapter Four: Biomarkers of Dietary Fat; Acute and Chronic 122

4.1	**Introduction**	123
4.2	**Methods**	125
4.2.1	Study design	125
4.2.2	Collection and storage of samples	125
4.2.3	Biochemical parameters investigated	125
4.2.4	Statistical analysis	126
4.3	**Results**	127
4.3.1	Platelet phospholipid fatty acid composition	127

4.3.1 (i) Platelet phospholipid saturated and monounsaturated fatty acid composition 127
4.3.1 (ii) Platelet phospholipid polyunsaturated fatty acid composition 128
4.3.2 Cholesteryl ester fatty acid composition 137
4.3.2 (i) Cholesteryl ester saturated, monounsaturated and polyunsaturated fatty acid composition 137
4.3.3 TRL fatty acid composition 141
4.3.3 (i) TRL saturated and monounsaturated fatty acid composition 141
4.3.3 (ii) TRL polyunsaturated fatty acid composition 141
4.3.4 Comparison; biomarkers of chronic fat intake 146
4.4 Discussion 148
4.4.1 Compliance 148
4.4.2 Platelet phospholipid fatty acid composition 148
4.4.3 CE fatty acid composition 152
4.4.4 TRL fatty acid composition 153
4.4.5 Comparison; biomarkers of chronic fat intake 154

Chapter Five: Fish oils and Alzheimer's disease 156
5.1 Introduction 157
5.2 Methods 161
5.3 Results 163
5.3.1 Subject information 163
5.3.2 Cholesteryl ester composition and Alzheimer's disease – The Mini-Mental Score 163
5.3.3 Cholesteryl esters and Alzheimer's disease – Clinical Dementia Rating 166
5.3.4 Cholesteryl ester composition and Alzheimer's disease; Clinical Diagnosis 168
5.3.5 Cholesteryl ester composition and Alzheimer's disease; Age- and Sex- matched patients and controls 170

5.3.6 Cholesteryl ester composition and Alzheimer's disease; Gender differences 170

5.3.7 Cholesteryl ester composition and Alzheimer's disease; Age differences 173

5.4 Discussion 174

Chapter Six: General Discussion 183

References 186

Appendix

Abbreviations

AD	Alzheimer's disease
AI	adequate intake
ALA	Alpha-linolenic acid
ANOVA	Analysis of Variance
apo	apolipoprotein
AUC	Area under the curve
BDRP	Blessed Dementia Rating Procedure
BHT	Butylated hydroxy toluene
BMI	Body mass index
BNF	British Nutrition Foundation
CDR	Clinical Dementia Rating
CE	Cholesteryl ester
CETP	Cholesteryl ester transfer protein
CHD	Coronary heart disease
CVD	Cardiovascular disease
CVS	Cardiovascular system
CM	Chylomicron
CSO	Central statistics office
CT	Computed tomography
DHA	Docosahexaenoic acid
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
FAO	Food and Agriculture Organisation
FSAI	Food Safety Authority of Ireland
GLC	Gas liquid chromatography
HCL	Hydrochloric acid
HDL	High density lipoprotein
HDS-R	Hasegawa's dementia rating scale

HIS	Hachinski Ischaemic Scale
HL	Hepatic lipase
HRPO	Horseradish peroxidase
HSL	Hormone sensitive lipase
IL-1β	Interleukin-1 beta
IADL	Instrumental Activities of Daily Living Scale
IS	Inflammatory system
ISSFAL	International Society for the Study of Fatty Acids and Lipids
LA	Linoleic acid
LCAT	Lecithin cholesterol acyl transferase
LDL	Low density lipoprotein
LNA	Linolenic acid
LSD	least significant difference
LPL	Lipoprotein lipase
MCT	Medium chain triglycerides
MMSE	Minimental score
MIRA	Mercers Institute for Research and Aging
MRI	Magnetic resonance imaging
MUFA	Monounsaturated fatty acids
n-3	omega-3 fatty acid
n-6	omega-6 fatty acid
NEFA	Non-esterified fatty acids
NIDDM	Non-insulin dependant diabetes mellitus
NMIC	National Medicines Information Centre
NSAID	Non-steroidal anti-inflammatory drugs
NSD	No significant difference
PBS	Phosphate buffer saline
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PI	phosphatidyllinositol
PRI	Population reference intake
PSMS	Physical Self-Maintenance
PUFA	Polyunsaturated fatty acid

RH	rheumatoid arthritis
rpm	revolutions per minute
Sf	Svedberg flotation units
SFA	Saturated fatty acids
SPECT	Single photon emission computed tomography
SS	Sensory system
TAG	Triacylglycerol
TLC	Thin layer chromatography
TMB	Tetramethylbenzadine free base
TPL	Triacylglycerol-poor lipoproteins
TRL	Triacylglycerol-rich lipoproteins
UK	United Kingdom
VLDL	Very low density lipoprotein
WHO	World Health Organisation

Chapter One

General Introduction

1.1 Fish oils and chronic diseases

Fish oils have an ameliorative effect on a myriad of human systems, including the cardiovascular system, sensory system, inflammatory system and nervous system. These oils are rich in the n-3 polyunsaturated fatty acids (PUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Vast amounts of research have been carried out on the benefits of these fatty acids on the cardiovascular system. The biochemical basis of the ameliorative effect of n-3 PUFA are thought to be inhibition of coagulation, promotion of vasodilation, attenuation of inflammation and modification of plasma lipid and lipoprotein concentrations (Roche and Gibney, 2000). The benefits of fish oils in the attenuation of inflammation has sparked interest in investigations into other inflammatory conditions such as Crohn's Disease, rheumatoid arthritis, asthma, psoriasis and Alzheimer's disease (AD).

The influences of n-3 PUFA during early development on growth and functional development of visual and neural tissues have been intensively researched (Koletzko, 1998). DHA plays a crucial role in the development of the brain and retina, particularly during the last trimester of pregnancy and during infancy when brain development is so rapid. Infants acquire this fatty acid and its precursor α-linolenic acid (ALA) *in utero* during pregnancy and from mother's milk during lactation. The conversion of ALA to DHA is poor particularly in the first few months of life (Farquharson *et al*, 1995), so the infant must rely on DHA in its pre-formed state. Deficits in n-3 PUFA during foetal and neonatal development can permanently disturb neural integrity and function (Anderson, 1994). During lactation, the mother's body loses ~ 70 - 80 mg/d DHA to breast milk (Makrides and Gibson, 2000). Independent research has shown that breast fed babies have higher levels of brain tissue DHA and enhanced mental ability later in life when compared to babies fed DHA-free formula (Horrocks and Yeo, 1999). Visual acuity and mental development are both positively affected by breast-feeding, which has been attributed to increased intake of n-3 PUFA, particularly DHA, from breast milk (Neuringer, 2000). For this reason, the composition of formula milk has been changed to closely resemble that of breast milk and DHA is now added to formulae in 40 countries world-wide (Haumann, 1998).

Long chain PUFA, particularly DHA, may reduce the development of unipolar depression (Hibbeln and Salem, 1995). The consumption of fish has been shown to correlate negatively with the incidence of depression in a number of countries (Hibbeln, 1998). Not only does fish consumption seem to be protective against depression but significant improvements in measures of depression and aggressive hostility have been found in patients in a five year follow up study when instructed to increase fish consumption (Weidner *et al,* 1992). Research into causes of depression postpartum and secondary to alcoholism also leans towards a deficit in n-3 PUFA (Hibbeln and Salem, 1995). Lactating women are at increased risk of having low DHA stores because they have high losses (70-80 mg/d DHA to breast milk), as well as their maintenance level of DHA to fulfil requirements and for oxiditation. Alcohol abuse is characterised by increased lipid peroxidation, leading to a decrease in DHA in the brain. It has been postulated that this depression may resolve in abstinence from alcohol (Hibbeln and Salem, 1995).

n-3 PUFA are also important for the brain during aging, however research in this area is largely still in preliminary stages. A 3-year follow-up study revealed that in elderly men (age 69 - 89 years), a diet high in n-3 PUFA intake through fish consumption was inversely related to cognitive impairment and cognitive decline (Kalmijn *et al,* 1997b). The prevalence of Alzheimer's disease has also been shown to correlate negatively with fish consumption and positively with high fat and high total calorie intake (Grant, 1997). Recent research has shown an inverse relationship between taking non-steroidal anti-inflammatory drugs (NSAIDs) and the development of AD (Stewart *et al,* 1997, Newman, 1998). There is also evidence for a therapeutic effect of these anti-inflammatory drugs in AD (Moller, 1998), so it is possible that the anti-inflammatory properties of fish oils explain their alleged benefits in reducing the incidence and severity of the disease (Nourhashémi *et al,* 2000). This again demonstrates that the benefits of n-3 PUFA on various systems are not mutually exclusive; in this scenario both inflammatory systems and nervous systems benefit from their therapeutic effects. A more extensive discussion of the link between fish oils and Alzheimer's disease and other neurological disorders is given later in this chapter (section 1.4).

Numerous studies have shown the benefits of fish oils in relation to CHD (Roche and Gibney, 2000), affecting several biochemical pathways. In populations where the consumption of fish is high (e.g. Iceland, Japan) there is a low incidence of CHD.

Cardiovascular disease is the leading cause of death in Western society (Nair, 1997). Low fat diets have traditionally been the first line of dietary intervention in relation to the treatment of CHD, because they caused a reduction in low density lipoprotein (LDL) cholesterol. However a low-fat, high carbohydrate diet is associated with an increase in triacylglycerol (TAG) levels and a reduction in cardio-protective high density lipoprotein (HDL) levels, which, taken together, may increase the risk of CHD. The addition of small amounts of fish oil (1 g/d) to a low fat high carbohydrate diet has been shown to counteract the negative effects of this diet (Roche and Gibney, 1996). Fish oils have also been attributed to having a positive effect on arterial hypertension (Endres *et al,* 1995). Hypolipidaemic pharmaceutical agents such as statins, fibrates, cholestyramine, colestipol and niacin are all effective in improving an atherogenic lipid profile at different levels, however they are expensive and have some disagreeable side effects including GI disturbances (cholestyramine, colestipol, statins), elevation of liver enzymes (statins), vasodilation and flushing (niacin). The use of clofibrate is contraindicated in those with hepatic or renal disorders. Fish oils effectively lower plasma TAG concentrations, both fasting and postprandially, and DHA raises the HDL / LDL cholesterol ratio and lowers the total cholesterol / HDL cholesterol ratio. They are also a natural food constituent, have no harmful side effects and are considered an effective non-pharmacological means for lowering plasma TAG (Harris, 1999). A more detailed discussion on the benefits of fish oils on CHD risk factors is fielded later in this chapter (section 1.2).

The merits of fish oils on inflammatory processes were already been mentioned with relation to CHD and Alzheimer's disease. Rheumatoid arthritis (RA) is the most widespread inflammatory disease affecting more than 2,500,000 Americans with 200,000 new cases each year (Horrocks and Yeo, 1999a). NSAID's are traditionally used long-term to treat RA, and as already mentioned, long-term use of these drugs may reduce the risk of Alzheimer's disease (Nourhashémi *et al,* 2000). NSAID's however, have their disadvantages. They are expensive and have considerable adverse side effects. Increased concentrations of EPA and DHA in the diet of a person with rheumatoid arthritis alleviate joint pain and inflammation (Horrocks and Yeo, 1999a). In fact, a 12-month double blind controlled study revealed that in patients with rheumatoid arthritis, n-3 PUFA supplementation not only resulted in significant clinical benefit for the patients but it also reduced the need for concomitant medications (Geusens *et al,* 1994). The benefits of fish oil supplementation have also been noted in relation to inflammatory

bowel disease. A double-blind, placebo-controlled cross-over study revealed that supplementation with fish oil (4.2g/d) not only caused a decline in disease activity but also reduced or eliminated the need for concurrent medication (Aslan and Triadafilopoulos 1992). Chronic inflammation is also a risk factor for certain types of cancer (Ames *et al,* 1995). NSAID's have again been shown to possibly protect against colon cancer through the suppression of hepatocyte growth factor expression (Ota *et al,* 1999), an effect that could also be achieved through the use of n-3 PUFA. Fish oils have also been shown to benefit the immune system through the modulation of host response to respiratory infection (Turek *et al,* 1996). In young adults with cystic fibrosis, supplementation with EPA-rich oil for 6 weeks resulted in improvements in disease activity score and forced expiratory volume and a reduction in sputum volume (Lawrence and Sorrell, 1993). Evidence is emerging that n-3 PUFA may also have a protective effect with regard to insulin resistance. Cells are thought to take up glucose more readily when their membranes contain more n-3 PUFA (BNF, 2000), however further research is warranted before any conclusions are drawn.

This chapter on the health benefits of fish oils serves to give a brief overview of the multitude of effects of n-3 PUFA on a wide range of systems and human diseases. In the scope of this thesis it would be impossible to provide a detailed and complete analysis of current research on the benefits of fish oils in health, and on the modulation and prevention of human diseases. A more comprehensive review of the focus of this thesis, that is, the merits of n-3 PUFA on CHD and AD, is provided in the following chapters.

1.2 Fish oils and coronary heart disease

1.2.1 Prevailing evidence

1.2.1 (i) Evidence from epidemiology

Following the emergence of the work by Bang *et al,* in the early 1970's observing that, despite similar total dietary fat intakes, Greenland Eskimos exhibited significantly lower rates of mortality from coronary heart disease compared with the Danes, a number of epidemiological studies on population fish intakes commenced. Evidence supporting the view that fish consumption reduces CHD risk currently abounds in the literature from such studies (Kromhout 1985 and 1995, Shekelle 1985 and 1993, Albert *et al,* 1998, Daviglus *et al,* 1997, Norell *et al,* 1986, Dolecek 1992). This theory is also supported by case-control studies (Gramenzi *et al,* 1990, Siscovick *et al,* 1995), and secondary prevention trials (Singh *et al,* 1997, Burr *et al,* 1989). Many of these were carried out in cultures with relatively low fish intakes. These large epidemiological studies revealed that men who did not eat fish were at a particularly high risk for CHD mortality compared with men who ate fish once or twice a week. In cohorts with a relatively high fish intake e.g. in Hawaii and Norway, no appreciable association was observed between fish consumption and CHD mortality (Vollset *et al,* 1985, Curb and Reed 1985). In these populations very few men did not eat fish, and thus the prevalence of persons who rarely or never ate fish may have been too small to show any significant effect. These results indicate that eating a small amount of fish compared with eating no fish at all is protective against CHD, but in populations with already high fish intakes, where very few people do not eat fish, increasing fish intake from one to three dishes per week does not reduce mortality from CHD. This theory has been further validated by the work of Ascherio *et al,* (1995) who concluded that increasing fish intake from one to two dishes per week to five to six per week does not substantially reduce risk of CHD. Most of the aforementioned studies have been carried out in men, however one Italian study focusing on women alone revealed that the risk of myocardial infarction (MI) was inversely associated with frequency of fish consumption, again showing the protective effect for a small amount of fish on CHD mortality (Gramenzi *et al,* 1990).

However, prevailing evidence is not absolutely conclusive. The US Physician's Health Study reported no link between moderate fish consumption and risk of cardiovascular disease after a 4 year follow-up period (Morris *et al,* 1995), but analysis of data from the same population with 11 year follow-up revealed that fish consumption at least once per week may reduce risk of sudden cardiac death (Albert *et al,* 1998), thus showing how length of follow-up, as well as baseline fish consumption levels can affect significance of results.

A cohort study of Seventh Day Adventists also found no statistically significant association between fish consumption and CHD risk (Fraser *et al,* 1992). However, in this predominantly lacto-ovo vegetarian population the prevalence of persons who consumed fish was low (about 10%), and the confidence intervals were very broad (0.42 - 1.33 for fatal events, and 0.55 - 1.96 for non-fatal events), which would render the results largely uninformative (Shekelle and Stamler 1993).

As little as 1-2 fish meals per week have been shown to reduce the risk of sudden cardiac death (Albert *et al,* 1998, Kromhout *et al,* 1985), sudden cardiac arrest (Siscovick *et al,* 1996) and also the risk of fatal MI (Daviglus *et al,* 1997). However, even amongst these studies we meet conflicting results; some showing that higher fish consumption is associated with a lower rate of sudden death (Albert *et al,* 1998, Siscovick *et al,* 1996), with another showing that fish consumption is related to a significantly lower rate of non-sudden but not sudden death (Daviglus *et al,* 1997). Variability in findings may be due, in part, to choice of end-point. The Western Electric Study (Daviglus *et al,* 1997) used death certificates alone to determine nature of death. On the other hand, Albert *et al,* analysing data from The Physician's Health Study, used a combination of next-of-kin reports, medical reports and autopsy data to determine nature and cause of death, as it has been shown that the rate of misclassification of cause of death from death certificates alone can be quite high (Kircher *et al,* 1985, Dobson *et al,* 1983). Overall, it can be concluded from these epidemiological studies that, in populations with low fish intakes, as little as one to two fish dishes per week may be protective against CHD.

1.2.1 (ii) Clinical trials / intervention studies

Following the revelation of the beneficial effects of fish and fish oils on CHD, clinical trials on normolipidaemic and in hyperlipidaemic volunteers began to emerge. A recent comprehensive review of 32 parallel placebo-controlled studies revealed that the average supplementation period in normal healthy volunteers was 7-10 weeks with mean n-3 fatty acid intakes 3-4g/d (Harris, 1997). Hyperlipidaemic volunteers were supplemented for an average of 19 weeks, but the supplementation dose was the same as that for normolipidaemic volunteers. In the same paper, data from 36 cross-over studies were also examined to reveal again a mean duration of supplementation of 7 - 10 weeks, with an average supplementation dose of ~ 4g n-3 fatty acids for both normo- and hyperlipidaemic volunteers. Overall, results from both groups of studies revealed that fish oil supplementation did not affect total cholesterol concentrations, in either normolipidaemic or hyperlipidaemic volunteers, however triacylglycerol (TAG) concentrations were reduced by an average of 25% in normolipidaemic subjects and by 34% in hyperlipidaemic groups. LDL-cholesterol concentrations increased in the hyperlipidaemic group, but to a lesser extent in the parallel studies owing to their longer supplementation period (19 weeks *v* 7 weeks in crossover studies). HDL-cholesterol concentrations remained largely unchanged.

From Harris' review of all intervention studies it emerged that olive oil was the most common placebo oil used, and no change in lipids or lipoproteins was observed in the placebo groups, confirming the lipid-neutral effect of olive oil at low doses used in these studies. This has been further substantiated by a meta-analysis published in the same year of 395 controlled dietary experiments examining the effects of fatty acids on blood lipids and lipoproteins, which again concluded that monounsaturated fatty acids had neutral effects (Clarke *et al,* 1997).

3 - 4g fish oil per day, as was the average supplemented dose in the studies used in Harris' review, is quite an unreasonably high amount to expect people to take on a daily basis. Short supplementation periods (average 7 - 10 weeks) have also been characteristic of many studies looking at the beneficial effects of fish oils on plasma lipids, lipoproteins, platelet aggregation, haemostasis and bleeding time in both fasting (Blonk *et al,* 1990, Sanders and Roshanai, 1983) and postprandial studies (Agren *et al,* 1996, Harris and Muzio, 1993, Williams *et al,* 1992). More recently, supplementation

with lower, more acceptable doses of fish oils have been explored with no beneficial consequences on fasting lipoprotein concentrations (Lervang *et al,* 1993, Davidson and Gold, 1988). These lower-dose studies have again used short supplementation periods, thus perhaps it is the length of the supplementation period that requires further investigation. This concept is further consolidated by the recent finding that 1g/d fish oil supplemented for 16 weeks showed a beneficial effect on postprandial triacylglycerolaemia in normolipidaemic volunteers (Roche and Gibney, 1996).

To date there have only been two randomised clinical trials with n-3 PUFA using "hard" end points, that is cardiac events and mortality. The Diet and Reinfarction Trial (Burr *et al,* 1989) was the first secondary prevention trial examining the effects of increasing fatty fish intake (by 200-400g/wk) on 2-year all cause mortality in a large cohort of 2033 men who had recovered from MI. They were randomised to receive or not to receive advice on each of three dietary factors:

1. reduction in fat intake and an increase in ratio of polyunsaturated fat to saturated fat
2. increase in fatty fish intake
3. increase in cereal fibre intake

The authors concluded that a modest intake of fatty fish amounting to two to three portions per week reduced mortality in men who recovered from MI by 29% compared with those not so advised.

The Indian Experiment of Infarct Survival (Singh *et al,* 1997) examined the effects of n-3 PUFA from either plant or marine origin on cardiac events in a secondary prevention trial. 360 volunteers with suspected MI were recruited and randomised to receive mustard oil (ALA), fish oil, or placebo (aluminium hydroxide). The fish oil group consumed 2g n-3 PUFA/d, and the mustard oil group consumed 20g of the oil per day, which would render 3g/d n-3 PUFA. The incidence of heart failure, arrhythmia and angina reduced in both intervention groups in comparison with control in the year following the trial. This reduction was greater in the fish oil group, again showing the benefits of n-3 PUFA from fish oils on secondary prevention.

Prevailing evidence shows that fish oil is a safe therapeutic modality for the reduction of CHD risk (Connor, 1999). Continuing research in the form of clinical trials and prospective cohort studies is warranted using hard end points to establish precisely

the optimal dose of n-3 PUFA which will reduce the risk of CHD in healthy populations and which will have an impact on the treatment of patients with CHD.

1.2.2 Coronary Heart Disease in Ireland

Diseases of the circulatory system form the largest category of deaths in Ireland, the largest proportion of these being due to ischaemic heart disease (IHD), with many of the remaining being due to cerebrovascular disease or stroke (Central Statistics Office, 1995). Table 1.2.1 shows mortality rates per 100,000 population for diseases of the circulatory system.

Table 1.2.1 Mortality rates per 100,000 population for diseases of the circulatory system and cancers

	Number of Deaths per 100,000			
	1966	**1976**	**1986**	**1996**
All Circulatory System Diseases	17,678	17,247	16,403	13,949
Ischaemic Heart Disease	5,930	8,840	8,805	7,605
Stroke	4,338	4,472	3,637	2,917
Cancer; All Malignant Neoplasms	5,173	6,284	6,890	7,306

As can be seen from this table, cancer deaths only make up just over half those from diseases of the circulatory system. In addition, diseases of the circulatory system are an important cause of morbidity. Tables 1.2.2 and 1.2.3 show the percentage of people who have a first degree relative who has had either heart disease or a heart attack and the numbers are remarkably high. Having said this, awareness for CHD prevention has

heightened in Ireland over the last number of years. The Kilkenny Health Project in 1992 had as its specific aim to "reduce the risk of heart disease among the people of the city and county of Kilkenny". Arising from that, recommendations were made to the Department of Health for the development of national strategies for disease prevention and health promotion, including the development of a national CHD prevention programme. Building on the experiences of the Kilkenny Heart Project, the Irish Heart Foundation established the Happy Heart Community Programme, and they published their report on "Health Behaviour in Ireland" in 1994. 1996 saw the arrival of a consensus statement by organisations in Ireland concerned with public health entitled "Nutrition and Heart Health". The number of deaths from IHD has shown an encouraging trend in the last decade, falling by 14% between 1986 and 1996.

Table 1.2.2 Kilkenny Health Project 1992

	Family History of Heart Disease %				
	Offaly		Kilkenny		*Average*
	1986	*1991*	*1985*	*1991*	
Men	38	32	50	36	*39*
Women	41	36	54	37	*42*
Average	*39*	*34*	*52*	*36*	

Table 1.2.3 Happy Heart National Survey 1994

	Near Relative who had a Heart Attack %				
Age (years)	***30 - 39***	***40 - 49***	***50 - 59***	***60 - 69***	*Average*
Males	27.9	31.9	39.2	34.1	*32.5*
Females	35.1	42.4	50.8	39.3	*41.2*
Average	*31.4*	*37.1*	*44.8*	*36.8*	*36.8*

We do not fare well in CHD incidence when we compare ourselves to our European neighbours (Fig. 1.2.1). We come a close second to Finland for having the highest

mortality rates from CHD in Europe. These results were compiled in 1995. However, with the correct implementation of "Heart Health" programmes, encouraging people to eat more oily fish or take small amounts of fish oil supplements, hopefully our downward trend in deaths from CHD will continue into the next century.

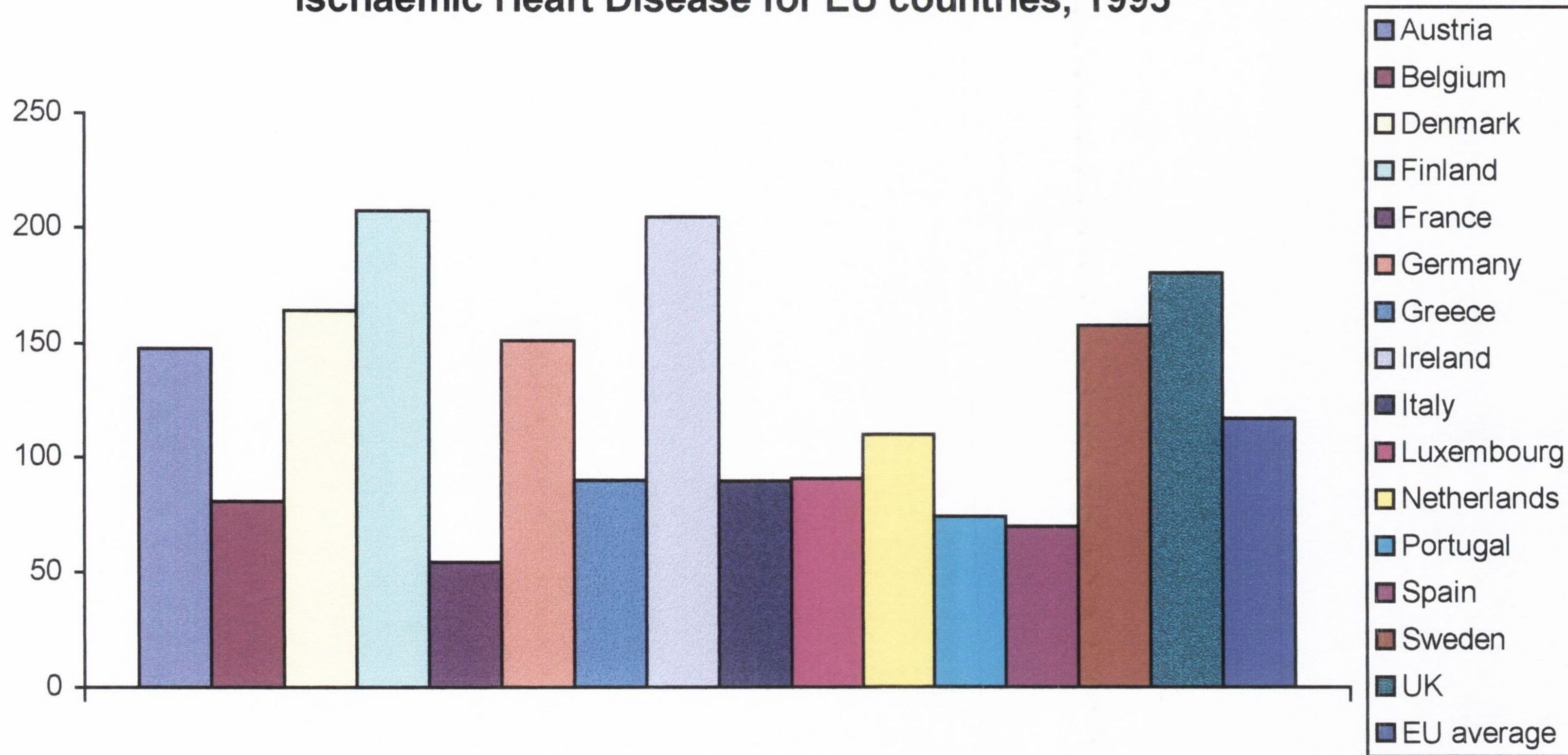


Fig 1.2.1 Age-standardised mortality rates per 100,000 population by Ischaemic Heart Disease for EU countries, 1995
250
200
150
100
50
0
Austria
Belgium
Denmark
Finland
France
Germany
Greece
Ireland
Italy
Luxembourg
Netherlands
Portugal
Spain
Sweden
UK
EU average

1.2.3 Current intakes and recommendations.

The therapeutic benefits of fish oils on CHD risk factors are undoubtedly clear, however intakes of 3 - 4g n-3 PUFA would be difficult to achieve in a population such as ours. Amongst 18 - 60+ year olds, the percentage consumers of fish ranges from 50 – 77 % of the total Irish population, and the average seafood intake is 23 – 39 g/d (Lee and Cunningham 1990). Specific intakes of fatty fish were not assessed in this study, but in the UK it is estimated that three-quarters of total fish consumed is white fish (Gregory *et al*, 1990). The Kilkenny Health Project estimated that current Irish intakes of n-3 PUFA are 0.14 % energy, which would be the equivalent of about 0.39g/d for men and 0.30 g/d for women (based on EAR for groups of males and females with a physical activity level (PAL) of 1.4 (DRV, 1991)). These intakes fall well below current recommendations from various scientific bodies, which are outlined in Table 1.2.4.

As can be seen from Table 1.2.4, there seems to be little consensus between scientific bodies with respect to recommendations for n-3 PUFA intakes. The Department of Health (1991 and 1994) and the Scientific Community for Food (SCF) (1993) base their recommendations on that amount which would prevent essential fatty acid (EFA) deficiency. The Food Safety Authority of Ireland (FSAI) recently reviewed all current recommendations and, recognising the importance of these substances in our food chain in relation to health and disease, made recommendations for n-3 PUFA intakes for Ireland (FSAI 1999). They based their recommendations on the 1993 SCF values. This was the first time that specific recommendations for intakes of these fatty acids have been made in Ireland. The BNF (1992) and Food and Agriculture Organisation/World Health Organisation (FAO/WHO) (1998) acknowledge that there are additional beneficial effects associated with greater intakes of these fatty acids and therefore their recommendations are quantitatively greater, as their aim is to promote health as well as prevent deficiency. The International Society for the Study of Fatty Acids and Lipids (ISSFAL) Working Group (1999) use what they call an "Adequate Intake" (AI) when making recommendations for intakes of n-3 fatty acids. The explanation of the AI is "*if sufficient scientific evidence is not available to calculate an Estimated Average Requirement, a reference intake called an Adequate Intake is used instead of a Recommended Dietary Allowance. The AI is a value based on*

experimentally derived intake levels or approximations of observed mean nutrient intakes by a group (or groups) of healthy people. The AI for children and adults is expected to meet or exceed the amount needed to maintain a defined nutritional state or criterion of adequacy in essentially all members of a specific healthy population".

Table 1.2.5 describes the fat and fatty acid content of commonly eaten fish in Ireland. One portion of oily fish will supply ~ 2.2g n-3 fatty acids (Holland *et al,* 1992). To achieve the recommendations set out by the ISSFAL 1999, which is 0.3% energy (0.65 -0.85 g/d), a weekly intake of about two portions oily fish would be necessary. An alternate method to achieving n-3 PUFA recommendations is the consumption of fish oil supplements, of which there is a wide range on the market (Table 1.2.6). Some of these are in liquid oil form, whilst others are in encapsulated tasteless form. They vary widely in recommended dose and also in fat-soluble vitamin content. Another feasible method for increasing n-3 PUFA intake to achieve recommendations is through the consumption of foods which have been fortified with these fatty acids, namely functional foods, however this is discussed in further detail in section 1.6. EPA and DHA can also be derived from the bioconversion of ALA, as is discussed below. However, the conversion process is not particularly efficient in humans thus contributing little to overall EPA and DHA intakes (Galli, 1999).

Table 1.2.4 Summary of recommendations for n-3 PUFA intakes

	Specific recommendations	n-3 PUFA (% en)	n-3 PUFA (g/d) males*	n-3 PUFA (g/d) females*
Food Safety Authority of Ireland (FSAI), 1999	0.5% energy n-3 PUFA	0.5	1.42	1.07
ISSFAL Working Group (1999)	DHA to be at least 0.22g/d; 0.1%energy EPA to be at least 0.22g/d; 0.1%energy	0.3	0.85	0.65
FAO / WHO (1998)	• 4-10% energy from linoleic acid • ratio of LA to ALA 5:1 to 10:1 *(calc. based on 7% en from LA and 7.5 : 1 ratio)*	0.93 †	2.64	1.98
Department of Health, UK (1991 and 1994)	1.5g EPA and DHA per week At least 0.2% energy from ALA	0.2 †	0.57	0.43
BNF (1992)	0.5% energy from EPA and DHA 1% energy from ALA	1.5 †	4.25	3.2
SCF (1993)	0.5% energy n-3 PUFA	0.5 †	1.42	1.07

* Calculations based on EAR for energy for adults with a PAR of 1.4 (DRV 1991)

† Roche, 1999

Table 1.2.5 Fat and n-3 fatty acid content of fish and shellfish*

Fish (100g edible portion, raw)	Fat (g)	n-3 fatty acids (g)
Anchovy	4.8	1.4
Cod, Atlantic	0.7	0.3
Haddock	0.7	0.2
Herring, Atlantic	9.0	1.6
Mackerel, Atlantic	13.9	2.5
Pike	1.2	0.3
Salmon,		
Atlantic	5.4	1.2
Pink	3.4	1.0
Sardines (in sardine oil)	15.5	3.3
Sole	1.2	0.1
Trout		
lake	9.7	1.6
rainbow	3.4	0.5
Tuna (fresh)	2.5	0.5
Crustaceans		
Crab	0.9	0.3
Lobster	0.9	0.2
Shrimp	1.1	0.3
Mollusks		
Mussel	2.2	0.5
Oyster	2.3	0.6
Scallop	0.8	0.2
Squid	1.1	0.3

* Hepburn *et al,* 1986

Table 1.2.6 Commonly used fish oil supplements

Brand Name	Type	Dose per day	n-3 fatty acids mg	EPA mg	DHA mg
Sanatogen	Super cod liver oil	10ml	1800	1200	600
Seven Seas	High-strength pure cod liver oil	10ml	1800	1200	600
	One-a-day pure cod liver oil	500mg capsule	90	83.2 EPA + DHA	
	Pure cod liver oil	10ml	1564	828	736
	Lemon flavour pure cod liver oil	10ml	1488	788	700
Boots	Cod liver oil	5ml	782	414	368
	Cod liver oil and orange syrup	10ml	782	414	368
	Super-strength concentration fish oils 60% ω-3	1 capsule	605	353	252
Pulse	High-strength tri-omega concentration fish oils	2 capsules	520	400	120

1.2.4 Elongation and desaturation of ALA to EPA and DHA

The elongation and desaturation of ALA to EPA and DHA is shown in Fig. 1.2.2. Previously it was thought that the conversion involved a Δ4-desaturase, however Sprecher *et al,* (1996) found that no detectable quantities of radiolabelled 22:6n-3 are produced when the supposed precursor of DHA, namely 22:5n-3, is labelled with ^{14}C and incubated with rat liver microsomal fraction. They proposed an alternative pathway in which DHA was formed from two sequential chain elongation steps from EPA, followed by a Δ6-desaturation to form 24:6n-3 with a final retroconversion step to form 22:6n-3. This pathway has now been accepted (Périchon *et al,* 1998, Gurr, 1999). The retroconversion process is catalysed by enzymes similar to those of β-oxidation, either in the mitochondria or in the peroxisomes of the cell (Gurr, 1999). Peroxisomal β-oxiditation shows specificity for very long chain PUFA (those with >/= 20C). These fatty acids are preferentially, if not exclusively, oxidised within the peroxisomes (Périchon *et al,* 1998), which explains why those with peroxisomal disorders are characterised by a severe deficiency in DHA.

Figure 1.4.2 Conversion of ALA into EPA and DHA

C18:3 n-3 (ALA)

⇓ *Δ6-desaturation*

C18:4 n-3

⇓ *elongation + C2*

C20:4 n-3

⇓ *Δ5-desaturation*

C20:5 n-3 (EPA)

⇓ *elongation + C2*

C22:5 n-3 (DPA)

⇓ *elongation + C2*

C24:5 n-3

⇓ *Δ6-desaturation*

C24:6 n-3

⇓ *retroconversion - C2*

C22:6 n-3(DHA)

1.3 Postprandial lipaemia and coronary heart disease

1.3.1 Relationship between postprandial lipaemia and CHD

Coronary heart disease (CHD) is a multi-factorial disorder, and fats have been implicated in the pathogenesis and progression of CHD. Earlier epidemiological investigations examining the effects of plasma lipids on CHD risk factors examined fasting lipid concentrations as these are not confounded by dietary factors and thus provide a more reproducible and better defined baseline for investigations. However, particularly in the light of the increasing "snacking phenomenon" world-wide, we are spending the majority of our time in a postprandial state (Williams, 1997). For this reason, fasting lipid profiles alone may be inadequate in assessment of CHD risk. This is not a novel idea as more than twenty years ago Zilversmit (1979) described atherogenesis as a "postprandial phenomenon". Postprandial lipaemia represents a series of metabolic events occurring in response to the ingestion of a fat-containing meal (Roche and Gibney, 1999). It is associated with a number of beneficial events including the stimulation of reverse cholesterol transport. This is an important physiological mechanism, where excess cholesterol is removed from extrahepatic tissues and is delivered to the liver for catabolism. High density lipoproteins (HDL) are the vehicle of cholesterol in this reverse transport and cholesteryl ester transfer protein (CETP) facilitates the process (Zampelas and Williams, 1994). However, prolonged postprandial lipaemia is also associated with a number of adverse lipoprotein changes including the formation of atherogenic small dense LDL particles, the accumulation of atherogenic chylomicron (CM) remnants and a reduction in the cardio-protective HDL fraction (Cohn, 1998). The magnitude and duration of the postprandial response varies greatly between individuals and is described below (section 1.3.4)

The postprandial response

1.3.2 (i) Digestion and absorption of dietary fat

In Western society it is estimated that about 100g fat is consumed daily (Frayn, 1998). The majority of this fat is in the form of TAG (95 - 98%), with cholesterol and phospholipids amounting to about 0.5g and 4g - 8g, respectively (Castelli, 1986). Fats are not appreciably altered in the mouth or stomach; lingual and gastric lipase only play a small part in their digestion, hydrolysing some of the lipases at the sn-3 position of the TAG molecule resulting in the release of a 1,2 diacylglycerol molecule and a free fatty acid (Small, 1991). The pancreas secretes pancreatic lipase (*EC* 3.1.1.3), cholesterol esterase (*EC* 3.1.1.13) and phospholipase (*EC* 3.1.4.4) and this is where the main fat digestion takes place (Small, 1991). TAG are hydrolysed sequentially, finally yielding *sn*-2 monoacylglycerols and free fatty acids. Less than 5% of the fat ingested remains in the form of di- and triacylglycerols (Sethi *et al,* 1993). Phospholipids are hydrolysed by pancreatic phospholipase, yielding glycerol, fatty acids, phosphate and nitrogenous bases. Only a very small portion of ingested phospholipid is absorbed intact.

Absorbed cholesterol esters are hydrolysed by cholesterol esterase, again secreted from the pancreas. Absorption is limited to about 2g per day in man, unlike the absorption of TAG, which is almost unlimited. Endogenous cholesterol, derived from bile, comprises half the daily absorption of this compound.

Monoglycerol and fatty acid components of dietary TAG are subsequently absorbed into the enterocyte. Here, their end metabolic fate is dependent on their chain length. Small and medium-chain fatty acids (< 10 - 12C) escape from mucosal cells without re-esterification and, bound to albumin, are transported as NEFA to the liver and other tissues. Free glycerol also evades re-esterification and is transported by portal blood. Fatty acids with more than 12 carbon molecules and absorbed cholesterol enter the endoplasmic reticulum of the enterocyte where they are re-esterified resulting in the formation of TAG molecules and cholesteryl esters (CE), respectively (Pfeffer *et al,* 1977).

1.3.2 (ii) TRL formation (chylomicron and VLDL)

Triacylglycerol-rich lipoproteins (TRL), chylomicrons (CM) and very low density lipoproteins (VLDL) are the transport vehicles for TAG in circulation. CM are used to ferry dietary TAG and are formed in the intestinal epithelial cells. They consist of a core of re-esterified TAG molecules, which constitutes 86 - 92% of the molecule by mass (Roche and Gibney, 1995). Cholesteryl esters are also core components of the CM. The core is wrapped in a monolayer of phospholipids that constitute about 8% of the CM by mass and the surface layer consists of apoproteins. Apolipoprotein (apo) B-48 is the major apoprotein of the chylomicron and is exclusively associated with this molecule (Karpe and Hamsten, 1995). It is synthesised within the enterocyte and remains in the core of the CM until uptake and removal by the liver (Sethi *et al,* 1993) and is necessary for CM secretion from mucosal cells. The enterocyte also synthesises apo A-I and apo A-IV which are also associated with the CM molecule. The final processing of the CM occurs in the Golgi apparatus, before it is released by exocytosis into the thoracic duct.

Chylomicrons are carried through the thoracic duct in the form of lymph and enter the circulation via the subclavian vein resulting in postprandial lipaemia. The rise in plasma TAG concentrations following the ingestion of a meal is largely due to an increase in particle number (71%) and to a smaller extent to an increase in the amount of TAG carried per particle (29%) (Poapst *et al,* 1985). However, this rise in particle number is not only due to an increase in CM, but also to an increase in hepatically derived VLDL. In fact, Schneeman *et al,* (1993) estimated that 80% of the increase in particle number following a meal was due to an increase in VLDL. However, chylomicrons are quantitatively a much larger carrier of TAG, representing 80% of the postprandial increase in TAG (Cohn *et al,* 1993). VLDL are transport vehicles for endogenous TAG and are recognised by the unique presence of an apo-B100 molecule. This endogenous TAG component is derived from three main sources; free fatty acids (NEFA) generated from hormone sensitive lipase (HSL) action in adipose tissue; secondly, reduced uptake by adipose tissue and muscle of NEFA generated from lipolysis of TRL shunts these NEFA to the liver. Finally, *de novo* synthesis in the liver of TAG provides substrate for VLDL (Karpe, 1997). The endogenous TAG is situated in the core of the VLDL molecule along with CE, and is secreted as nascent VLDL.

There are many similarities between the metabolism of chylomicrons and VLDL (Havel, 1997). Once in circulation, TRL acquire apo CII, CIII and apo-E from HDL with the reciprocal transfer of some apo-AI and apo-AIV. It is the acquisition of the apo CII that permits the subsequent hydrolysis of TAG from the lipoprotein. The presence of apo E acts as a ligand for receptor recognition of the molecule which permits removal by the liver. The presence of apo CIII is thought to influence events in two ways; firstly it counteracts the lipolysis-stimulating function of apo CII and secondly it inhibits the receptor-mediated uptake of the lipoprotein (Karpe *et al*, 1993). CM are the largest lipoprotein molecule present in circulation (carrying 80% TAG) and appear about 60 minutes after the ingestion of a fat-containing meal. They remain in circulation for about 6 - 8 hours, after which time the major TAG component has been removed.

1.3.2 (iii) TRL catabolism

Lipoprotein lipase (LPL) is the rate-limiting enzyme responsible for the removal of TAG from TRL in circulation. Because of this its activity is an important determinant of the magnitude and duration of postprandial lipaemia. Other physiological and dietary factors also affect postprandial lipaemia, but these are discussed in further detail later in this chapter (section 1.2.4). LPL is present on the surface of capillary endothelial cells of adipose tissue (Goldberg, 1996), but is also found in other tissues with a high requirement for fatty acids including cardiac muscle, skeletal muscle and mammary gland (Eckel, 1989). Apo CII acts as a co-lipase increasing the interaction of LPL with the substrate at the interface of the lipoprotein. LPL has a diverse range of actions, depending on its location. In the postprandial state, insulin increases the synthesis and secretion of adipose tissue LPL, thus promoting the uptake of fatty acids, however skeletal muscle LPL is inhibited by insulin so its uptake of fatty acids occurs only in the post-absorptive state.

TRL interact with multiple LPL molecules, resulting in the hydrolysis of TAG in the chylomicron producing monoacylglycerols and free fatty acids. These free fatty acids have a number of metabolic fates; they are used as an energy source for cells, re-esterified to form TAG and stored in adipose tissue, used in the synthesis of lipid in the mammary gland or are incorporated into membrane lipid (Gurr, 1988). A considerable

amount of these free fatty acids escape into circulation where, bound to albumin, they are transported to the liver. In the absence of albumin, these circulating free fatty acids interfere with the binding of LPL to TAG, and thus inhibit LPL activity (Eckel, 1989). Hydrolysis of TAG from the lipoprotein particle by LPL renders a smaller lipid-poor, protein rich entity known as a "chylomicron remnant" or a "VLDL remnant".

1.3.2 (iv) Reverse cholesterol transport

As previously explained (section 1.3.1), reverse cholesterol transport involves the removal of cholesterol from extrahepatic tissues and the delivery of this cholesterol to the liver for catabolism. High density lipoproteins (HDL) are the vehicle for cholesterol transport and lecithin cholesteryl acyl transferase (LCAT) acts as the rate-limiting enzyme. Cholesteryl ester transfer protein (CETP) facilitates the process. HDL has the ability to promote cholesterol efflux from the arterial wall (Fielding and Havel, 1996), however, the exact mechanism by which this happens is unknown (Reichl and Miller, 1986). It is thought that cells loaded with CE have apo-A1 receptors, which recognise the HDL molecule, which in turn activates the hydrolysis of the fatty acid from CE and promotes its efflux from peripheral cells (Fielding and Havel, 1996). The free cholesterol is then incorporated into HDL and esterified by LCAT where it is then forced from the outer polar coat into the apolar core of the HDL particle. This then creates a free 'slot' on the HDL coat for accepting a new free cholesterol particle, thus creating a gradient for cholesterol efflux from peripheral tissues for subsequent hepatic degradation. HDL particles are recognised by their apo E component through the apo E receptors on hepatocytes, resulting in endocytosis and catabolism. Uptake of CE from HDL can also occur without the particle being taken up directly by the liver (Miller, 1987).

Whilst in circulation, 'neutral lipid exchange' occurs amongst lipoproteins. This is the mechanism whereby cholesteryl esters are transferred from HDL and LDL particles to TRL with the reciprocal equimolar transfer of TAG. This action is mediated through the action of CETP and endothelial lipases. Subsequently, TRL become relatively enriched with CE but relatively depleted of TAG and are taken up by hepatic parenchymal cells by receptor-dependent endocytosis.

Exaggerated postprandial lipaemia can occur for a number of reasons (sections 1.3.4 and 1.3.5), with adverse consequences. Impaired LPL activity, particularly in adipose tissue in the postprandial state, will effectively increase the residence time of large TRL. This may be also due to overproduction of CM or VLDL. Exaggerated neutral lipid exchange then occurs. Once enriched with TAG, HDL and LDL become excellent substrates for hepatic lipase, resulting in atherogenic small dense LDL particles which not only have the ability to induce foam cell formation, but are also are more susceptible to oxidation (Chait *et al,* 1993). Small dense HDL_3 particles are quickly taken up by the liver, thereby reducing the overall concentration of the cardio-protective HDL fraction. TRL remnants are also considered atherogenic as they can penetrate the endothelium in the same way as LDL and HDL particles (Mamo and Wheeler, 1994). Thus, for a number of reasons, exaggerated postprandial lipaemia can lead to an unfavourable lipid profile.

1.3.2 (v) TRL clearance

Following neutral lipid exchange, TRL remnant particles have lost 80 - 90% of their original TAG content (Cohn, 1998) but contain CE and apo B-48 (chylomicron) or CE and apo B-100 (VLDL). In the formation of the remnant, apo E is transferred from HDL which allows the remnant particle to be recognised by various ligands on the surface of hepatocytes. Hepatic lipase also aids in the final degradation process by hydrolysing residual TAG and phospholipid in the remnant particle. This permits further exposure of apo E to the hepatocytes, promoting hepatic uptake through the receptor-dependant process. Once bound to the hepatic receptor, the remnant particle is delivered to hepatic lysosomes where hydrolysis of lipid and protein leads to complete degradation.

Larger VLDL remnants (Svedberg flotation units (Sf) 60-400), containing apo B-100 are thought to be catabolised and cleared via the same mechanisms as described above. They too are delipidated via LPL action, with the reciprocal transfer of apo E from HDL which allows remnant particle recognition by hepatocytes leading to subsequent degradation. Smaller VLDL remnants (Sf 20-60) are not taken up by the

liver as they are further hydrolysed to form intermediate density lipoprotein (IDL) and ultimately LDL (Shepherd and Packard, 1987).

1.3.3 Atherogenicity of remnant lipoproteins

Evidence from animal and human studies linking TRL remnants and atherosclerosis has been recently reviewed (Cohn, 1998). It is thought that not only do the presence of a large number of TRL remnants represent an atherogenic profile in which there is a "TAG intolerance", but the remnants themselves contribute to atherogenesis through a number of mechanisms. The 'remnant hypothesis' was initiated by Zilversmit in 1979 through his work with rabbits, and has more recently been verified from evidence showing that TRL can be directly incorporated into human atherosclerotic plaques (Rapp *et al,* 1994) and can initiate the formation of foam cells (Chung and Segrest, 1991). Conversely, more recent research has shown that CM remnants are removed from circulation before they are delipidated to a size small enough to allow them to penetrate vascular endothelium (Karpe and Hamsten, 1995). More significantly, the presence of a large number of TRL remnants in the postprandial period, whether due to reduced clearance or the overproduction of TRL represents an 'atherogenic profile'. The characteristics of such a profile include; an increase in CM and CM-remnants, an increase in VLDL and their remnants, a preponderance of atherogenic small dense LDL particles (which leads to increased susceptibility to oxiditation), a reduction in the cardio-protective HDL_2 (with the associated increase in HDL_3 which are rapidly broken down resulting in lower HDL concentrations) and increased CE transfer to TRL, resulting in a decrease in HDL cholesterol. Laboratory and clinical studies linking the accumulation of TRL remnants with CAD have recently been reviewed (Cohn, 1998), all evidence suggesting that these lipoproteins play a significant role in the progression of atherosclerosis. However, many factors influence the magnitude and duration of postprandial lipaemia and these are discussed below.

1.3.4 Physiological factors affecting the magnitude and duration of the postprandial response

Numerous studies have examined the factors affecting the magnitude and duration of postprandial lipaemia, and the general consensus seems to be that fasting TAG concentrations are the most consistent predictor of the magnitude of the postprandial response (Roche and Gibney, 1995). An inverse relationship has also been shown between levels of HDL_2 cholesterol and magnitude of postprandial lipaemia (Patsch *et al,* 1983). Rapid clearance of TRL promotes formation of HDL_2 which may protect the arterial wall. Conversely, delayed lipolysis increases the transfer of TAG from TRL into HDL and the reciprocal transfer of CE into lipoprotein fractions, which is associated with high CHD risk (Patsch, 1994). This process has previously been described in further detail (section 1.3.2).

There is considerable heterogeneity between individuals in the magnitude and duration of the postprandial response, but certain trends prevail, which will be explained below.

1.3.4 (i) The effect of age and gender

Gender differences in the magnitude of the postprandial TAG response have been displayed by many authors (Redard *et al,* 1990, Cohn *et al,* 1988, Kashyap *et al,* 1983, Baggio, 1980), all showing that male volunteers are the greater responders. The extent of the between-gender difference in the magnitude of postprandial response varies between studies, from a three- to four-fold increase in TAG area under the curve (AUC) (Kashyap *et al,* 1983) to a more modest 30% increase in peak TAG concentrations in males when compared with female values (Cohn *et al,* 1988). However, in the study by Kashyap and colleagues male subjects were older and had higher fasting TAG concentrations than females. The two other studies referred to here did not mention exact values for TAG AUC but Redard *et al* quoted a significant overall P value for gender difference of 0.002, and Baggio and colleagues explained that their male volunteers showed a significantly greater TAG concentration at 4.5 hours postprandially

from baseline, with female volunteers not displaying any statistically significant rise at any time point.

Plasma lipid concentrations also vary during phases of the menstrual cycle, being highest around mid-cycle and at time of ovulation. Cholesterol and TAG concentrations also rise during pregnancy, peaking around 36th - 39th week (Durrington, 1990).

Cohn *et al,* (1988) showed that older subjects (66 ± 2 years) had higher peak TAG concentrations than younger subjects (29 ± 2 years), despite the fact that there was no statistically significant difference in fasting TAG concentrations between older and younger volunteers. The type of plasma TAG response (i.e. the number of postprandial peaks) was not associated with age or gender in this study but other studies have shown that it is affected by meal content and by timing of meals, but these will be discussed below.

1.3.4 (ii) The effect of lipoprotein lipase (LPL) activity

LPL is the key hydrolytic rate-limiting enzyme for TAG removal from circulation (Grundy and Mok, 1976). The importance of LPL in relation to the magnitude of the postprandial response is clearly demonstrated by looking at the differences in the magnitude of the postprandial response in families who are affected by a genetic deficiency of LPL. Carriers of a missense mutation of the LPL gene have been identified in two Austrian families. Despite the fact that they had normal fasting TAG concentrations, they were found to display an exaggerated postprandial TAG response which was attributed to their low LPL activity (Miesenbock *et al,* 1993).

Variations in LPL activity could be responsible for the varying postprandial TAG responses in males and females (Despres *et al,* 1999), and in older (> 40 years) and younger (< 30 years) volunteers (Weintraub *et al,* 1987). In the acute setting, LPL activity is also increased following exercise training in endurance-trained people (Hardman, 1998) and in untrained people (Seip *et al,* 1997), leading to lower postprandial TAG responses. Athletes also display greater LPL activity than sedentary controls (Weintraub *et al,* 1989).

Previously it has been shown that PUFA - rich diets, particularly n-3 PUFA diets, cause a reduction in concentration and residence time of TRL particles and their

remnants in the postprandial period. As has been previously described, LPL is the rate-limiting enzyme responsible for the removal of TAG from circulation and therefore would influence the magnitude and duration of the postprandial response. Studies examining LPL activity in animals and in humans are summarised in Tables 1.3.4 (i) and (ii). There seems to be a general consensus from animal studies that background diets rich in PUFA and MUFA increase post-heparin LPL activity compared to background diets rich in SFA (Groot *et al,* 1988, van Heek and Zilversmit, 1990). It is thought also that the larger CM produced from PUFA-rich diets are preferred substrates for LPL and are therefore cleared more quickly (Groot *et al,* 1988, Levy *et al,* 1991). Results from human studies are more ambiguous. Some studies have found an increase, although non-significant, in post-heparin LPL activity following background diets rich in n-3 PUFA (Harris *et al,* 1988, Nozaki *et al,* 1991, Kasim-Karakas *et al,* 1995), whereas some have found no change in activity of this enzyme (Weintraub *et al,* 1988, Westphal, 2000). A study looking at the acute effects of test meal fatty acid composition on 9 hours post-heparin LPL activity has shown elevated activity following the high n-3 PUFA test meal when compared to meals containing mixed oil or corn oil (n-6 PUFA) (Zampelas *et al,* 1994). More recently it has been shown that endogenous LPL activity is increased following n-3 PUFA supplementation in both normo- and hyper-lipidaemic volunteers (Harris *et al,* 1997a), however it is as yet unclear whether endogenous LPL activity reflects LPL activity *in vivo* (Roche and Gibney, 1999). It would seem that the difference observed in these studies lies in the methodology used in the assessment of LPL activity. Most authors used heparin stimulation to release the endothelium-bound enzyme, whereas Harris did not use any stimulation, which may better reflect *in vivo* LPL activity (Westphal, 2000).

Table 1.3.4 (i) Effects of dietary fatty acids on LPL activity - animal studies †

Reference	Animal	Test meal	LPL measurement
Groot *et al,* 1988	rat	diets rich in palm oil (SFA) or sunflower seed oil (n-6 PUFA)	Post-heparin LPL activity (10 hours and 12 hours after meal) significantly higher in sunflower seed oil - fed rats
van Heek and Zilversmit 1990	rabbit	diets rich in coconut oil (SFA) or olive oil (MUFA)	Post-heparin LPL activity significantly higher in olive oil - fed rabbits after 49 days
Levy *et al,* 1991	rat	diets rich in coconut oil (SFA), safflower oil (PUFA), MCT*	Adipose tissue LPL activity significantly higher in safflower-oil - fed rats
Murphy *et al,* 1993	rat	mixed oil, maize oil or fish oil diet	Adipose tissue LPL mRNA significantly higher in fish oil fed rats

* MCT = Medium Chain Triglycerides

† Williams, 1997

Table 1.3.4 (ii) Effects of dietary fatty acids on LPL activity - human studies

Reference	Test meal	LPL measurement
Weintraub *et al,* 1988	Background diets rich in SFA, n-6 PUFA or n-3 PUFA	Post-heparin LPL activity higher (NS) on n-3 PUFA diet
Harris *et al,* 1988	Background diets rich in SFA, n-6 PUFA or n-3 PUFA	NSD* between meals in post-heparin LPL or HL activity
Nozaki *et al,* 1991	Background diets supplemented with 10g/d n-3 PUFA for 4 weeks (hypertriacylglycerolaemic patients)	NSD* between groups in post-heparin LPL or HL activity
Kasim-Karakas *et al,* 1995	Background diets supplemented with 3.3g/d n-3 PUFA for 1 month (hypertriacylglycerolaemic patients)	Increase in post-heparin LPL activity (NSD) in fish oil group
Harris *et al,* 1997	Background diets supplemented with placebo (olive oil; MUFA); 5.0 g/d n-3 PUFA for 3 weeks (healthy); 5.0g.70kg/body weight/d (hypertriacylglycerolaemic patients)	Endogenous LPL activity Significantly higher in fish oil fed normo- and hyper-triacylglycerolaemic volunteers

* NSD = No Significant Difference

1.3.4 (iii) The effect of exercise

Several studies have shown the beneficial effect of acute and chronic bouts of exercise on fasting and postprandial lipids and lipoproteins in both normolipidaemic and hyperlipidaemic volunteers. In the chronic setting, endurance-trained athletes exhibit low levels of postprandial lipaemia, which is reported to be due to an increased rate of TAG removal when compared to sedentary people (Cohen *et al,* 1989, Hardman, 1998). Weintraub *et al,* (1989), controlling for diet composition and weight change, found that regular aerobic exercise significantly reduced total postprandial lipoprotein levels by 32%, reduced fasting TAG levels by 16% and also increased LPL activity by 16% in normolipidaemic volunteers. Other studies have also reported increased muscle LPL activity following exercise training in endurance-trained people (Hardman, 1998) and in untrained people (Seip *et al,* 1997), which would account for the low levels of postprandial lipaemia in both groups. The intensity of exercise has also been examined with relation to attenuating postprandial response. Low to moderate intensity exercise in normolipidaemic volunteers who are physically active (but not endurance trained), taken the day prior to the fat-rich test meal has been shown to attenuate postprandial lipaemia when compared to controls in a balanced, cross-over design (Tsetsonis and Hardman, 1996).

In the acute setting, exercise both before and after the test meal has been shown to attenuate the postprandial response. A single period of walking after the test meal for 1.5 hours at a low intensity exercise (40 % maximal oxygen consumption), has been shown to attenuate postprandial lipaemia (Hardman and Aldred, 1995) in sedentary normolipidaemic volunteers. The acute effects of exercise on a day prior to a test day on postprandial lipaemia, in trained and untrained middle-aged women have also been examined. The volunteers were asked to either walk for 90 min at 60% maximal oxygen uptake (exercise group) or refrain from exercise (control group) on the day prior to the test day. Exercise decreased lipaemia in both groups; the trained group showed a mean TAG AUC of 6.96 ± 0.48 mmol.h/L in the control period, versus a mean of 4.87 ± 0.33 mmol.h/L after the exercise, and the untrained group dropping from a mean TAG AUC of 8.36 ± 0.83 to 7.01 ± 0.79 mmol.h/L after the test meal (Tsetsonis *et al,* 1997), demonstrating that the athletic group could catabolise fat to a greater extent than their

sedentary counterparts. The same phenomenon has also been described in trained and sedentary men (Cohen *et al,* 1989).

These results confirm that exercise training decreases postprandial lipaemia, to a greater extent in trained than untrained individuals (Cohen *et al,* 1989). In light of this, the influence of alcohol intake on postprandial lipid metabolism was studied in relation to exercise status of subjects, as alcohol has a known hypertriacylglycerolaemic effect (Hartung *et al,* 1993). They showed that consumption of alcohol was associated with increased postprandial lipaemia and retarded TAG clearance in inactive, but not exercise-trained subjects. They proposed that the increased LPL activity in trained people induces a protective mechanism to guard against the hypertriacylglycerolaemic effect of alcohol in these volunteers.

1.3.4 (iv) The effect of obesity and weight reduction

Obesity is often associated with hypertriacylglycerolaemia, which is thought to result from either an increase in TAG production rate and/or impaired removal (Huttunen *et al,*. 1975). Lewis *et al,* selected two normolipidaemic groups, one obese (BMI 43.7 ± 2.81 kg/m^2) and one normal weight (BMI 23.6 ± 0.72 kg/m^2), and examined their postprandial response over a 24 hour period to a mixed high fat meal. The obese group showed a plasma TAG cumulative increment that was 3.35-fold greater than controls, while that of retinyl ester was 1.63 - fold greater, suggesting that a significant portion of the postprandial TAG response is due to endogenous hepatic lipoproteins. However, even though volunteers were selected under the criteria that they have normal blood lipid profiles, the obese group had significantly higher fasting TAG concentrations (1.35 ± 0.12 *v* . 0.68 ± 0.08 mmol/l) than the normal weight group, which could account somewhat for the increased postprandial response. This group also displayed insulin resistance which is associated with increased hepatic VLDL production (Roche and Gibney, 1995). HDL cholesterol levels were also significantly lower in the obese group than the normal weight (0.94 ± 0.08 *v* 1.35 ± 0.11 mmol/l).

Results from a meta-analysis of studies on the effects of weight reduction on plasma lipids and lipoproteins demonstrated that each kilogram weight loss resulted in 0.015 mmol/l decrease in TAG concentrations (Dattilo and Kris - Etherton, 1992). LPL

activity has also been shown to increase with weight loss, particularly once weight stabilises (Schwartz and Brunzell, 1981).

1.3.4 (v) Effect of insulin and glucose metabolism

Chronic insulin deficiency, as displayed by patients with diabetes, is associated with a decrease in LPL activity in humans (Taylor *et al,* 1979). In the acute state, administration of insulin in such individuals increases LPL activity (Sadur and Eckel, 1982). Muscle LPL activity has been shown to be lower in obese, hyperinsulinaemic males than in either a group of equally obese males with normal insulin levels or a normal weight control population (Pollare *et al,* 1991). Another study, controlling for the effect of four potentially confounding factors; gender, obesity, hyperglycaemia and hypertriacylglycerolaemia, has also shown that degree of insulin sensitivity affects LPL activity (Chen *et al,* 1994), which would in turn affect the magnitude of the postprandial TAG response. Indeed the same group also examined the postprandial response in a group of non-obese normotriacylglycerolaemic diabetics and controls and found that the postprandial increase in TRL was significantly greater in diabetics than controls (Chen *et al,* 1992).

Lewis *et al,* (1991) completed a similar study with obese subjects. They had two subject groups, one being normotriacylglycerolaemic and one hypertriacylglycerolaemic. They found a slower clearance rate of CM in the non-insulin dependant (NIDDM) group with hypertriacylglycerolaemia, and also a late and prolonged surge in NEFA concentrations. They concluded that moderate fasting hypertriacylglycerolaemia in diabetic patients was predictive of a constellation of postprandial lipid and lipoprotein changes which would potentiate the already unfavourable atherogenic lipid profile in these patients.

1.3.4 (vi) Effect of Apo E phenotype

Apolipoprotein E (apo E) plays an important role in the receptor-mediated clearance of lipoproteins from circulation. Three alleles exist, apo E2, apo E3 and apo E4 with the apo E3/3 phenotype being the most common (60% population), followed by the apo E2/3 phenotype (carried by 20 - 25% population) (Roche and Gibney, 1995). Homozygotes for apo E2 have impaired TAG clearance in the postprandial phase and are described as having Type III Hyperlipidaemia. Brown and Roberts, (1991) have shown that subjects with the apo E4 phenotype display the lowest plasma TAG and chylomicron TAG response, followed by those with the apo E3 phenotype, with impaired clearance in those either homo- or heterozygous for the apo E2 allele. Controlling for apo E phenotype could therefore help to reduce the interindividual variation observed in studies on postprandial lipaemia.

1.3.5 Dietary factors affecting the postprandial lipaemic response

The effects of diet, particularly dietary fatty acids on postprandial lipid metabolism are of extreme importance, as it is estimated that we spend approximately 17 hours of our 24 hour day in the postprandial state (Williams, 1997). Current dietary recommendations to the general public for developing a healthier lifestyle concentrate on the reduction of fat intakes, particularly saturated fats, however there is a paucity of dietary studies evaluating postprandial as well as fasting lipoproteins. Most epidemiological studies measured lipoprotein concentrations following a 12 hour fast, where TAG levels would reflect what we would experience somewhere between the hours of 3 am and 6 am when people normally would be experiencing a 12 hour fast. As previously explained, and as first noted by Zilversmit in 1979, atherogenesis is a postprandial phenomenon as prolonged postprandial lipaemia is associated with a number of adverse metabolic events including the formation of small dense LDL particles, an accumulation of atherogenic CM remnants (Karpe *et al,* 1994) and a

reduction in the cardioprotective HDL fraction (Miesenbock and Patsch, 1992). CHD patients have a more prolonged postprandial response when compared with healthy controls (Braun *et al,* 1997, Patsch *et al,* 1993). Epidemiological studies have highlighted the importance of background dietary fatty acid composition on CHD; there is a low prevalence of CHD in Mediterranean countries (Keys *et al,* 1986) where the consumption of MUFA is relatively high (Keys *et al,* 1970, Kafatos and Mamalakis, 1993) and in countries where intakes of n-3 PUFA (oily fish) is high (Bang *et al,* 1971, Kromhout *et al,* 1985). Therefore it is important that we evaluate the effects of varying dietary fatty acids on postprandial lipaemia, in both acute and chronic states.

Studies in the acute phase - test meal composition

1.3.5 (i) Test meal fat quality composition

Results from studies examining the effect of fatty acids on the postprandial response are difficult to interpret as there are disparities regarding the amount and type of fat used in these tests. Varying amounts of fats have been used with different compositions. One study used a liquid emulsion (de Bruin *et al,* 1993), whilst two other studies used liquid formula mixed meals (Shishehbor *et al,* 1999, Harris *et al,* 1988), with a mixed meal consisting of solid foods being the most popular choice. Length of follow-up between studies is diverse, varying from 6 - to 24 - hours, with the majority of groups choosing 8 - to 12 - hour follow-up periods. The amount of fat used also affects the magnitude of the postprandial response (Dubois *et al,* 1998, Cohen *et al,* 1988). Results of MUFA-containing test meals are inconsistent between studies. De Bruin *et al,* fed their volunteers test meals containing cream (SFA), olive oil (MUFA) and soyabean oil (PUFA) using a cross-over design and found that CM from volunteers after the MUFA meal had higher concentrations of retinyl palmitate and apo B-48 than those fed the SFA or PUFA test meals. However, the same hyperchylomicronaemic effect of MUFA-containing meals was not observed by Zampelas and colleagues (1994) or by Jackson *et al,* (1999). Jackson and associates fed volunteers three test meals, a 12 % MUFA (i.e. 17.2 % SFA, 12 % MUFA, 5.0 % PUFA), a 17 % MUFA (12.3 % SFA,

17.1 % MUFA, 4.6 % PUFA) and a 24 % MUFA meal (5.4 % SFA, 24.1 % MUFA and 4.6 % PUFA). PUFA concentrations remained constant between test meals, so they varied only in their MUFA and SFA compositions, the 12 % MUFA reflecting the habitual fatty acid intake of the UK population (Gregory *et al,* 1990), the 17% MUFA meal representing a meal characteristic of the Mediterranean and the 24 % MUFA meal being distinctive of the Cretans at the time of the Seven Countries Study (Keys *et al,* 1970). They examined the postprandial response to each test meal over a 9 hour period. Their results showed that intestinally derived lipoproteins resulting from test meals of varying MUFA concentration did not vary according to the composition of the test meal. The amount of fat in each test meal (40g) reflects the amount associated with a typical main meal in the UK.

Volunteers in this study displayed a monophasic TAG response to the 12 % and the 17 % MUFA meals, however a biphasic response was displayed following the ingestion of the 24 % MUFA meal. It was hypothesised that the biphasic response observed was due to either;

1. differences in gastrointestinal handling of the high MUFA meal,
2. clearance of the MUFA - rich CM, or
3. differences in fatty acid induced secretion of VLDL particles in the late postprandial phase.

Previous studies have shown a biphasic TAG response following a fat-containing meal when the test meal is given in the afternoon or evening as the second surge of TAG observed is thought to be due to VLDL TAG (Schneeman *et al,* 1993). However, in the study by Jackson and colleagues the test meal was consumed after a 12 hour fast, so it unlikely that the second TAG peak was acquired from a previously consumed meal. Another study compared the postprandial response to a similar mixed test meal (24% MUFA) with a high - (similar to that used by Jackson *et al*, 1999) or low - carbohydrate content (136 g *v.* 23 g) (Shishehbor *et al,* 1998). The higher carbohydrate meal produced a biphasic TAG response, as was observed by Jackson *et al,* however the low-carbohydrate meal produced a monophasic TAG response. This suggests that the carbohydrate content of a test meal given following a 12 hour fast, may determine the nature of the postprandial TAG response.

Zampelas *et al,* (1994) examined the postprandial lipaemic response to three test meals given in the evening time, one SFA meal (palm-coconut-olive-sunflower oil mix reflecting the typical UK diet (Gregory *et al,* 1990)), one n - 6 PUFA meal (maize oil) and one n - 3 PUFA meal (fish oil). They followed the postprandial response over an 11 hour period, and found that the fish oil meal attenuated the postprandial lipaemic response as demonstrated by a significant reduction in the TAG I- AUC compared with the other two meals. A strong trend towards a reduction in the TRL TAG fraction was also observed in the n - 3 PUFA meal compared to the other two meals. More recently, it has been shown that the addition of even small amounts of fish oil to a standard fat test meal attenuates the postprandial lipaemic response compared with a meal that has been supplemented with olive oil (Yahia and Sanders, 1996).

Mero *et al,* (1998) investigated the postprandial response to three test meals of varying fatty acid composition but with the same quantity of fat (63 g). A mixed meal, a liquid cream meal (SFA) and a soybean oil meal (n - 6 PUFA) were used. They observed no difference in the magnitude and duration of the postprandial response between meals in plasma TAG or apo B-48 concentrations. This finding is in agreement with other studies examining the effects of varying meal fatty acid content on the postprandial TAG response, where no difference has been observed between the response to n-6 PUFA-containing meals and SFA meals when the quantity of fat given has remained constant (Weintraub *et al,* 1988, Zampelas *et al,* 1994).

In conclusion, the main consensus is that *n* - 3 PUFA-containing test meals produce an attenuated lipaemic response when compared to meals consisting predominantly of SFA, n-6 PUFA or MUFA, when the quantity of fat administered remains constant.

1.3.5 (ii) Test meal fat quantity composition.

Recently, Dubois *et al,* examined the effects of graded amounts (0 - 50g) of dietary fat (n - 6 PUFA, sunflower oil) on postprandial lipaemia and lipoproteins in normolipidaemic adults (Dubois *et al,* 1998). Serum and CM TAG-AUC rose significantly from baseline in the postprandial phase and correlated positively with the amount of fat ingested. A no-effect level of dietary fat was observed on postprandial

lipids and lipoproteins with the meals containing 0 g and 15 g fat, as reflected in the low serum and CM TAG-AUC, which did not change significantly from baseline. In an earlier study the same group examined the postprandial lipaemic response to two meals, one containing 31 g fat and the other 42 g fat in the form of sunflower oil (n-6 PUFA). The 42 g fat meal produced a significantly higher TAG AUC response than the lower-fat test meal, again showing that the magnitude of the postprandial response is related to the amount of fat ingested. An earlier study examined the effects of extremely high doses of fat (40 g, 80 g, 120 g) in the form of cream (SFA) on the postprandial response (Cohen *et al,* 1988). They again concluded that the magnitude of lipaemia is directly proportional to the fat content of the meal. Taken together these results show that the ingestion of a meal containing 30 g - 40 g of dietary fat attenuates the postprandial response when compared with higher fat meals. This should be taken into consideration when giving dietary advice, in the light of the fact that postprandial events are the first metabolic responses to the ingestion of dietary lipids and play a key role in the process of atherogenesis.

1.3.5 (iii) Test meal carbohydrate composition

As already explained, the addition of carbohydrate to a test meal alters the nature of the postprandial response (Shishehbor *et al,* 1998), but not necessarily the magnitude. Not all forms of carbohydrate affect the postprandial response in the same fashion. The postprandial TAG response is greater after the addition of sucrose to a test meal where an isocaloric amount of glucose is used in place of sucrose (Mann *et al,* 1971). Grant *et al,* (1994) have also shown that the addition of sucrose to a fat-rich test meal prolongs the duration of the postprandial response. The effects of glucose, sucrose and fructose ingestion on the serum TAG responses to meals containing 40 g fat were also studied in 21 normolipidaemic volunteers (Cohen and Schall, 1988). The glucose / fat meal and the fat - alone test meals produced the lowest postprandial responses, with a significant increase in the magnitude of postprandial lipaemia being observed following the fat / sucrose and the fat / fructose meals. The authors proposed that fructose stimulates the influx of TAG into circulation from endogenous sources. This effect would appear to be transient, as Swanson *et al,* (1992) showed that a background diet providing 20 %

energy as fructose does not cause an overall increase in plasma TAG when compared to an iso-caloric low-fructose diet, except in the first day when a temporary increase in plasma TAG was observed.

Early studies have shown a hypotriacylglycerolaemic effect of fibre-containing test meals in animals (Kritchevsky and Story, 1986), but more recent studies have failed to show this effect in human studies. Dubois *et al,* (1993) examined the effect of adding 10g of soyabean fibre or pea fibre to a 70g fat test meal on the postprandial lipaemic response, and compared results to the response observed following consumption of a similar, but low fibre test meal. They found no significant difference between test meals in TAG AUC concentrations. However, using a 70g fat test meal supplemented with 10g of oat bran, rice bran or wheat fibre, they found that TAG responses were lower after each fibre-containing meal when compared to the low fibre (2.8g dietary fibre) control, and CM TAG were also reduced with wheat fibre (Cara *et al,* 1992). Further work is necessary to elucidate whether similar amounts of fibre would attenuate the postprandial TAG response to lower amounts of fat (40g) normally associated with a Western main meal.

1.3.5 (iv) Test meal protein composition

Dietary protein does not affect the magnitude or duration of the postprandial response as was demonstrated by Cohen, (1989), having fed their volunteers a 40g fat meal with and without 23g casein. They found no difference between meals in postprandial lipaemia or in CM TAG clearance, and concluded that protein ingestion has a neutral effect on the postprandial response.

Studies in the chronic phase - background diet composition

1.3.5 (v) Manipulation of background dietary fatty acid composition

There are a number of studies evaluating the impact of altering the background diet on postprandial lipaemia (Harris and Connor, 1980, Weintraub *et al,* 1988, Harris *et al,* 1988, Demacker *et al,* 1991, Bergeron and Havel 1995, Roche and Gibney, 1996, Roche and Gibney, 1998). Other studies have used supplements to manipulate dietary fatty acid intake. These have been reviewed extensively in this chapter (section 1.2.1 (ii)). One study employed the use of manufactured foods consisting of microencapsulated oils incorporated in to the foods (Lovegrove *et al,* 1997). Two of these studies have examined both the acute and chronic effects of fat saturation on postprandial lipoprotein metabolism (Demacker *et al,* 1991, Weintraub *et al,* 1988), both concluding that the main determinant of lipoprotein levels was the type of fat that was chronically fed. Weintraub *et al,* fed their volunteers isocaloric diets for 25 days, one SFA - diet, one n - 3 PUFA diet and one n - 6 PUFA diet in a crossover design. Compared with the SFA diet, the postprandial response was reduced following the n - 6 PUFA diet and reduced to an even greater extent by the n -3 PUFA diet. They also found a significant effect of the acute dietary fat load, concluding that it was the varying susceptibility to lipolysis of chylomicrons produced by SFA as opposed to PUFA fat loads that accounted for the difference. Demacker *et al,* studied the effects of two diets, one rich in SFA and the other rich in *n* - 6 PUFA on the postprandial processing of exogenous and endogenous TAG for 9 days using a cross - over design. They found that the PUFA-rich diet resulted in 43 % lower daily concentrations of CM's their remnants and a 20 % reduction in VLDL and their remnants.

The overall conclusion that can be taken from these results is that PUFA - rich diets, particularly n -3 PUFA-rich diets, cause a reduction in concentration and residence time of TRL particles and their remnants in the postprandial period. Background dietary fatty acid composition affects the magnitude and duration of the postprandial response to the magnitude n - 3 PUFA < n - 6 PUFA < MUFA < SFA (Williams 1998). Mechanisms underlying the mode of action remain unclear, but are hypothesised to be due to a change in LPL activity, preferential hydrolysis of large-CM-containing-PUFA,

or due to a change in the production and/or secretion of VLDL from the liver, as is discussed in further detail in this chapter (section 1.3.6).

1.3.5 (vi) Comparison of populations with varying habitual dietary fat intake

The aforementioned studies examining the effects of adaptation responses to background diet manipulation have used a wide range of response periods, from 9 days to 16 weeks. For some of these studies it is likely that the manipulation period was too short to reveal the full effect on lipoprotein metabolism which occurs when background dietary fatty acid composition is altered. One study has examined the postprandial response to two standard test meals in populations consuming different diets, one from Northern Europe and one from Southern Europe, both consuming diets habitual to their country of origin (Zampelas *et al,* 1998). The test meals consisted of one SFA meal which resembled the fatty acid composition of a typical UK diet, and a MUFA - rich meal, resembling the typical fatty acid composition of a Mediterranean diet. Volunteers' background diets varied in that the Southern Europeans had a greater total fat content in their diet, a greater MUFA content, but lower PUFA concentrations than their Northern European peers. In both groups there was no significant difference in the postprandial TAG response between meal types, but a significant difference was observed between groups in postprandial TAG, apo B-48, NEFA and insulin concentrations and in factor VII activity. Post heparin LPL activity was also lower in Southern Europeans compared to their Northern counterparts. The authors concluded that the differences in background diet may account for the differences observed in the postprandial handling of lipoproteins and this may account for the varying susceptibility between the two populations for CHD. This is supported by the view that elevated postprandial TAG concentrations in the late postprandial period (6 hours - 10 hours postprandially), as was observed in the Northern Europeans, has been shown to be a significant risk factor for CHD (Groot *et al,* 1991, Patsch *et al,* 1992).

1.3.5 (vii) Dietary carbohydrate intake

Traditionally, high carbohydrate, low fat diets have been prescribed as the first therapeutic mechanism in the treatment of hyperlipidaemia, diabetes and obesity, however they are associated with an increase in TAG concentrations and a reduction in the cardioprotective HDL fraction (Roche, 1999, Gibney, 1992). Populations consuming high carbohydrate diets have higher levels of fasting TAG concentrations (West *et al,* 1990). It has been shown that the increased postprandial triacylglycerolaemia following a high carbohydrate, low fat diet is due to increased VLDL TAG (Melish *et al,* 1980). Fat is the last fuel which is oxidised by humans, with carbohydrate, protein and alcohol preceding it. The body adapts to a low fat diet by suppressing the oxiditation of NEFA, which in turn leads to increased hepatic TAG synthesis and VLDL production, resulting in elevated TAG concentrations (Roche and Gibney, 1995). However, the type of carbohydrate consumed when changing from a high fat to a high carbohydrate diet is also of importance, as Turley *et al,* (1998) have recently shown. Their results displayed that the replacement of saturated fat with carbohydrate from grains, vegetables, legumes and fruit reduces total and LDL cholesterol and has only a minor effect on HDL cholesterol and on plasma TAG. The addition of low doses of fish oil (1 g/d) has also been shown to counteract the hypertriacylglycerolaemic effect of low fat, high carbohydrate diets on fasting lipid concentrations (Roche and Gibney, 1996).

Another group (Dubois *et al,* 1995) examined the effects of chronic oat bran intake on postprandial lipaemia, giving three different experimental sequences to each volunteer:

1. usual low-fibre diet and low-fibre test meal,
2. usual low-fibre diet and oat bran test meal (40g), or
3. 14 days oat bran supplemented diet and oat bran (40g) test meal.

TAG AUC was greatest after the supplemented diet with oat bran test meal, and after the usual low fibre diet with the oat bran test meal than the control diet. This effect was only slight when fasting concentrations were examined but became more remarkable in the postprandial period.

1.3.6 Fish oils and TAG concentrations

The hypotriacylglycerolaemic effect of fish oils has been well reviewed (Roche and Gibney, 2000; Harris, 1997), but the mechanisms involved have until recently remained elusive. The effect must be due to reduced TRL synthesis, increased removal of TRL, or a combination of both. Reduced chylomicron synthesis is unlikely as a study by Harris *et al,* (1997b) showed that in rats, fish oil supplementation had no effect on CM synthesis or secretion. However, fish oil supplementation causes a decrease in rate of secretion of VLDL from the liver (Harris, 1999). Experimental studies have also found that EPA inhibits TAG synthesis/secretion in rat and in rabbit hepatocytes, in HepG2 cells and in perfused rat livers (Lang and Davis, 1990, Rustan *et al,* 1988, Benner *et al,* 1990, Wong *et al,* 1989, Wong *et al,* 1984, Zhang *et al,* 1993). This phenomenon has also been observed in human studies (Harris *et al,* 1990, Phillipson *et al,*, 1985). Lipoprotein lipase (LPL) is the rate-limiting hydrolytic enzyme for TRL. VLDL and chylomicrons compete for the LPL-mediated removal from circulation (Grundy and Mok, 1976), therefore a reduction in VLDL concentrations would give CM particles increased opportunity to interact with LPL, thus increasing their rate of clearance from circulation due to reduced competition for lipolysis. Changes in LPL activity would also affect CM and VLDL clearance rate (Williams, 1998). Post-heparin LPL activity has been shown to be significantly greater after an n-3 PUFA-rich meal than after a saturated-fat-rich test meal (Zampelas *et al,* 1994). When looking at the chronic effect of manipulating background dietary fat intake on LPL activity, rat studies have shown that expression of adipose tissue LPL mRNA is higher following fish oil consumption (Murphy *et al,* 1993). Therefore, it is probable that fish oils exert their hypotriacylglycerolaemic effect in two ways, that is, by reducing the production of hepatic VLDL and also by increasing concentrations of LPL, which effectively increases the rates of removal from circulation of TRL.

1.4 Fish oils and neurological disorders

1.4.1 Introduction

Recent studies have shown that low levels of long chain PUFA seem to be associated with psychiatric disorders including depression (Hibbeln and Salem, 1995, Adams *et al,* 1996, Maes *et al,* 1996, Edwards *et al,* 1998, Hibbeln 1998) and schizophrenia (Mellor *et al,* 1995, Laugharne *et al,* 1996, Horrocks and Yeo, 1999a). Deficiency of EPA and DHA in serum CE (Maes *et al,* 1996), in red blood cell membranes (Edwards *et al,* 1998) has been shown to be characteristic of patients with major depression. Weidner *et al,* (1992) carried out a five-year intervention study in patients suffering from depression in which they were instructed to increase their oily fish consumption. Measures of depression and aggressive hostility were reduced significantly as a result of intervention. Societies consuming large amounts of fish seem to have lower rates of depression, and the prevalence of depression decreases with increasing fish consumption (Hibbeln, 1998). For example, in Japan where fish consumption rates are 67 kg per person, the incidence of major depression is 0.12%, whereas in USA the average consumption per head is a third that of Japan at 22 kg per head, while the incidence of depression is 3.0% (Horrocks and Yeo, 1999a). A high serum n-6: n-3 ratio has been shown in violent impulsive offenders in Finland in comparison with diet-matched controls (Virkkunen *et al,* 1987). A depletion in serum DHA concentrations as a result of chronic alcohol abuse, or as a result of pregnancy due to demands on the mother for developing the foetal nervous system, is reputedly associated with the depression secondary to alcoholism or to post natal depression (Hibbeln and Salem, 1995). Red cell membrane DHA and arachidonic acid compositions have been shown to be depleted in patients with schizophrenia (Laugharne *et al,* 1996). Supplementing a cohort of 20 schizophrenic patients with 10g per day MaxEPA over a six-week period resulted in an improvement in schizophrenic symptoms and a reduction in severity of abnormal involuntary movements (tardive dyskinesia) (Mellor *et al,* 1994). DHA supplementation has also been shown to prevent aggression enhancement at times of mental stress (Hamazaki *et al,* 1996). Evidence for the link between fish oils and AD is emerging and is discussed in further detail below.

1.4.2 Fish oils and Alzheimer's disease - prevailing evidence

1.4.2 (i) Cross-sectional studies

Interest in dietary habits of patients with Alzheimer's disease was first examined about ten years ago (Burns *et al,* 1989, Broe *et al,* 1990) with the investigation of total fat and oily fish intakes in the elderly. Burns *et al,* examined nutritional status and dietary intake in three groups of volunteers; hospitalised patients with senile dementia (n = 21), demented patients living in the community (n = 28) and age-matched controls free from cognitive impairment (n = 29). Dietary data was collected from a randomly selected group from within each of the three main groups, and involved a three-day weighed intake, where each constituent of every food offered was weighed to within ± 1g and any leftovers/waste was also weighed out of sight of the volunteer. Results showed that hospitalised patients were more cognitively impaired than the community-based patients. Both patient groups had higher fat intakes than the control group (78g/d v 59g/d respectively), but this difference did not reach statistical significance. Energy and protein intakes were also higher in patients than in controls. Individual fat classes or specific fatty acid profiles were not examined in this study.

Broe *et al,* (1990) conducted a case-control study of clinically diagnosed Alzheimer's disease in Australia on 170 cases and 170 controls matched for age, sex and, where possible, the general practice of origin. A "Risk Factor Interview" was administered by medically naïve professional interviewers, which included questions on history of starvation, malnutrition and absence of fish in the diet. Abalan (1984) had previously proposed that exposure to starvation is a risk factor for Alzheimer's disease, but this association was not paralleled in this study. Whilst the authors noted that there is a plausible association between absence of fish in the diet and Alzheimer's disease, they did not find any significant relation between the two. No specific dietary analysis was performed in this study, nor was a biomarker of fish intake examined, and as the authors themselves concluded, "a more carefully designed and detailed interview might elicit significantly different responses in cases and controls".

1.4.2 (ii) Prospective studies

Recently, two large prospective population-based studies have further examined the theory that high fish, low saturated fat intakes seem to be protective against dementia, particularly that of Alzheimer's disease. Kalmijn *et al,* (1997a) performed comprehensive dietary analysis and monitored the onset of dementia in a large group of elderly volunteers in Rotterdam. Over 5,000 non-demented participants were screened for dementia using a three-step protocol at baseline and at an average of 2.1 year follow-up. Detailed dietary analysis was performed at baseline using a semi-quantitative food frequency questionnaire (FFQ), with modifications using additional more detailed questions on vegetable, fruit and meat consumption. High intakes of total fat, saturated fat and cholesterol were related to increased risk of dementia. Focusing on fish intake, an inverse relation was seen between fish consumption and risk of dementia, primarily of AD. This inverse association remained strong even with relatively low fish intakes. High total and saturated fat intakes were particularly related to dementia with a vascular component and low fish intakes were particularly associated with dementia of AD. The authors of this paper noted that whilst they discovered a relationship between saturated fat and oily fish intakes and incident dementia, they do recognise that there are some methodological problems associated with their study design. Dietary self-reported data from elderly patients with cognitive impairment are unreliable (van Staveren *et al,* 1994), and was not validated in this study by the use of a biomarker, by performing a weighed intake or by using a surrogate respondent (next-of-kin interview) as has been previously used (Amaducci *et al,* 1986) in similar studies. However the authors feel that it is unlikely that volunteers with cognitive impairment would have consistently over-reported total and saturated fat intakes and under-reported fish intakes, especially when such large numbers were involved. However, results cannot be rendered totally conclusive, particularly as the dietary assessment was only done on one occasion.

Another large prospective study closely examined the link between progression of cognitive impairment and diet, focusing on polyunsaturated fatty acid intake, in 342 men (Kalmijn *et al,* 1997b). This study was part of the large Zutphen Elderly Study, which is a longitudinal study on the risk factors for chronic diseases in men in eastern Holland. This study is a continuation of the Dutch contribution to the Seven Countries Study which was initiated in 1960. Participants were assessed in 1990 for cognitive

impairment using the Mini-Mental State Examination (MMSE) and were followed up three years later to assess cognitive decline again using the same score. Cognitive decline was defined as a drop in more than two points in the MMSE over a 3 year period. Qualitative and quantitative analysis of food intake was assessed using the cross-check dietary history method with additional information on n-3 fatty acid content of food (Hepburn *et al,* 1986). Dietary intake was assessed in 1985 and 1990 and cognitive function was examined in 1990 and 1993. All subjects were interviewed at home, preferably in the presence of a partner to reduce the possibility of under- or over-reporting associated with elderly people with cognitive impairment (van Staveren *et al,* 1994). The reliability and reproducibility of this method was further validated by the fact that the change in dietary intake between 1985 and 1990 was not different in volunteers who were cognitively impaired in 1990 compared with those who showed no cognitive impairment. Dietary and cognitive function data from 1990 were used to assess the association between diet and cognitive function and all sets of data were employed to examine cognitive decline. In agreement with the other studies on dietary intakes and dementia, volunteers who displayed cognitive impairment had higher total fat intakes than those who were free from cognitive impairment. Fish consumption was shown to be inversely associated with both cognitive impairment and decline. EPA and DHA intakes were also lower in cognitively impaired volunteers, although this reduction only reached significance in DHA intakes. Again in this study the authors noted that whilst these results would seem to render fish protective against cognitive decline, modest associations are difficult to detect with dietary data in a relatively small study, particularly as even detailed dietary data would not be precisely indicative of actual intakes. As with the other study reporting the protective effect of oily fish on cognitive function, a biomarker of fatty acid intake was not assessed.

A short report on a study looking at low serum DHA and risk of AD was recently published (Kyle *et al,* 1999). Serum DHA status of 1,188 elderly Americans (mean age 75 years) was assessed using phosphatidylcholine (PC) as a biomarker. Patients were followed up over the following 10 years looking a blinded analysis of the clinical outcomes (AD diagnosis and MMSE score). There was a two-fold higher frequency of those who had AD at baseline in subjects whose PC-DHA was in the lower half of the distribution. Subjects who were free from AD at baseline but who had DHA concentrations in the lower half of the distribution had a 67% greater chance of

developing AD in the subsequent 10 years than those in the upper half of the distribution. Women possessing the apo E4 allele had a four-fold greater risk of having low scores on the MMSE if they were also in the lower half of the PC-DHA distribution. The authors concluded that low levels of circulating PC-DHA was a significant risk factor for low MMSE score, and the development of AD.

1.4.2 (iii) Intervention studies

The hypothesis that an increase in n-3 PUFA in the diet of a patient with AD could improve the disease state of the patient was first published in Australia in 1990 (Peers, 1990). This was a single-case observation of a 77 year old farmer whose restless and destructive behaviour settled following admission to a nursing home where the diet included weekly dishes of oily fish which had been absent from his diet prior to admission. He regained the ability to dress himself and fine motor skills also improved. The author advised that patients with Alzheimer's disease should be checked for excessive intakes of n-6 PUFA intake that is not balanced by adequate supply of n-3 PUFA in the diet, with the obvious treatment being to give fish oil. Over the past 50 years the ratio of n-6: n-3 fatty acids has increased in Europe (Sanders, 2000), so the changes observed in the above patient in Australia and the treatment used could also apply to other patients. A ratio of 4: 1 of n-6: n-3 fatty acids (LA to LNA) has been shown in rats to be the optimal proportion in improving learning tasks (Yehuda *et al,* 1997). Using an oil mixture in this ratio, 60 Alzheimer's disease patients were supplemented with 2ml of this oil and placebo oil was given to 40 patients with the disease (Yehuda *et al,* 1996). At the end of the study period an overall improvement was seen in 49 of the 60 treated patients in mood, co-operation, appetite, sleep, ability to navigate in the home and short-term memory. The remaining 11 in the intervention group showed no change. The placebo group showed no change in 30 supplemented patients, with 5 showing an improvement and 5 showing deterioration.

The most recently published intervention study on the effects of n-3 PUFA supplementation and dementia is a pilot study looking at the effects of DHA on a group of elderly patients (n = 20) with senile dementia of CVD (Terano *et al,* 1999). The patients were divided into two groups according to age, baseline scores on psychometric

tests, and serum fatty acid composition. All were living in a home for the elderly and had mild to moderate dementia of CVD. One group received 6 DHA capsules (0.72g DHA) daily for 12 months, which were administered by nursing staff who also supervised the consumption of the supplements. The other group were registered as controls. After the 12-month period scores of Hasegawa's dementia rating scale (HDS-R) and MMSE improved in the DHA group, but remained unchanged or deteriorated in the control group. Serum DHA and EPA concentrations increased in the supplemented groups while fatty acid concentrations in the placebo group remained unaltered. The authors concluded that further work is necessary to examine the effects of DHA supplementation on other forms of Alzheimer's disease and to clarify the results of the present study by repeating using a double-blind design.

1.4.3 Association between fish oils and Alzheimer's disease

As already discussed, Broe *et al,* (1990) remarked that there is a plausible association between AD and absence of fish in the diet, however in their case-control study they failed to confirm this association. It is probable that the methodology used was inadequate to detect any association. In fact, a recent meta-analysis of epidemiological studies on the incidence of dementia concluded that until more refinement in diagnostic procedures occurred it would not be possible to compare such studies from various parts of the world (Ineichen 1998). Recent studies have shown a positive effect of increased fish consumption on reduction in AD incidence (Kalmijn *et al,* 1997a, Kalmijn *et al,* 1997b). There are a number of plausible reasons for this association owing to the biological properties of n-3 polyunsaturated fatty acids (PUFA), the naturally occurring fatty acids found in fish. Firstly, fish intake may protect against dementia by reducing cardiovascular disease risk. There is a well-established inverse relation between fish consumption and cardiovascular disease risk from epidemiological, case-control, secondary prevention and intervention studies, as has been discussed extensively in this thesis (section 1.2). Cardiovascular disease has been shown to be associated with increased risk of dementia and its major subtypes Alzheimer's disease and vascular dementia (Hofman *et al,* 1997). These results were taken from the large

population-based Rotterdam study, which assessed 284 patients with dementia, 207 of whom had Alzheimer's disease, and 1698 non-demented controls. As the degree of atherosclerosis increased, so did the degree of dementia, particularly that of Alzheimer's disease. This association between atherosclerosis and dementia is particularly strong in those with the apolipoprotein-E ε4 genotype. Individuals who are homozygous for the ε4 allele have a greater risk of developing AD than heterozygous men with the same allele (Hendrie, 1998).

Secondly, immune processes are believed to be involved in the pathogenesis of Alzheimer's disease (Kalmijn *et al,* 1997a). Increased concentrations of acute phase proteins and other markers of immune function such as pro-inflammatory cytokines (interleukin-1 beta (IL-1β)) are characteristic of patients with AD. n-3 PUFA modulate the synthesis of cytokines (Blok *et al,* 1997, Caughey *et al,* 1996, Billiar *et al,* 1988) and therefore could reduce inflammatory processes associated with the disease. A recent study has shown that even low doses of fish oil supplemented over a short period of time (0.6 g/d, 6 weeks) are sufficient to beneficially affect the immune responses of elderly subjects (age > 70 years) (Bechoua *et al,* 1999). Recent research has shown an inverse relationship between taking NSAIDs and the development of AD (Newman, 1998). There is also evidence for a therapeutic effect of these anti-inflammatory drugs in AD (Moller, 1998), so it is possible that the anti-inflammatory properties of fish oils explain their alleged uses in reducing incidence and severity of the disease.

Thirdly, there is strong experimental and clinical evidence indicating that dietary n-3 fatty acids are essential for normal nervous system development in the human (ISSFAL Board Statement, 1995). Neurological differences have been found between 9-year-old children fed breast milk as babies and those fed formula-milk (Lanting *et al,* 1994). It was hypothesised in this study that the neurological differences were due to a disparity between the two feeds. Breast milk contained preformed EPA and DHA which have undergone Δ6-desaturation, in contrast to conventional infant formula milks which contain only ALA, thus requiring the infant to perform its own desaturation process in order to obtain the EPA and DHA which are vital for brain development (Horrobin, 1993). Rhesus monkeys who are deficient in DHA display reduced learning ability, abnormal electroretinograms, impaired vision and polydipsia (Connor *et al,* 1992). Most expert nutrition committees have used the fatty acid composition of breast

milk as a basis for recommendations for infant formulas (Gibson and Makrides, 2000). n-3 PUFA can still be incorporated in the brains of animals after a period of brain development (Neuringer and Connor, 1986), and therefore can be important in nerve cell regeneration (Compston, 1994). Several studies have shown altered fatty acid composition of brains in patients with Alzheimer's disease in comparison with controls. Prasad *et al,* (1998) examined membrane phospholipid fatty acid concentrations in Alzheimer's disease patients and controls and found that DHA concentrations were significantly decreased in both phosphatidylethanolamine (PE) and phosphatidylinositol (PI) fractions of Alzheimer's disease patients. Another study also showed a decrease in DHA in brain PE in Alzheimer's disease patients compared with controls which was not due to ageing, as ageing has been shown to have no influence on fatty acid composition of the brain (Soderberg *et al,* 1991).

1.4.4 Diagnosis of Alzheimer's disease

Diagnosis of AD cannot be confirmed until the characteristic lesions are verified by pathologic examination of the brain, either by autopsy or by the examination of brain biopsy material, which is rarely performed. Therefore, accuracy of clinical diagnosis relies on an informed history from a reliable surrogate, as, by the very nature of the disease, the patient is not a reliable historian. Tests routinely performed include cognitive assessments using a qualitative test e.g. Mini-Mental State Examination (MMSE - Table 1.4.1) (Folstein *et al,* 1983), which is performed on the patient, and a Clinical Dementia Rating (CDR - Table 1.4.2), which is performed on the next-of-kin. The MMSE consists of eight sections out of which the patient can score a maximum of 30 points. These questions examine a range of basic functions including orientation, registration, attention and calculation, recall, language (naming and repeating), reading and writing, three-stage command and construction (copying a drawing). A cut-off point of 26 is generally used to assess absence or presence of cognitive impairment, the lower the score denoting the more severe impairment. The CDR is administered to the next-of-kin and consists of six subject areas including memory, orientation, judgement and problem solving, community affairs, home and hobbies and personal care. Each

topic has five statements from which the one which best describes the patient is selected. An average of the scores allows the patient to be categorised (see Table 1.4.2). Neither of these tests is conclusive in diagnosing AD; a general physical and neurological examination is also performed and a battery of biochemical tests including thyroid function is executed. These investigations, combined with CT scan of the brain, MRI, and a cerebral single photon emission computed tomography (SPECT) scan can be 90 - 95 % accurate in making a diagnosis (Anon, 1999).

Table 1.4.1 Mini-Mental State Examination (MMSE)

Disease category	Score Values (0-30 points)
No/little cognitive impairment	26 - 30
Mild cognitive impairment	19-25
Moderate cognitive impairment	11-18
Severe cognitive impairment	0-10

Table 1.4.2 Clinical Dementia Rating (CDR)

Severity of AD	Score Values (0-3 points)
Mild AD	0.5
Moderate AD	1
Moderate - severe AD	2
Severe AD	3

1.5 Qualitative biomarkers of fat intake

Traditionally, studies of food intake have relied on conventional dietary assessment methods such as retrospective diet histories, 24-hour recall and food frequency questionnaires and prospective food diaries. Whilst all of these are useful in the quantitative assessment of macronutrient intakes, they are less accurate in providing us with precise qualitative information. They are fraught with potential errors of validity and reproducibility, including reactive changes in usual eating habits when under scrutiny, under- and over-reporting and recall memory (van Staveren *et al,* 1994). For these reasons, these methods provide crude indicators of fat quality intake, specifically fatty acid intake.

The overall lack of valid and reproducible methods for assessment of dietary intake, particularly that of individual fatty acids, has promoted interest in the use of objective biochemical variables reflecting diet quality, or 'biomarkers'. The use of these biomarkers has become increasingly prevalent. Reliable biomarkers of dietary intake are frequently used as alternatives to or complimentary to conventional dietary assessment methods (Satia *et al,* 1999, Zock *et al,* 1997, Subbaiah *et al,* 1993, Anttolainen *et al,* 1996, Andersen *et al,* 1996). They are also used to validate these methods (Lemaitre *et al,* 1998). The most commonly used biomarkers of diet fat quality are fatty acid content of serum TAG, phospholipids, CE, of erythrocytes and platelets, and of adipose tissue. These have all been extensively validated to reflect dietary fat intake. Fatty acid composition of human serum TAG reflects dietary fatty acid composition of the last few meals, and therefore is of use in short-term acute studies. CE, phospholipid and erythrocyte membrane fatty acid composition reflect the dietary intake of the preceding weeks or months, whereas that of human subcutaneous adipose tissue is a valid index of the fatty acid composition of the habitual diet over the past 2-3 years (Glatz *et al,* 1989). Study design and population type are two variables that determine which biomarker of fat intake is most appropriate to use. Serum TAG have a short half life and their composition reflects recent meal fatty acid composition, and for this reason are useful specifically in acute studies where the postprandial handling of fat quality intake is paramount. Owing to the longer half-lives of serum CE, phospholipid or erythrocyte membranes compared with serum TAG, fatty acid compositions of these fractions more

accurately reflect habitual dietary fat intake. Specifically, CE fatty acid composition can be precisely measured and are frequently used to reflect dietary intake patterns (Simon *et al,* 1995), particularly in the elderly. Table 1.6.2 summarises CE EPA and DHA concentrations from recently-published work. A strong, significant correlation has been shown between the fatty acid composition of serum CE in a large group of men (*n* 855) taken at 50 years and 70 years old (Ohrvall *et al,* 1996), which indicating that men at this age have well-formed dietary fat quality intake habits, or at least keep to their ranking in their group over a 20 year period. CE fatty acid analysis therefore reflects habitual dietary fat quality intake over a long period of time, particularly in an elderly population. Phospholipids are essential constituents of cell membranes and are more resistant to compositional changes than stored triacylglycerols. For this reason, phospholipid fatty acid composition is a more appropriate and accurate method for measuring the change of fat quality in the diet in a chronic intervention study where the manipulation of fat quality is being assessed, and has been used in many such circumstances (Roche and Gibney, 1994, Prisco *et al,* 1996, Lands, 1995). Table 1.6.1 reviews work from other authors using phospholipid fatty acid concentrations as biomarkers of fat quality intake. The collection of human subcutaneous adipose tissue samples is intrusive and invasive and requires specialised training and equipment and therefore is frequently unfeasible, particularly in an elderly population. The measurement of CE, phospholipid or erythrocyte membrane fatty acid composition has many practical advantages over adipose tissue sampling and for these reasons are more frequently used and are more appropriate in prospective intervention studies. Adipose tissue fatty acid composition is therefore best used in retrospective dietary analysis surveys, particularly in non-elderly populations.

EPA and DHA differ not only in the rates of incorporation into various lipids but also in the magnitude of incorporation. One study has shown that EPA is incorporated more quickly into phospholipids and CE than DHA (Subbaiah *et al,* 1993). However the supplement used in that study had a greater amount of EPA than DHA (3.48g EPA v 2.28g DHA), which could somewhat account for this difference. The magnitude of incorporation of EPA and DHA into lipid classes is not consistent across the classes. EPA is distributed equally between phospholipid and CE classes (Vidgren *et al,* 1997) whereas DHA is poorly incorporated into CE (Subbaiah *et al,* 1993, Rapp *et al,* 1991, Holub *et al,* 1987). This would indicate that DHA is a poor substrate for lecithin-

cholesterol acyl transferase (LCAT), as most CE EPA and DHA are derived from the action of this enzyme on plasma phosphatidylcholine (Subbaiah *et al,* 1993). It has been surmised that the preferential incorporation of particular fatty acids into plasma lipid fractions may be reflective of their biological effects. For example DHA may be a more powerful anti-atherosclerotic agent than EPA, as DHA-rich oils more effectively increase HDL_2 and decrease LDL: HDL ratio than EPA-rich oils (Sanders *et al,* 1989, Childs *et al,* 1990). CE is the major lipid of LDL (47% of the total LDL lipid) and phospholipid and CE are the major lipids of HDL (contributing 47% and 31% respectively of the total HDL lipid) (Chapman, 1986). n-3 PUFA may be preferentially incorporated into phospholipids as they are the preferred source of substrates for eicosanoid production, producing weakly inflammatory eicosanoids as opposed to strongly inflammatory eicosanoids which are produced from arachidonic acid (Gurr 1999), which may explain the difference in incorporation rates of DHA into phospholipids and CE.

Table 1.6.1a Review of platelet phospholipid concentrations (w/w%) in n-3 PUFA supplementation studies.

Author	Dose	Study period	EPA pre-trial	EPA post-trial	DHA pre-trial	DHA post-trial
Roche and Gibney, 1994	low fat spread (8% fish oil)	12 weeks	0.8 ± 0.3	1.5 ± 0.5	2.3 ± 0.5	2.9 ± 0.9
	butter (8% fish oil)	12 weeks	0.6 ± 0.4	2.4 ± 1.3	2.3 ± 0.6	3.1 ± 0.9
	usual spread	12 weeks	0.9 ± 0.2	1.0 ± 0.3	2.2 ± 0.2	2.4 ± 0.5
Li and Steiner 1991	3g/d EPA	21 days	0.3 ± 0.1	4.8 ± 0.6	0.2 ± 0.1	4.6 ± 0.6
	6g/d EPA	21 days	0.3 ± 0.2	5.9 ± 0.9	0.3 ± 0.2	4.9 ± 0.6
Schmidt *et al*, 1992	4g n-3 PUFA	6 weeks	0.8 ± 0.2	2.9 (range 2.4-3.3)	2.1 (range 2.0-2.4)	2.4 (range 2.2-2.9)
	4g n-3 PUFA	9 months	0.8 ± 0.2	3.8 (range 3.1-4.3)	2.1 (range 2.0-2.4)	2.8 (range 2.3-3.3)
Gibney & Hunter 1993	15g/d n-3 PUFA	2 weeks	0.7 ± 0.1	2.6 ± 0.5	3.1 ± 0.5	2.5 ± 0.2
	15g/d n-3 PUFA	12 weeks	0.7 ± 0.1	2.4 ± 1.2	3.1 ± 0.5	2.9 ± 0.5

Table 1.6.2 Review of cholesteryl ester concentrations (w/w%).

Author	Study description		EPA (w/w%)	DHA (w/w%)
Cambien *et al*, 1988	Prospective study, 3,348 middle-aged men		0.8 ± 0.4	0.5 ± 0.2
Maes *et al*, 1996	prospective study,			
	36 major depressed,		0.37 ± 0.22	0.37 ± 0.18
	14 minor depressed,		0.50 ± 0.23	0.53 ± 0.30
	24 healthy controls		0.53 ± 0.36	0.35 ± 0.13
Sarkkinen *et al*, 1994	intervention study			
	saturated fat diet (n 37),	0 months	1.64 ± 0.9	0.80 ± 0.26
		6 months	1.81 ± 0.9	0.79 ± 0.24
	low-fat diet (n 40),	0 months	1.78 ± 0.79	0.92 ± 0.33
		6 months	2.24 ± 0.99	0.97 ± 0.33
Subbaiah *et al*, 1993	6 g/d n-3 PUFA,	0 days	0.2 ± 0.2	0.5 ± 0.3
		28 days	0.4 ± 0.2	2.3 ± 0.8

1.6 Functional Foods

More than 2,500 years ago, Hippocrates advised, "Let food be thy medicine and medicine thy food". This philosophy is still relevant in today's modern age in an era where food technologists are adding nutrients to foods to a level which wouldn't naturally occur, to increase our intake of these particular nutrients. One such instance is the development of foods with added n-3 PUFA. Current Irish recommendations for n-3 PUFA intake are based on EU Population Reference Intake (PRI) recommendations, or 0.5 % energy (Food Safety Authority of Ireland (FSAI) 1999). It is estimated that our current intakes are about 0.14% en (Gibney, 1997), or just over a quarter of what is recommended. Attaining the proposed intakes would require a large increase in oily fish consumption. Even these estimates of fish consumption presented here are extremely generous as they are based on the assumption that all fish eaten is oily. However, most fish eaten in the UK is white fish (BNF, 1999). A survey of 1,400 Irish adults revealed that two-thirds "do not intend to increase their fish consumption over the next 3 years" (Kearney, 1994). Alternative strategies to achieving recommended intakes, without radical changes in eating habits, include food enrichment of frequent and commonly consumed food products and/or the use of biotechnology to manipulate EPA and DHA as well as ALA levels in the food supply. The enrichment of foods with n-3 PUFA or the development of "functional foods" is a focal point of intense scientific scrutiny as it is a potentially effective and viable way of increasing n-3 PUFA intakes.

Infant nutrition is already advanced in this field, as the importance of n-3 PUFA with respect to neural and visual development in the foetus and infant has lead to the fortification of infant formulae with these fatty acids in 40 countries world wide (Haumann, 1998). The development of wide range of acceptable, palatable and commonly eaten foods fortified to a significant level is not straightforward. Problems associated with the enrichment of foods with n-3 PUFA include their susceptibility to oxidation and their unpleasant fishy taste and odour, which can be difficult to mask. A recent study found that food product palatability was affected very differently by enrichment with n-3 fatty acids (Kolanowski *et al,* 1999). In that study, powder milk-based formulae concentrates and products of high sweetness and flavours intensity were best able to mask the unpleasant odour and were fortified up to a level of 18% which

provided 1.8% EPA and DHA. Enrichment of low-flavoured intensity products like milk strongly decreased its palatability. Conversely, other authors have recently shown that it is technologically possible to fortify common foods at a significant level (bread, biscuits, cakes, ice-cream, orange juice, milk shake, spreads, pasta, mayonnaise and vinaigrette) with long-chain PUFA such that they are indistinguishable from control non-fortified foods, and lead to a doubling of platelet phospholipid n-3 PUFA in just 21 days (Lovegrove *et al,* 1997).

Fatty acid composition of animal products is affected by the quality of the animals diet (Simopoulos, 1999), therefore by altering this we can alter the fatty acid composition of the final product. "Edison eggs" and "Einstein milk" are now being produced in Korea through altering the feed supplement of animals (Horrocks & Yeo, 1999b). The "Einstein milk" contains 0.2% each of EPA and DHA, and the "Edison eggs" contain at least 300 mg DHA per 100g of egg. Research has shown that consuming three Edison eggs a day for 4 weeks lowers cholesterol and reduces platelet aggregation values when compared to consuming three generic eggs in young (20 year old) women (Horrocks & Yeo 1999b). Consumption of Einstein milk has also been shown to reduce platelet aggregation values when compared with consumption of generic milk, and it also leads to an increase in plasma DHA content (Horrocks & Yeo 1999b).

From the 1960's, attempts have been made to manipulate our diets due to the unmitigated recommendation to lower our saturated fat intake in favour of vegetable oils high in n-6 PUFA (Simopoulos, 1999). n-3 PUFA have diminished in our food chain with the frequent use of vegetable oils used in both the food industry and by ourselves, such as corn oil, safflower oil, sunflower oil and cottonseed oil, which are very low in n-3 PUFA. Also, grain-fed animals have much lower n-3 PUFA in their carcasses than animals in the wild (Crawford, 1968). Farmed fish, poultry and eggs also follow this trend (Simopoulos, 1999). The Seven Countries Study highlighted the effect that changing dietary fatty acid ratio can have on chronic disease. The people of Crete had an n-6: n-3 ratio of just over 1 compared with that of countries in Western Europe where the ratio is 10-20:1 (Simopoulos, 1998). They also had lower rates of cancer, heart disease and increased longevity than other European countries, Japan and America. n-3 fatty acids maintain their preventative and therapeutic properties when packaged in a food other than fish (Simopoulos, 1999). It is now believed that 'the time has come to

return the n-3 fatty acids into the food supply' (Simopoulos, 1999). The way in which to do this seems to be the manufacture of functional commonly-eaten foods rich in these fatty acids, or at least containing a finer balance of n-6: n-3 fatty acids. The level to which a food can or should be fortified remains elusive as the minimal effective dose of n-3 PUFA is not clear. Investigations in this thesis attempt to clarify this dilemma, which then allows the technology and quality consortium of the 'Nutrifish' project to further probe into the development of a range of palatable, commonly-eaten foods with an acceptable shelf-life which are fortified with an effective amount of n-3 PUFA.

Chapter Two

Materials and Methods

2.1 Collection and storage of samples

Collection and storage of blood samples were followed according to the recommendations of Mills *et al* (1984). The blood was drawn into evacuated lithium heparin tubes for plasma lipid investigations, into evacuated tubes containing 0.105M sodium citrate (v/v : 1:10) to investigate platelet phospholipid fatty acid composition, into EDTA (K_2) tubes for full blood count (FBC) investigation and into serum tubes containing no preservative for cholesteryl ester fatty acid composition. Plasma rather than serum was used for lipid and lipoprotein determination, minimising the enzymatic degradation that can occur while the clot is forming.

Lithium heparin tubes were centrifuged immediately at 2500 rpm to avoid the spontaneous hydrolysis of TAG that can occur if blood samples are left at room temperature for too long. Plasma was subsequently taken off, vortexed, aliquoted and stored at -20^{0}C until required for analysis, with the exception of plasma used for HDL and TRL isolation which was stored at 2^0 - 5^{0}C at this point. Whole blood from EDTA (K_2) tubes was used immediately for FBC. Platelets were isolated from citrated tubes as described below in section 2.3. Serum tubes were centrifuged at 2000 rpm for 15 minutes. Serum was subsequently taken off, vortexed, aliquoted and stored at -20^{0}C until required for analysis.

2.2 Triacylglycerol - Rich Lipoprotein (TRL) separation

Plasma TRL were separated using the method described by Grundy and Mok (1976) with some modifications. Two 3.2ml polycarbonate centrifugation tubes (Beckman instruments, USA) were required for each sample. In each tube 1.6 ml plasma was overlaid with the same volume of saline solution (d = 1.006 g/ml). The saline solution was prepared by dissolving 9.6 g sodium chloride in 1L distilled water and its density

was verified using a hydrometer. Samples were ultracentrifuged at 100,000 rpm (Optima ™ TLX Ultracentrifuge, Beckman USA) for 22 minutes to separate TRL. Approximately 0.6 ml of the supernatant was aspirated from each tube using a fine - tipped glass pasteur pipette and duplicate samples were pooled, vortexed and aliquoted. Duplicate infranatant samples were also pooled, vortexed and aliquoted. Both fractions were immediately frozen for subsequent cholesterol, TAG and fatty acid composition analysis.

TAG and cholesterol concentrations of TRL and of TPL were determined using the methods described in sections 2.5 and 2.6. Actual concentrations were adjusted according to the dilution factor.

Dilution factor for TRL:

Concentration TRL mmol/l = (Volume TRL taken off / volume plasma used) * concentration TRL mmol

Dilution factor for TPL:

Concentration TPL mmol/l = (Volume TPL taken off / volume plasma used) * concentration TRL mmol

To assure accuracy, TAG and cholesterol concentrations of all samples of TRL and TPL fractions were determined and their sum was compared with the total plasma TAG and cholesterol concentrations, respectively. TAG samples showed a mean difference of 0.089 mmol/l (SD 0.216) and cholesterol samples showed a mean difference of -0.004 mmol/l (SD 0.672).

2.3 Platelet phospholipid, cholesteryl ester and TRL fatty acid composition

2.3.1 Isolation of plasma and platelets

Four 4.5ml siliconised vacutainers containing 0.105M sodium citrate (v/v: 1:10) filled with venous blood were immediately centrifuged at 1100 rpm (140g) for 10 minutes. Platelet rich plasma (PRP) was removed using a transfer pipette into a 15ml conical

polypropylene centrifuge tube and centrifuged at 5,000 rpm (670g) for 15 minutes to separate out the platelet pellet from the platelet poor plasma (PPP). The PPP was divided into 0.5ml aliquots and immediately frozen at -20^{0}C. The platelet pellet was washed three times in 2mls of Tris:EDTA (ph 7.5), using a plastic pasteur pipette in the 15ml conical polypropylene tube. The Tris:EDTA/pellet suspension was sedimented each time after washing by centrifugation at 5,000 rpm (670 g) for 15 minutes and the supernatant discarded after each centrifugation. After the three washes, the pellet was re-suspended in 1ml Tris-EDTA buffer, pH 7.5 and extracted immediately.

2.3.2 Lipid Extraction

The method of lipid extraction used for both the platelet pellet suspended in 1 ml of Tris-EDTA buffer (pH 7.5) and for 400μl TRL or 400μl serum was similar (Folch Method, 1957). The platelet pellet suspension, TRL or serum was transferred into a 16 x 100 mm borosilicate glass tube, to which 2.5 ml chloroform: methanol (2: 1) with 0.01% (w/v) butylated hydroxy toluene (BHT) was added. The mixture was vortexed for 60 seconds and centrifuged for 10 minutes at 2,500 rpm to separate the aqueous and organic phases. The organic phase (infranatent) was removed and added to a 12 x 75 mm borosilicate glass test tube. 2 ml chloroform with 0.01% BHT was added to the remaining sample in the 16 x 100 mm borosilicate test tube, vortexed for 30 seconds and centrifuged (2,500 rpm, 5 minutes) to again separate the aqueous from the organic phase. The infranatent was pooled with the first extract and evaporated to dryness in a vortex evaporator (AGB Scientific Ltd., Dublin, Ireland). When completely dried, each tube containing the dried lipid sample was flushed with nitrogen and stored at -20^{0}C.

2.3.3 Isolation of phospholipids & cholesteryl esters

Thin layer chromatography (TLC) was used to extract total phospholipid and cholesteryl ester fractions from platelets and serum, respectively. The solvent system, which was

used for both fractions, was as follows (Gibney and Bolton Smith, 1988):

- 80 Petroleum ether (v/v) (bp 40 - 80)
- 20 Diethyl ether (v/v)
- 2 Formic acid (v/v)

The TLC tank was lined with two sheets of filter paper, the mixed solvent system mixture was poured into the tank and allowed to equilibrate for a minimum of 1 hour before use. A fresh system was made up daily. Silica 60 LKD 19 Lane TLC plates (Whatman, 9 Bridewell Pl., Clifton, New Jersey, U.S.A.) were activated by heating in an oven at 120^0C for one hour. Lipid extracts were reconstituted in 30μl chloroform, vortexed and added in aliquots of 5μl to the origin of the lane on the TLC plate. A hairdryer was used to dry the area after each application. To ensure maximal lipid recovery, a further 30μl chloroform was added to each tube, vortexed and applied to the TLC plate as described above. A phospholipid standard (5μl), phosphatidyl choline (Sigma P2772) and a cholesteryl ester standard, cholesteryl oleate (10μl) (Sigma C9253) were applied to the first lane of the TLC plate as markers.

The TLC plate was carefully placed in the TLC tank and allowed to run for 35 minutes. It was then removed from the tank and allowed to dry in the fume cupboard for 30 minutes. To identify the separated lipid fractions, the plate was liberally sprayed with 0.1% (w/v) solution of 2,7 dichlorofluorosein in 95% methanol and examined under an ultra-violet lamp (Hanori Lamps, Slough, U.K.). Phospholipids remained at the origin of the neutral solvent system, which was confirmed by comparison with the phospholipid standard, and cholesteryl esters gravitated close to the top of the TLC plate, as was again confirmed by comparison with the CE standard.

The corresponding silica was carefully scraped off the TLC plate and transferred to a 16 x 100 mm borosilicate test tube, to which 3 ml chloroform: methanol (2: 1) was added and vortexed for 60 seconds to elute the phospholipids or cholesteryl esters. Each tube was centrifuged at 2,000 rpm for 5 minutes and the supernatant removed using a glass pasteur pipette which was transferred to a 12 x 75 mm glass borosilicate

test tube. This procedure was repeated once to ensure near maximal phospholipid or CE recovery. Both supernatants were pooled and evaporated to dryness in the vortex evaporator as before. The dried samples were then flushed with nitrogen and stored at -20^{0}C.

2.3.4 Generation of methyl esters of fatty acids for gas liquid chromatography (GLC).

Platelet phospholipids, TRL lipids and CE were transesterified to allow identification of fatty acids by Gas Liquid Chromatography (GLC).

Cholesteryl esters only:

To hydrolyse CE, 2 ml of 0.5M NaOH in methanol was added to each dried sample and was vortexed for 30s. A heating block was pre-heated to 80 ^{0}C and each tube was sealed with a small marble and incubated in this block for 30 minutes.

Platelet phospholipids, TRL fatty acids and cholesteryl esters:

Boron Trifluoride Methanol (0.5 ml of 14% BF_3, Sigma B1127) was added to each dried sample (Platelet phospholipids, TRL fatty acids) and to the hydrolysed CE in 0.5M NaOH/L in methanol mixture, and was vortexed for 30 seconds. A heating block was pre-heated to 75^{0}C, each tube was sealed with a small marble and incubated in this block for 30 minutes. After this time 0.5 ml hexane was added to each tube and vortex mixed for 40 seconds. The supernatant was then removed using a glass pasteur pipette and transferred to a 12 x 75 mm borosilicate glass test tube. This extraction was repeated twice to ensure maximal extraction, pooling supernatants with the first extraction. These were then evaporated in the vortex evaporator, flushed with nitrogen and stored at -20^{0}C until subsequent identification by Gas Liquid Chromatography (GLC).

2.3.5 Determination of fatty acid composition of platelet phospholipids, TRL or cholesteryl esters by GLC

Gas liquid chromatography was used to identify fatty acid methyl esters of TRL and platelet phospholipids using a Shimadzu GC-14A Series Gas Chromatograph (Mason Technologies, Dublin, Ireland). The gas chromatograph (GC) was fitted with a BP 21 polar aluminium coated silica column (Scientific Glass Engineering Pty. Ltd., 1 Potters Lane, Kiln Farm, Milton Keynes, MK11 3LA, U.K.). The split ratio was set at 1/60 and the analytical conditions were as follows:

Make-up gas:	Oxygen free nitrogen	1.0 kg/cm^3
Column gas:	Oxygen free nitrogen	1.25 kg/cm^3
	Hydrogen	0.6 kg/cm^3
	Dry air	0.5 kg/cm^3

Temperature programme:	Column initial temperature	120^0C
	4 ^0C/min for 20 minutes to 200^0C	
	Hold at 200 ^0C for 12 minutes	
	4 ^0C/min for 5 minutes to 220^0C	
	Hold at 220^0C for 12 minutes	
	Injection temperature	240^0C
	Detection temperature	280^0C

A flame ionisation detector identified the fatty acid methyl esters, they were recognised by their retention times and identified using known Sigma standards. Peak areas were calculated using a Shimadzu C-R6A chromatograph integrator (Mason Technologies, Dublin, Ireland).

On a daily basis prior to running samples, two hexane washes (1.5 µl) were injected in to the column and run using the conditions described above as a means of checking the baseline. Next, 1.5µl standard was injected as a means of confirming the retention times and identification of fatty acids in the samples.

Platelet phospholipid samples were reconstituted in 100 µl hexane and 6 µl was injected onto the column while TRL and CE fatty acid samples were reconstituted in 80 µl hexane, with 8 µl injected onto the column for TRL and 6.5 µl being injected onto the column for CE samples.

2.4 Separation of plasma HDL, HDL_2 and HDL_3

Materials

Quantolip Reagent A (Immuno AG reference no. 8252015)
Quantolip Reagent B (Immuno AG reference no. 8225016)
Immunolip control serum (IMMUNO art. no. 8220005)
Lyotrol N control serum (bioMerieux, reference no. 62 373)

Plasma total HDL and HDL_3 were separated using the Quantolip HDL (HDL_2/HDL_3) Cholesterol Precipitation kit (Allphar Services Ltd., Dublin, Ireland). The kit consists of two reagents, Reagent A and Reagent B, which contain polyethylene glycol (PEG) of different concentration and pH, dissolved in 0.1 mol phosphate buffer.

Reagent A: 9.5% (w/v) PEG 20.000 in 0.1 mol/l Na-Phosphate buffer, pH 6.5
Reagent B: 15% (w/v) PEG 20.000 in 0.1 mol/l Na-Phosphate buffer, pH 7.5

The addition of these PEG solutions of different concentrations and pH results in the selective precipitation of the different lipoprotein fractions; Reagent A precipitating the VLDL and LDL fractions, leaving HDL in the supernatant while Reagent B precipitates along with the VLDL and LDL, also the HDL_2 subclass leaving only the HDL_3 subfraction in the supernatant. 200 ml Reagent A was added to 100 ml plasma and 200 ml Reagent B was added to another 100 ml plasma. Both samples were vortexed and incubated at room temperature for 10 minutes. They were then centrifuged at 3000 rpm for 15 minutes and the supernatants were removed, vortexed

and frozen for subsequent cholesterol determination using the RA-XT Autoanalyser (Bayer Diagnostics, Dublin, Ireland).

Calculation of HDL cholesterol

HDL cholesterol =

(Absorption of sample + Absorption of standard) * 1.29 mmol/l † * 3‡

† Cholesterol concentration of Preciset standard no.1 (Boehringer Mannheim)

‡ Plasma was diluted 1 in 3 with the precipitation solution, therefore must multiply by a factor of 3 to get the actual concentration.

Calculation of HDL_3 cholesterol

HDL_3 cholesterol =

(Absorption of sample + Absorption of standard) * 1.29 mmol/l † * 3 ‡

† Cholesterol concentration of Preciset standard no.1 (Boehringer Mannheim)

‡ Plasma was diluted 1 in 3 with the precipitation solution, therefore must multiply by a factor of 3 to get the actual concentration.

Calculation of HDL_2 cholesterol

HDL_2 cholesterol = total HDL cholesterol - HDL_3 cholesterol

2.5 Measurement of TAG concentration

Materials

Triglycerides Enzymatique PAP 1000 (bioMerieux, reference no. 61 238)
Lyotrol N control serum (bioMerieux, reference no. 62 373)
Lipid - Unitrol control serum (bioMerieux, reference no. 62 211)
Calimat calibration serum (bioMerieux, reference no. 62 321)

Analysis of plasma, TRL and TAG-poor lipoprotein (TPL) concentrations was determined using enzymatic colourimetric assays (Biomerieux, 69280 Marcy-l'Etoile, France) on a RA-XT Autoanalyser (Bayer Diagnostics, Dublin).

The Triglycerides Enzymatique PAP 1000 consists of the following reagents:

Reagent 1 buffer	tris buffer pH 7.6 parachlorophenol magnesium	100 mmol/l 2.7 mmol/l 4 mmol/l
Reagent 2 enzymes	aminoantipyrine lipase glycerolkinase glycerol-3-phosphate oxidase peroxidase ATP	0.4 mmol/l = 1,000 U/l = 200 U/l = 2000 U/l = 200 U/l 0.8 mmol/l

TAG present in the sample is determined according to the following reactions: TAG is converted to glycerol and fatty acids by the action of lipase. ATP in combination with this glycerol in the presence of glycerokinase forms glycerol 3 phosphate and ADP. Glycerol 3 phosphate oxidase then acts on this glycerol 3 phosphate, forming

dihydroxyacetone phosphate and H_2O_2. Parachlorophenol reacts with 2 H_2O_2 and amino-4-antipyrine, in the presence of peroxidase to form quinoneimine, a colour product. TAG concentration can be calculated from the absorption value read at a wavelength of 505 nm.

2.6 Measurement of cholesterol concentration

Materials

Cholesterol Enzymatique PAP 1200 (bioMerieux, reference no. 61 226)
Lyotrol N control serum (bioMerieux, reference no. 62 373)
Lipid - Unitrol control serum (bioMerieux, reference no. 62 211)
Calimat calibration serum (bioMerieux, reference no. 62 321)

Analysis of plasma, TRL, TPL and HDL cholesterol concentrations was determined using enzymatic colourimetric assays (Biomerieux, 69280 Marcy-l'Etoile, France) on a RA-XT Autoanalyser (Bayer Diagnostics, Dublin).

The Cholesterol Enzymatique PAP 1200 consists of the following reagents:

Reagent	Component	Concentration
Reagent 1	phosphate buffer	0.1 mol/l
buffer	phenol	15 mmol/l
	sodium cholate	3.74 mmol/l
	surfactant	
Reagent 2	4-aminoantipyrine	0.5 mmol/l
enzymes	peroxidase	= 1,000 U/l
	cholesterol oxidase	= 200 U/l
	cholesterol esterase	= 125 U/l

Cholesterol present in the sample is determined according to the following reactions : cholesterol ester is converted to cholesterol and fatty acids by the action of cholesterol esterase. In the presence of cholesterol oxidase, cholesterol is converted into cholest-4-en-3-one and H_2O_2. This H_2O_2, in combination with phenol and 4-amino antipyrine, in the presence of peroxidase, forms $4H_2O$ and quinoneimine, a colour product. Cholesterol concentration can be calculated from the absorption value read at a wavelength of 500 nm.

2.7 Measurement of NEFA concentration

Analysis of plasma NEFA concentration was determined using an enzymatic colourimetric assay (Randox Laboratories Ltd., N. Ireland) on a RA-XT Autoanalyser (Bayer Diagnostics, Dublin).

Materials NEFA kit (Randox Laboratories Ltd., Ardmore, Diamond Road, Crumlin, Co Antrim) (Table 2.7.1)

Wako Control Serum II (Wako Chemicals GmbH, Germany)

Table 2.7.1 **Reagent Content of NEFA Kit**

Contents	Initial Concentration of Solutions
1. Buffer	
Phosphate buffer	0.04 mmol/l, pH 6.9
Magnesium chloride	3 mmol/l
Surfactant	
2. Enzyme/Coenzymes	
Acyl Coenzyme A Synthetase	= 0.3 U/ml
Ascorbate oxidase	= 1.5 U/ml
Coenzyme A	0.9 mmol/l
ATP	5.0 mmol/l
4-aminoantipyrine (4-AAP)	1.5 mmol/l
3. Enzyme Diluent	
Phenoxyethanol	0.3 % (w/v)
Surfactant	
3a. Maleimide	10.6 mmol/l
4. Enzyme Reagent	
Acyl Coenzyme A Oxidase	= 10 U/ml
Peroxidase	7.5 U/ml
TOOS*	1.2 mmol/l
5. Standard	1 mmol/l

*N-ethyl-N-(2 hydroxy - 3 - sulphopropyl) m-toluidine)

NEFA present in the sample is determined according to the following reactions:

NEFA in the sample, along with ATP and Coenzyme A are converted to Acyl Coenzyme A and AMP and PPi by the action of Acyl Coenzyme A Synthetase. This Acyl Coenzyme A reacts with O_2 in the presence of Acyl Co enzyme A Oxidase to form 2,3,-trans-Enoly-CoA and H_2O_2. The H_2O_2 reacts with TOOS and 4-AAP, in the presence of Peroxidase forming a purple adduct and H_2O. The absorbance of the sample is read against the reagent blank at 550 nm.

2.8 Measurement of glucose concentration

Materials

Glucose Enzymatique PAP 1200 (bioMerieux, reference no. 61 273)
Lyotrol N control serum (bioMerieux, reference no. 62 373)
Calimat calibration serum (bioMerieux, reference no. 62 321)

Analysis of plasma glucose concentrations was determined using enzymatic colourimetric assays (Biomerieux, 69280 Marcy-l'Etoile, France) on a RA-XT Autoanalyser (Bayer Diagnostics, Dublin). Table 2.8.1 shows the reagent content of the Glucose Enzymatique PAP.

Table 2.8.1 Reagent Content of Glucose Enzymatique PAP 1200

Reagent 1 buffer	phosphate buffer phenol	150 mmol/l 10 mmol/l
Reagent 2 enzymes	amino-antipyrine peroxidase glucose oxidase	0.4 mmol/l = 300 U/l = 15,000 U/l

Glucose present in the sample is determined according to the following reactions: glucose is converted to gluconic acid and H_2O_2 by the action of glucose oxidase. This H_2O_2, in combination with phenol and 4-amino antipyrine, in the presence of peroxidase, forms $4H_2O$ and quinoneimine, a colour product. Glucose concentration can be calculated from the absorption value read at a wavelength of 505 nm.

2.9 Measurement of insulin concentration

A Phadeseph™ Insulin RIA kit (Pharmacia Diagnostics AB, Uppsala, Sweden) was used to determine the concentration of plasma insulin. This is a radioimmunoassay based on a double antibody solid phase technique was used to measure plasma insulin concentration. The principle behind the assay is that insulin in an unknown sample is allowed to compete with a fixed amount of an ^{125}I labelled insulin for binding sites on the highly specific antibodies. The concentration of insulin is then determined by comparing its competitive capacity to that of insulin standards of known concentration.

100μl of plasma, standards and controls (low, medium and high) were incubated with 100μl Insulin-^{125}I solution and 100μl anti-insulin solution at room temperature for 2 hours. Adding a double antibody suspension, followed by incubation at room temperature for a further 30 minutes, separated bound and free insulin. The tubes were then centrifuged at 1500xg for 15 minutes and immediately decanted. The radioactivity of the solid phase pellet was measured using a gamma counter (Automatic Gamma Counter 1470, WIZARD, Wallac). The amount of bound radioactivity is inversely proportional to the amount of insulin present in the sample.

The standard curve was constructed using a computer attached to the gamma counter (Multicalc, Immunoassay Management Porgram, Pharmacia, Wallac oy, Finland) and plasma insulin concentrations were calculated from this. Quality control was assured using low, medium and high control samples (Ligand 1, 2, 3 CIBA-CORNING, Ciba Corning Diagnostics Corp., Medfield, MA USA).

2.10 Apo-B48 preservative preparation

This highly toxic preservative was prepared freshly each week. The components as shown in Table 2.10 were placed in a beaker and 20 ml distilled waster was added whilst stirring, 3MNaOH was added drop wise to adjust the pH to 7.4 until the solid dissolved. This solution was then made up to 25 ml with distilled water. The preservative was added to the appropriate tubes before the addition of plasma in order to have a final concentration of 5% (v/v).

Table 2.10 Materials for Apo B-48 preservative

Stock Solutions	Final concentration in sample	Amount for 25 ml of preservative
NaCl (0.3 M, pH 7.4)	0.15 M	4.4 g
EDTA (0.2 M, pH 7.4)	1.2 g/l	0.6 g
Chloramphenicol (200 mg/ml)	80.0 mg/l	40.0 mg
Sodium azide (2.5%)	0.1 g/l	0.05 g
Gentamicin Sulphate (10mg/ml)	80.0 mg/l	40.0 mg
Kallikrien inactivator	10,000 U/l	5,000 U
(Aprotinin - 4 TIU/mg)		(1.375 mg)
Benzamidine	0.16 g/l	0.08 g

2.11 Measurement of apo-B48 concentration

Plasma apo B-48 concentration was measured using the method described by Lovegrove *et al* 1996. This method uses an antiserum, specific for apolipoprotein (apo) B-48, in a competitive, enzyme-linked immunosorbent assay (ELISA) for apo B-48 in fasting and postprandial plasma samples. A heptapeptide-thyroglobulin conjugate of the carboxyl-terminal residues of the apo B-48 molecule is used as the coating material. Fasting and postprandial plasma samples are pre-incubated with specific anti-serum before adding to the pre-coated ELISA plate. A horseradish peroxidase (HRPO)-conjugated antibody is

used to detect antibodies attached to the coating material through the action of TMB substrate. Absorbance measured at 450 nm is inversely proportional to the concentration of apo B-48 in plasma.

2.11.1 Materials

Reagent	Grade	Supplier
Disodium hydrogen orthophosphate	AnalR	Fisons Scientific Equipment, Loughborough, U.K.
Dimethylformamide (DMF)		
Gelatine		
Hydrogen peroxide (30%)		
Hydrochloric acid		
Potasium chloride		
Potassium dihydrogen orthophosphate		
Sodiun hydrogen carbonate		
Sodium carbonate		
Sodium chloride		
Citric acid	AnalR	BDH Limited, Poole, U.K.
Human Serum Albumin (HSA) (Fraction V 96 - 99% purity)		Sigma Chemical Company, St. Louis, U.S.A.
Tween 20		
Dimethylsulfoxide (Sigma Product No. D-5879)		
3,3',5,5'-Tetramethlbenzadine (TMB)		Boehringer Mannheim GmbH, Germany

Coating conjugate

An heptapeptide was custom synthesised at the National Institute of Medical Research (Mill Hill, London, UK) consisting of an N-terminal cysteine residue attached to a C-terminal hexpeptide, mimicking residues 2147 - 2152 of apo B-48 (H_3N^+Cys-Leu-Gln-

Thr-Tyr-Met-Ile-COO^{-}). In order to absorb the heptapeptide to the microtitre plate it was conjugated to thyroglobulin using succinimidly 3-(2-pyridyldithio) propionate. This was stored at -20^{0}C and diluted in coating buffer at a dilution of 1:80,000 for use in the ELISA format.

The coating conjugate, lymph standard, apo B-48 antisera, detecting antisera and quality controls (low, medium and high) were kindly supplied by Prof. C.M. Williams Department of Food Science and Technology, University of Reading, Reading, Berkshire RG6 2AH, UK.

Buffers

Reverse osmosis distilled water was used in the preparation of all buffers.

Coating buffer - *carbonate/bicarbonate buffer pH 9.6, 0.1 M*

3.18 g Na_2CO_3

5.88 g $NaHCO_3$

Both salts were dissolved in distilled water and made up to 1 litre. pH was checked and solution was stored at 4^{0}C.

Stock buffer - *phosphate buffer saline (PBS) pH 7.4, 0.2M*

160 g NaCl

58 g Na_2HPO_4

4 g KCl

4 g KH_2PO_4

All salts were dissolved in distilled water and made up to 2 litres, pH was checked and was stored at room temperature. On a daily basis, this stock solution (0.2M) was diluted 1: 10 with distilled water, resulting in a 0.02M solution.

Wash and diluent buffer (PBS-GT) - *0.02 M PBS, 0.1% gelatin and 0.05% Tween 20, pH 7.4*

100ml PBS stock solution

1.0g gelatin

0.5ml Tween 20

100ml of 0.2M PBS was added to 900 ml distilled water. 100 ml of this solution was removed, to which 1g gelatin was added and stirred on a heating block until dissolved. The Tween 20 was then added, mixed and added back to the remaining PBS 0.02M. This was then stirred well to give the working solution.

Substrate buffer - *Citrate phosphate buffer 0.05M, pH 5.0, for TMB substrate*

0.73 g Na_2HPO_4

0.51 g citric acid

Both salts were dissolved in 100ml deionised water and stored at 4^0C.

TMB Substrate

9ml 0.05 M citrate-phosphate (substrate) buffer

1ml Dimethylsulphoxide (DMSO, Sigma Product No. D-5879)

2μl H_2O_2

1 Tetramethylbenzadine Free Base (TMB) tablet (Sigma Product no. T- 5525)

One TMB tablet was dissolved in 1ml of DMSO. The substrate buffer was added. Immediately prior to use, H_2O_2 was added and mixed.

Stop solution - *1 M HCL*

50ml concentrated HCL. Add to 500ml distilled water.

2.11.2 Procedure

Day 1: A 1: 1000 dilution of coating conjugate was made in coating buffer and was pipetted into all wells of a flat-bottomed polystyrene microtitre plate (Nunc,

Roskilde, Denmark). The plate was placed into a damp storage box and stored overnight (16 hours) at 4^0C.

Day 2: An 8-point standard curve and a zero standard were prepared using the lymph standard and standard diluent. A 1 : 125,000 dilution of apo B-48 antisera in PBS-GT was prepared and an equal volume was added to the lymph standards, QCs (low, medium, high) and test samples (150µl of antiserum was added to 150µl of samples). These were vortexed and incubated at room temperature for 1 hour.

Immediately, the coated plate was washed three times in PBS-GT using an automated plate washer (Lifesciences International, UK) and banged dry on paper towels several times to remove any remaining washing buffer. 125µl of PBS-GT was added to each well and incubated in a damp storage box at 37^0C for 1 hour.

The plate of block was emptied and banged dry. The pre-incubated samples, standards and quality controls were vortexed and added, 100µl, per well to the plate. The plate was incubated at 37^0C for 2 hours.

The incubated plate was washed three times with PBS-GT and banged dry, as previously described. A 1 : 15,000 dilution of Donkey anti-rabbit antibody (HRPO) was prepared and 100µl added to each well. The plate was again incubated at 37^0C for 2 hours.

After this incubation period the plate was washed three times in PBS-GT and banged dry. The TMB substrate was prepared, and 100µl added to each well. The plate was incubated at 37^0C until colour development (blue; about 30 minutes).
50µl 1 M HCL was added to each well and the yellow product was read at 450nm using an automated plate reader (Mulitskan Biochromatic Plate Reader, Lifesciences Inernational, UK). Using Genesis software (Lifesciences International, UK) a standard curve was drawn and concentrations of apo B-48 were determined.

2.12 Statistical analysis

All statistical analyses were completed with the Apple Macintosh compatible statistical package Datadesk 5.0.1 (Data Description, Inc., Ithaca, N.Y., USA.). Results are presented as mean values with their standard deviations. Statistical significance was accepted at a level of $P \leq 0.05$. The area under the postprandial response curve was calculated using the trapezoidal method (Mathews *et al,* 1990). Subjects were divided according treatment group. Analysis of variance (ANOVA) with least significant difference contrasts was used to assess differences between groups. Repeated measures analysis of variance was used when investigating postprandial values. The association between fasting & postprandial values was assessed using Pearson Product-Moment correlation coefficient. Paired t-Test was used to assess the effect of within-group variation for a single measurement and 2 sample t-Test was used to assess differences between platelet phospholipid and cholesteryl ester fatty acid concentrations post-trial.

Chapter Three

The Effect of Low Doses of Fish Oils on Postprandial Lipaemia

3.1 Introduction

The magnitude and duration of the postprandial response varies greatly between individuals as it is affected by a multitude of physiological and dietary factors, which are discussed in Chapter 1. The elevation of lipid concentrations in the postprandial state for a prolonged period has a well-documented affinity with coronary heart disease risk due to its association with a cascade of pro-atherogenic metabolic events, including the formation of atherogenic CM remnants, formation of highly atherogenic small dense LDL particles, a reduction in the concentration of cardio-protective HDL_2 particles and the activation of coagulation factor VII (Cohn, 1998, Roche and Gibney, 1999). However, in the normolipidaemic state, the postprandial response is associated with a phenomenon known as reverse cholesterol transport. In this beneficial process TRL are enriched with cholesteryl esters within the circulation by transfer of cholesteryl esters from HDL, and this appears to be the major route by which cholesteryl esters are returned to the liver for excretion. When TAG concentrations are elevated postprandially and TRL are present in high concentrations for a prolonged time extensive remodelling of many lipid fractions occurs. TRL become progressively enriched with cholesteryl esters from HDL and LDL with the reciprocal transfer of TAG to LDL and HDL particles. These TAG-enriched LDL and HDL particles are then susceptible to hydrolysis by lipases, reducing their size leading to a preponderance of small dense atherogenic LDL particles (Karpe *et al,* 1993) which are associated with an increased risk of CHD (Griffin *et al,* 1994). The TAG-enriched HDL particles are delipidated by hepatic lipase, resulting in the formation of HDL_3 particles with the subsequent reduction in the concentration of cardio-protective HDL_2 particles and an elevation in concentration of HDL_3 particles (Patsch *et al,* 1983). At the same time, TRL are hydrolysed by the enzyme lipoprotein lipase (EC 3.1.1.34) located on the endothelial surface of capillaries in extrahepatic tissues, resulting in the delivery of free fatty acids to tissues and the formation of chylomicron remnant particles (Redgrave 1970). The progressive enrichment of these particles with esterified cholesterol has been shown to be atherogenic in the experimental setting in both animals and humans (Zilversmit, 1979,

Karpe *et al,* 1994) as they easily penetrate arterial tissues and can be found in fatty lesions (Proctor and Mamo 1996).

Long-chain n-3 PUFA, EPA and DHA, from oily fish and fish oils have previously been shown to be powerful hypotriacylglycerolaemic agents in the experimental setting in both acute and chronic studies (Zucker *et al,* 1988, Williams *et al,* 1992). In the acute setting, the postprandial response to meals containing predominantly fats of different saturation levels has been shown to vary according to saturation level in the order n-3 PUFA < n-6 PUFA < MUFA = SFA (Williams, 1998). The underlying mechanism is thought to be elucidated through the preferential hydrolysis of n-3 PUFA-containing chylomicrons and greater activation of LPL. The effects of test meal fatty acid composition on magnitude and duration of postprandial lipaemia is further discussed in Chapter 1.

Previous studies have shown that the degree of fat saturation in the background diet correlates with postprandial lipaemic response. Weintraub *et al,* (1988) examined this more closely looking at the effects of fats of different degrees of saturation on lipoprotein levels. They randomly assigned their volunteers to receive three isocaloric background diets for 25 days over a period of 12 weeks; one rich in SFA, one rich in n-6 PUFA and one rich n-3 PUFA. They remarked that, relative to the postprandial response observed after the SFA-background diet, the postprandial response was attenuated most by the n-3 PUFA background diet, and to a lesser extent by the n-6 PUFA diet. The authors concluded that n-3 PUFA rich diets exert their lipoprotein-lowering effects through reduced synthesis and/or increased catabolism of hepatically derived PUFA-containing lipoproteins. Further evidence from both rat (Phan *et al,* 1999) and human (Demacker *et al,* 1991) studies suggests that the hypotriacylglycerolaemic effect of PUFA is elucidated through increased removal of CM remnants from diets rich in these fatty acids in comparison with diets rich in saturated fats with higher melting points. Zampelas *et al,* (1994) also examined the postprandial lipid response in a group of people randomly assigned on three separate occasions to receive a SFA-rich meal, which was designed to mimic the fatty acid composition of the British diet, a n-6 PUFA-rich meal or a n-3 PUFA-rich meal. They found that the fish oil meal decreased postprandial lipaemia relative to the SFA-rich meal and the n-6 PUFA meal had moderate postprandial TAG-lowering effect.

The majority of studies examining this hypotriacylglycerolaemic effect of n-3 PUFA have used unphysiologically high doses over short supplementation periods. A comprehensive review of 32 parallel placebo-controlled studies revealed that the average supplementation period in normal healthy volunteers was 7-10 weeks with mean n-3 fatty acid intakes 3-4 g/d (Harris, 1997). This would be the equivalent to 10 portions of cod or one small portion of salmon daily. Beneficial effects of supplementation with n-3 PUFA on plasma lipids, lipoproteins, platelet aggregation, haemostasis and bleeding time have been reported in both fasting (Sanders and Roshanai, 1983, Blonk *et al,* 1990) and postprandial studies (Williams *et al,* 1992, Harris and Muzio, 1993, Agren *et al,* 1996). Conversely, supplementation with lower, more acceptable doses of fish oils have been explored with no beneficial consequences on fasting lipoprotein concentrations (Davidson and Gold, 1988, Lervang *et al,* 1993). These lower-dose studies have again used short supplementation periods. Thus perhaps it is the length of the supplementation period that requires further investigation. This concept is further consolidated by the recent finding that 1 g/d fish oil supplemented for 16 weeks showed a beneficial effect on postprandial triacylglycerolaemia in normolipidaemic volunteers (Roche and Gibney, 1996). The lowest beneficial dose of fish oils is unknown and consequently the aim of the present study is to find the optimal level and length of time of fish oil supplementation which would have a beneficial effect on postprandial lipids and lipoprotein profiles. The postprandial response to standard test meals rich in n-6 PUFA in normolipidaemic subjects taking their usual diets is investigated in the present study pre- and post-supplementation.

3.2 Methods

3.2.1 Study design

This study was approved by The Ethics Committee of the Federated Dublin Voluntary Hospitals and was conducted in the Nutrition Laboratory, Department of Clinical Medicine, at the Trinity Centre Medical School, St. James' Hospital. All volunteers were recruited according to the following exclusion criteria:

- BMI < 20 or > 30
- Vegan
- Smoker
- Currently adhering to a special diet (e.g. cholesterol lowering or weight loss)
- Heavy exerciser (> 3 * 30 minutes strenuous exercise per week)
- Heavy drinker (men > 28 units alcohol per week; women > 21 units alcohol per week)
- Diabetic or presence of other endocrine / liver disease
- Pregnancy or planning pregnancy
- Dietary supplement user (fatty acid supplement or vitamin / mineral supplements)
- Habitual diet rich in n-3 PUFA

Habitual dietary intake of n-3 PUFA was assessed using a food frequency questionnaire, which identified rich sources of EPA, DHA and ALA (Appendix (i)). Details regarding age, weight and height were also recorded on screening. All subjects were healthy and were not taking any medications known to interfere with lipid metabolism. They gave written informed consent prior to participation.

A biochemical screen was completed following an overnight (12 hour) fast to ensure that all subjects had fasting blood lipid profiles and glucose concentrations within the following ranges:

• Plasma triacylglycerol (TAG)	0.5 - 2.3 mmol/L
• Plasma cholesterol	3.2 - 6.8 mmol/L
• Gamma glutamyl transpeptidase (γ GT)	5 - 60 IU
• Plasma glucose	2.8 - 8.3 mmol/L
• Haemoglobin	13.0 - 18.0 g/dl male
	11.5 - 13.5 g/dl female
• Platelet phospholipid EPA	< 1.0 w/w%

Volunteers were recruited from the academic and administrative staff and undergraduate and postgraduate students in Trinity College Dublin, who were approached using the internal email system. Altogether, the totals approached were:

Administrative staff	433
Academic staff	951
Postgraduate students	2147
Undergraduate students	9040

Volunteers were also recruited from the staff of St. James' Hospital, Dublin. In total over 500 replies were dealt with but many of these were excluded due to age, weight, smoking habit, alcohol intake, pregnancy or lack of time to do the study.

62 volunteers completed the study, which was conducted in the Nutrition Laboratory, Trinity Centre for Health Sciences, St. James' Hospital, Dublin 8. Subjects were stratified according to fasting TAG concentrations and randomised accordingly to one of four treatment groups. As the study was double blind this randomisation and stratification was carried out at the University of Ulster, Coleraine, Co. Derry.

The four treatment groups, including composition of the supplements are described in Table 3.2.2:

All supplements contained novel refined marine oil (1000 mg, Roche Lipid Technologies, Switzerland) stabilised with ascorbyl palmitate, lecithin and tocopherol, with the exception of the placebo which contained refined olive oil (1000 mg) stabilised with ascorbyl palmitate, lecithin and tocopherol. Volunteers were asked to consume three capsules daily, preferably before meals and at a regular time every day to ensure regular daily intake.

Table 3.2.2: Treatment Groups

Treatment	Control	Low Dose	Medium Dose	High Dose
Fish Oil	0.0g/d	0.3g/d	0.6g/d	0.9g/d
Olive Oil	0.9g/d	0.6g/d	0.3g/d	0.0g/d
EPA (%)	0.0	5.6	11.4	17.2
DHA (%)	0.0	3.6	7.2	11.0
Total n-3 content (%)	0.0	11.8	22.7	33.7
Actual n-3 content per capsule (mg)	0.0	0.118	0.227	0.337

*** % = % total oil, per capsule**

The intervention period was 16 weeks, during which time volunteers were contacted every four weeks where all remaining supplements were taken from each volunteer and counted and were replaced with supplements for the forthcoming 4-week period.

During the study period four volunteers dropped out, two due to intolerance to the supplements, one due to pregnancy and one due to anaemia.

Postprandial Study Days

Postprandial response to a test meal was determined pre- and post-intervention. Volunteers attended the Nutrition Laboratory on the morning of the investigation between 7:45 am and 9 am following their overnight fast. They were cannulated and two fasting samples were drawn before ingestion of a fat-rich test meal. This standardised meal was consumed within twenty minutes and was well tolerated. Test meal composition is outlined in Table 3.2.3. Each test meal was freshly prepared on the morning of postprandial investigation. During the postprandial study day volunteers avoided strenuous activity and abstained from food and drinks with the exception of water, weak black tea, black decaffeinated coffee and diet minerals, which were allowed *ad libidum*.

Table 3.2.3 Test meal composition

Contents	Protein (g)	Carbohydrate (g)	Fat (g)	Energy (kcals)
Skimmed milk	4.95	7.5	0.15	50
Skimmed milk powder	5.25	8	0.165	54
Sunflower oil	0.0	0.0	39.96	360
Flavouring*	trace	2.5	0	15
Total	10.2	18.0	40.28	497
Percentage energy	**9%**	**15%**	**76%**	

* SHS Modjul System, Orange Flavour

3.2.2 Collection and storage of samples

All subjects fasted for 12 hours before investigations and abstained from alcohol and vigorous exercise for 24 hours beforehand. Fasting blood samples were drawn at weeks 0, 4, 8 and 16.

A 21 gauge, 40 mm vacutainer "precision glide" needle was used for blood collection. Subjects remained seated during blood collection. The blood was drawn

into evacuated lithium heparin tubes for plasma lipid investigations, into evacuated tubes containing 0.105 M sodium citrate (v/v: 1:10) to investigate platelet phospholipid concentration. Lithium heparin and sodium citrate tubes were centrifuged immediately at 2500 rpm to avoid the spontaneous hydrolysis of TAG that can occur if blood samples are left at room temperature for too long. Plasma was subsequently taken off, vortexed, aliquoted and stored at -20^0 C until required for analysis, with the exception of plasma used for HDL and TRL isolation, which was stored at 2^0 - 5^{0}C at this point. TRL and HDL isolation from plasma was performed within 24 hours of collection and subsequent samples were stored at -70° C. Plasma and TRL samples for apo B-48 analysis were stored in apo B-48 preservative (5% v/v). Whole blood from EDTA (K_2) tubes was used immediately for FBC.

3.2.3 Biochemical parameters investigated

The following parameters were investigated pre- and post-trial in the fasting state:

- Total plasma glucose concentration,
- Total plasma insulin concentration,
- Total plasma HDL cholesterol concentration,
- Plasma HDL_2 and HDL_3 cholesterol concentration,
- Platelet phospholipid fatty acid composition
- Cholesteryl ester fatty acid composition
- Full blood count.

The following parameters were investigated pre- and post- trial at 0- , 2- , 4- , 6- and 8- hours postprandially:

- Total plasma TAG concentration,
- Total plasma cholesterol concentration,
- Total plasma NEFA concentration,
- TRL & TPL TAG concentration,
- TRL & TPL cholesterol concentration

Plasma apo B-48 concentration was investigated at 0- , 4- , 8- hours, both pre- and post-trial and TRL fatty acid composition was investigated at 0- and 4- hours, again at week 0 and at week 16.

A detailed description of all analytical methodologies is described in Chapter 2.

3.2.4 Statistical analysis

All statistical analyses were completed with the Apple Macintosh compatible statistical package Datadesk 5.0.1 (Data Description, Inc., Ithaca, N.Y., USA.). Results are presented as mean values with their standard deviations. All data was transformed to give the data a normal Gaussian distribution (Table 3.2.2).

Table 3.2.4 Data transformations

Parameter	Transformation
Plasma TAG	1/√
Plasma Cholesterol	Natural log (ln)
Plasma Glucose	1/√
Plasma NEFA	√
Haemoglobin	no transformation
TRL & TPL TAG	Natural log (ln)
TRL & TPL Cholesterol	√
Total HDL, HDL_2 & HDL_3 cholesterol	1 /
Apo B-48	Natural log (ln)

Statistical significance was accepted at a level of $P \leq 0.05$. The total study group was divided according to supplementation level. Repeated measures analysis of variance (ANOVA) with least significant difference contrasts was used to assess differences between groups, using group as the dependent variable. Two-way analysis of variance investigated any differences between biological parameters, pre- and post-trial.

3.3 Results

3.3.1 Subject information

Information pertaining to volunteers' age, weight, height and BMI who participated in the intervention is presented in Table 3.3.1. This information was gathered at each blood-sampling period. BMI did not change significantly in any group throughout the study period except for the group consuming 0.6g fish oil, which got slightly heavier ($P < 0.05$) at week 8 which coincided with the Christmas period, but returned to baseline weight by week 16.

Table 3.3.1 Subject information

Group	0.0g/d	0.3g/d	0.6g/d	0.9g/d
n	14	17	17	14
Age (years)	40.29 (12.77)	32.76 (9.14)	35.06 (11.33)	39.79 (13.19)
Wt (kg) ***Pre-trial***	78.56 (12.56)	75.60 (12.73)	76.29 (15.70)	71.24 (13.87)
Wt (kg) ***Post-trial***	78.51 (13.01)	75.88 (13.46)	77.21 (16.01)	72.12 (14.33)
Height (m)	1.74 (0.11)	1.74 (0.12)	1.76 (0.11)	1.71 (0.10)
BMI (kg/m^2) ***Pre-trial***	25.77 (2.28)	24.95 (2.04)	24.52 (3.10)	24.20 (2.93)
BMI (kg/m^2) ***Post-trial***	25.76 (2.95)	25.03 (2.23)	24.82 (3.19)	24.57 (2.90)

Values reported represent mean (standard deviation) of study groups.

3.3.2 Fish oil consumption

Mean daily n-3 PUFA consumption in each group throughout the study period is summarised in Table 3.3.2. Overall supplements were well tolerated, with the exception of one subject in the high-dose group who dropped out due to dislike of taste of supplements.

Table 3.3.2 Supplement intake

Group	0.0g/d	0.3g/d	0.6g/d	0.9g/d
No. capsules consumed	321.93 (36.18)	326.53 (24.59)	328.41 (28.10)	317.71 (33.71)
Days on study	115.36 (4.91)	115.41 (10.84)	118.76 (11.39)	115.36 (4.14)
No. capsules per day	2.79 (0.32)	2.84 (0.22)	2.78 (0.28)	2.76 (0.29)
Fish oil g/day	0.00 (0.00)	0.28 (0.02)	0.56 (0.06)	0.83 (0.09)
% Compliance	95.8%	97.2%	97.74%	94.56%

Values reported represent mean (standard deviation) of study groups.

3.3.3 Plasma glucose, insulin and haemoglobin concentrations

Mean plasma glucose, insulin & haemoglobin concentrations pre- and post-trial are presented in Table 3.3.3. Plasma glucose and insulin concentrations were not affected by supplementation. Haemoglobin concentrations changed significantly pre- versus post- trial (P = 0.0043), but no change was observed between groups at baseline or following intervention.

Table 3.3.3 Plasma glucose, insulin and haemoglobin concentrations pre- and post-trial.

n-3 PUFA		0.0g	0.3g	0.6g	0.9g
Glucose (mmol/l)	*Pre-*	5.17 (0.45)	5.38 (0.75)	5.36 (0.55)	5.05 (0.70)
	Post-	5.21 (0.54)	4.98 (0.43)	4.91 (1.19)	5.38 (0.90)
Insulin (μIU/ml)	*Pre-*	7.33 (2.50)	7.82 (3.12)	8.52 (2.42)	7.15 (3.62)
	Post-	7.83 (2.04)	8.00 (2.17)	8.84 (3.57)	7.51 (2.22)
Haemoglobin (g/dl)	*Pre-*	14.88 (1.55)	14.34 (1.28)	14.99 (1.25)	14.68 (1.0)
	Post-	14.71 (1.26)	13.98 (1.41)	14.57 (1.63)	14.55 (0.85)

Values reported represent mean (standard deviation) of study groups.

NSD between groups.

3.3.4 Plasma triacylglycerol concentrations

Fasting & postprandial plasma triacylglycerol (TAG) concentrations following the ingestion of the PUFA-rich test meal pre- and post-intervention are presented in Table 3.3.4 (i). Each group demonstrated monophasic mean plasma TAG response. Peak plasma TAG concentrations were achieved by all groups at 4 hours postprandially, where a significant change from baseline was observed using post-hoc testing ($P \leq 0.05$) in all groups except the placebo group where peak TAG concentration was not statistically significantly greater than baseline.

Repeated measures analysis of variance showed a significant change following intervention between groups, as displayed by a significant treatment * repeat effect ($P = 0.0347$). The group consuming 0.9g fish oil showed the greatest reduction at all time points, both fasting and postprandially.

Significance of changes of the postprandial triacylglycerolaemic response following dietary intervention was investigated when the response was expressed in summary form (Table 3.3.4 (ii)). Fasting TAG values correlated significantly with summary variable for postprandial values, TAG AUC ($r = 0.872$, $P < 0.0001$). No significant group * repeat interactions were observed for either AUC per hour (TAG AUC) or incremental AUC per hour (TAG I-AUC) as demonstrated by repeated measures analysis of variance. Despite this fact, a 17% reduction was observed in both AUC and I-AUC in the highest dose fish oil group. Maximum TAG concentrations were reduced following intervention in the highest dose fish oil group, showing a mean significant reduction of 17% ($P = 0.0257$) as demonstrated using a paired t-Test. The time taken to reach peak concentrations however did not change significantly as a result of intervention in any group.

Table 3.3.4(i) **The postprandial TAG response (mmol/l)**

Dose n-3 PUFA		0.0g fish oil	0.3g fish oil	0.6g fish oil	0.9g fish oil
0 hours	*Pre-*	1.15 (0.39)	0.89 (0.39)	0.95 (0.31)	1.20 (0.62)
	Post-	1.06 (0.37)	0.97 (0.32)	1.00 (0.50)	0.98 (0.44)
2 hours	*Pre-*	1.33 (0.60)	0.97 (0.39)	1.25 (0.49)	1.38 (0.59)
	Post-	1.15 (0.43)	1.12 (0.42)	1.17 (0.45)	1.13 (0.45)
4 hours	*Pre-*	1.48 (0.66)	1.07 (0.50)	1.22 (0.40)	1.47 (0.59)
	Post-	1.35 (0.61)	1.22 (0.61)	1.15 (0.47)	1.26 (0.50)
6 hours	*Pre-*	1.48 (0.67)	0.97 (0.48)	1.06 (0.32)	1.36 (0.69)
	Post-	1.29 (0.52)	1.00 (0.51)	1.06 (0.41)	1.11 (0.56)
8 hours	*Pre-*	1.01 (0.59)	0.74 (0.30)	0.77 (0.24)	1.48 (0.67)
	Post-	1.16 (0.38)	0.79 (0.39)	0.84 (0.32)	1.29 (0.52)

Values reported represent mean (standard deviation) of study groups.

NSD between groups.

Table 3.3.4(ii) Summary variables for postprandial TAG response (mmol/l)

		0.0g fish oil	0.3g fish oil	0.6g fish oil	0.9g fish oil
TAG AUC (mmol.h/L)	*Pre-*	9.96 (3.59)	7.64 (3.22)	8.77 (2.52)	10.71 (4.87)
	Post-	9.80 (3.46)	8.44 (3.56)	8.60 (3.07)	8.89 (3.54)
TAG I-AUC (mmol.h/L)	*Pre-*	8.81 (3.25)	6.75 (2.89)	7.83 (2.24)	9.51 (4.27)
	Post-	8.74 (3.15)	7.47 (3.26)	7.60 (2.63)	7.91 (3.16)
Maximum value (mmol/L)	*Pre-*	1.48 (0.53)	1.15 (0.48)	1.42 (0.41)	1.62 (0.58)
	Post-	1.53 (0.49)	1.32 (0.59)	1.29 (0.45)	1.35 (0.55) †
Time to peak concentration (hours)	*Pre-*	4.62 (1.26)	4.35 (1.62)	3.50 (1.71)	4.50 (1.24)
	Post-	4.43 (1.60)	3.65 (1.27)	4.38 (1.50)	4.55 (2.02)

Values reported represent mean (standard deviation) of study groups. Significance of difference during supplementary period investigated using two-way analysis of variance (ANOVA), with Least Significance Difference (LSD) post hoc testing.

† $P \leq 0.01$ - significant change from baseline

3.3.5 Postprandial cholesterol response

Fasting and postprandial cholesterol values are presented in Table 3.3.5. Repeated measures analysis of variance revealed significant variability between individuals ($P \leq 0.0001$) and also a significant ($P \leq 0.0001$) group * repeat interaction, displaying the significance of change following intervention between groups. A significant treatment * repeat interaction was observed in fasting plasma cholesterol concentrations ($P = 0.024$) demonstrating how the groups responded differently to intervention, even though there was no significant difference between groups. The group consuming the placebo (0.9g olive oil) showed a significant reduction ($P \leq 0.05$) in cholesterol concentrations at all time points except at 6h following intervention.

Significance of changes of the postprandial cholesterol response following intervention was investigated when the cholesterol response was expressed in summary form. Fasting cholesterol values were significantly related to cholesterol AUC ($r = 0.959$, $P \leq 0.0001$). No significant group, repeat or group * repeat interactions were observed for AUC per hour (cholesterol AUC) in any group, as demonstrated by repeated measures analysis of variance.

Table 3.3.5 Fasting & postprandial cholesterol values (mmol/l) pre- and post-intervention.

Dose fish oil		0.0g	0.3g	0.6g	0.9g
0 hours	*Pre-*	5.435 (1.115)	5.034 (0.944)	5.162 (1.199)	5.223 (0.952)
	Post-	5.031 (1.161) †	5.121 (0.831)	5.151 (1.171)	5.460 (0.954)
2 hours	*Pre-*	5.374 (1.223)	4.973 (1.008)	4.859 (1.045)	5.196 (1.139)
	Post-	4.981 (1.223) †	5.120 (0.757)	4.933 (1.141)	5.237 (0.979)
4 hours	*Pre-*	5.118 (1.032)	4.963 (0.982)	4.948 (0.990)	5.213 (0.897)
	Post-	4.839 (1.076) †	5.199 (0.882)	4.968 (1.150)	5.483 (0.954)
6 hours	*Pre-*	5.228 (1.187)	5.038 (1.067)	4.982 (1.082)	5.199 (1.050)
	Post-	4.998 (1.154)	5.194 (0.910)	5.081 (1.211)	5.453 (0.946)
8 hours	*Pre-*	5.117 (1.282)	5.171 (1.057)	4.902 (1.080)	5.617 (1.019)
	Post-	4.695 (0.797) †	5.159 (0.956)	5.153 (1.126)	5.437 (0.949)
AUC (mmol.h/L)	*Pre-*	41.819 (8.901)	40.154 (7.950)	39.375 (7.805)	42.057 (7.467)
	Post-	39.362 (8.476) †	41.306 (6.728)	40.168 (9.066)	43.243 (7.388)

Values reported represent mean (standard deviation) of study groups. Significance of difference during supplementary period investigated using repeated measures analysis of variance (ANOVA), with Least Significance Difference (LSD) post hoc testing and two-way ANOVA was used for summary variable (AUC) also with post hoc testing
† $P \leq 0.01$ - significant change from baseline

3.3.6 Postprandial NEFA response

Postprandial plasma non-esterified fatty acid (NEFA) concentrations following the ingestion of the PUFA-rich test meal pre- and post-intervention are presented in Table 3.3.6 (i). Repeated measures analysis of variance showed a significant ($P \leq 0.0001$) person variability and also a significant repeat effect ($P \leq 0.0001$) as all groups showed a depression in plasma NEFA concentration at 2 hours postprandially in comparison with values at 0 -, 6 - or 8 -hours both pre- and post-trial. Plasma NEFA concentrations were not affected by intervention in any group, as demonstrated by repeated measures ANOVA.

Significance of changes of the postprandial NEFA response following dietary intervention was investigated when expressed in summary form (Table 3.3.6 (ii)). Fasting values were strongly related to NEFA AUC ($r = 0.423$, $P \leq 0.0001$). No significant group, repeat or group * repeat interactions were observed for either AUC per hour (NEFA AUC) or incremental AUC per hour (NEFA I-AUC), as demonstrated by 2-way analysis of variance (ANOVA). Minimum NEFA concentrations, maximum NEFA concentrations, time to peak or time to reach minimum concentrations were not affected by intervention in any group, again as shown by 2-way ANOVA.

Table 3.3.6 (i) The postprandial NEFA response (mEq/l)

Dose fish oil		0.0g	0.3g	0.6g	0.9g
0 hours	*Pre-*	0.453 (0.240)	0.476 (0.176)	0.416 (0.167)	0.446 (0.254)
	Post-	0.417 (0.224)	0.515 (0.208)	0.414 (0.167)	0.405 (0.148)
2 hours	*Pre-*	0.251 (0.108)	0.294 (0.116)	0.276 (0.104)	0.220 (0.134)
	Post-	0.262 (0.147)	0.298 (0.147)	0.275 (0.138)	0.283 (0.125)
4 hours	*Pre-*	0.471 (0.155)	0.506 (0.186)	0.604 (0.207)	0.486 (0.189)
	Post-	0.477 (0.365)	0.515 (0.258)	0.450 (0.163)	0.446 (0.234)
6 hours	*Pre-*	0.612 (0.227)	0.675 (0.222)	0.552 (0.199)	0.662 (0.367)
	Post-	0.598 (0.340)	0.553 (0.236)	0.651 (0.248)	0.587 (0.176)
8 hours	*Pre-*	0.548 (0.204)	0.672 (0.272)	0.644 (0.310)	0.634 (0.223)
	Post-	0.608 (0.404)	0.621 (0.331)	0.591 (0.279)	0.565 (0.203)

Values reported represent mean (standard deviation) of study groups. NSD between groups (ANOVA)

Table 3.3.6 (ii) Summary variables for postprandial plasma NEFA (mEq/l)

Dose fish oil		0.0g	0.3g	0.6g	0.9g
NEFA AUC (mEq.h/L)	*Pre-*	3.664 (0.917)	4.022 (0.988)	3.828 (1.205)	3.854 (1.354)
	Post-	3.697 (1.882)	3.868 (1.320)	3.745 (1.099)	3.601 (1.144)
NEFA I-AUC (mEq.h/L)	*Pre-*	3.211 (0.677)	3.546 (0.811)	3.408 (1.033)	3.408 (1.100)
	Post-	3.280 (1.658)	3.353 (1.112)	3.331 (0.932)	3.196 (0.996)
Maximum value	*Pre-*	0.670 (0.207)	0.738 (0.224)	0.691 (0.348)	0.772 (0.388)
	Post-	0.699 (0.360)	0.746 (0.289)	0.750 (0.264)	0.657 (0.169)
Time to maximum value (h)	*Pre-*	6.615 (1.261)	6.588 (1.372)	6.250 (1.770)	7.00 (1.301)
	Post-	6.143 (1.834)	6.706 (1.572)	6.625 (1.408)	7.077 (1.320)
Minimum value	*Pre-*	0.243 (0.098)	0.287 (0.114)	0.276 (0.103)	0.215 (0.123)
	Post-	0.237 (0.124)	0.272 (0.125)	0.262 (0.129)	0.272 (0.126)
Time to minimum value (h)	*Pre-*	2.615 (1.710)	2.353 (1.057)	2.250 (1.000)	2.143 (0.535)
	Post-	3.143 (2.316)	2.588 (1.698)	2.500 (1.549)	2.462 (0.877)

Values reported represent mean (standard deviation) of study groups. NSD between groups (ANOVA)

3.3.7 Plasma apo B-48 concentrations

Mean ± standard deviations for plasma apo B-48 concentrations are presented in Table 3.3.7. Repeated measures analysis of variance showed a significant individual variability ($P \leq 0.0001$) and a significant repeat (time) variability ($P \leq 0.0001$) as demonstrated by concentrations rising from baseline at 4 hours and returning almost to baseline values by 8 hours postprandially both pre- and post- trial. Plasma apo B-48 concentrations were not affected by intervention in any group as displayed by repeated measures ANOVA and also by the observation of no significant change in apo B-48 AUC, as demonstrated by 2-way ANOVA. Summary postprandial apo B-48 concentrations portrayed a distinct relationship with fasting values ($r = 0.816$, $P \leq 0.0001$).

Table 3.3.7 The postprandial apo-B48 response

		0 hours	4 hours	8 hours	AUC
0.0g fish oil	*Pre-*	0.435 (0.153)	0.535 (0.192)	0.490 (0.152)	3.870 (1.183)
	Post-	0.408 (0.112)	0.509 (0.182)	0.442 (0.138)	3.737 (1.170)
0.3g fish oil	*Pre-*	0.358 (0.098)	0.549 (0.231)	0.428 (0.141)	3.767 (0.741)
	Post-	0.428 (0.106)	0.516 (0.166)	0.423 (0.172)	3.764 (1.075)
0.6g fish oil	*Pre-*	0.384 (0.130)	0.499 (0.174)	0.450 (0.202)	3.703 (1.278)
	Post-	0.440 (0.181)	0.532 (0.208)	0.460 (0.169)	3.781 (1.412)
0.9g fish oil	*Pre-*	0.418 (0.169)	0.460 (0.150)	0.436 (0.088)	3.371 (0.572)
	Post-	0.397 (0.105)	0.499 (0.148)	0.469 (0.112)	3.776 (0.877)

Values reported represent mean (standard deviation) of study groups. NSD between groups (ANOVA).

3.3.8 a Plasma lipoprotein concentrations – TRL & TPL

TRL & TPL TAG and cholesterol concentrations are presented in Tables 3.3.8 (i) a - 3.3.8 (v) a. TRL TAG concentrations demonstrated significant subject variability ($P \leq 0.0001$), significant difference between treatment groups ($P \leq 0.0001$) and a significant change following intervention ($P \leq 0.05$). However no significant treatment * repeat effect was observed which reflects the fact that groups had different TRL TAG concentrations at baseline rather than a difference as a result of intervention. The group consuming 0.9g fish oil showed the greatest reduction in TRL TAG concentration in the fasting state, amounting to an average of 39%, however this reduction did not reach statistical significance. 2-way ANOVA of summary variables for TRL TAG showed no significant transformation as a result of intervention for TRL TAG AUC, TRL TAG I-AUC, for maximum TRL TAG concentrations and for time to peak. Fasting TRL TAG concentrations correlated strongly with TRL TAG AUC ($r = 0.878$, $P \leq 0.0001$). In all groups, TRL TAG concentrations rose significantly from baseline, peaked at 4 hours postprandially and returned to baseline by 8 hours.

TRL cholesterol concentrations showed a highly significant subject variation ($P \leq 0.0001$), and also a significant treatment * repeat effect which demonstrated a difference as a result of intervention. The highest dose fish oil group showed significantly greater TRL cholesterol concentrations post-trial at 2- , 6- , and 8- hours ($P \leq 0.05$). 2-way ANOVA of summary variables also showed a significant treatment, repeat and treatment * repeat effect for TRL cholesterol AUC as reflected in the significantly greater TRL cholesterol AUC for the highest dose fish oil group following intervention. Overall, fasting TRL cholesterol concentrations showed a powerful association with TRL cholesterol AUC ($r = 0.817$, $P \leq 0.0001$).

Repeated measures ANOVA of TPL TAG showed a highly significant subject variability ($P \leq 0.0001$), however no differences between groups were observed or no differences as a result of intervention were revealed at any time point, or when 2-way ANOVA investigated the area under the curve (TPL TAG AUC). However, overall, TPL TAG AUC was significantly correlated with fasting values ($r = 0.826$, $P < 0.0001$). Highly significant subject variability was observed in TPL cholesterol concentrations ($P \leq 0.0001$), however concentrations were not significantly altered as a result of

intervention in any group. Again, fasting values showed a strong relationship with postprandial values (TPL cholesterol AUC) ($r = 0.922$, $P \leq 0.0001$).

Table 3.3.8 (i) a Postprandial TRL TAG response (mmol/l)

Dose fish oil		0.0g	0.3g	0.6g	0.9g
0 hours	*Pre-*	0.375 (0.244)	0.206 (0.097)	0.231 (0.144)	0.383 (0.272)
	Post-	0.331 (0.173)	0.266 (0.200)	0.270 (0.177)	0.275 (0.132)
2 hours	*Pre-*	0.549 (0.305)	0.352 (0.335)	0.333 (0.219)	0.523 (0.327)
	Post-	0.451 (0.218)	0.409 (0.283)	0.449 (0.216)	0.409 (0.131)
4 hours	*Pre-*	0.593 (0.436)	0.339 (0.176)	0.319 (0.157)	0.605 (0.365)
	Post-	0.639 (0.368)	0.394 (0.310)	0.452 (0.269)	0.403 (0.176)
6 hours	*Pre-*	0.573 (0.340)	0.319 (0.216)	0.262 (0.122)	0.603 (0.447)
	Post-	0.501 (0.259)	0.287 (0.280)	0.366 (0.216)	0.408 (0.209)
8 hours	*Pre-*	0.306 (0.255)	0.164 (0.160)	0.141 (0.065)	0.428 (0.429)
	Post-	0.429 (0.228)	0.213 (0.236)	0.232 (0.149)	0.284 (0.166)

Values reported represent mean (standard deviation) of study groups. NSD between groups (ANOVA)

Table 3.3.8 (ii) a Summary variables for postprandial TRL TAG response (mmol/l)

Dose fish oil		0.0g	0.3g	0.6g	0.9g
TRL TAG AUC (mmol.h/L)	*Pre-*	3.975 (2.455)	2.465 (1.453)	2.195 (1.030)	4.075 (2.796)
	Post-	3.891 (1.782)	2.578 (1.963)	3.007 (1.572)	3.004 (1.065)
TRL TAG I-AUC (mmol.h/L)	*Pre-*	3.630 (2.212)	2.254 (1.366)	1.978 (0.911)	3.728 (2.554)
	Post-	3.560 (1.627)	2.312 (1.771)	2.737 (1.416)	2.715 (0.972)
Maximum value	*Pre-*	0.685 (0.382)	0.473 (0.309)	0.391 (0.196)	0.740 (0.398)
	Post-	0.723 (0.333)	0.508 (0.309)	0.527 (0.237)	0.524 (0.169)
Time to maximum value (h)	*Pre-*	4.50 (1.732)	4.0 (1.754)	3.625 (1.668)	4.545 (2.018)
	Post-	5.143 (1.875)	3.750 (1.770)	4.143 (1.658)	4.20 (2.201)

Values reported represent mean (standard deviation) of study groups. NSD between groups (ANOVA)

Table 3.3.8 (iii) a Postprandial TRL cholesterol response (mmol/l)

Dose fish oil		0.0g	0.3g	0.6g	0.9g
0 hours	*Pre-*	0.145 (0.082)	0.076 (0.031)	0.102 (0.072)	0.128 (0.106)
	Post-	0.136 (0.053)	0.104 (0.080)	0.112 (0.071)	0.165 (0.133)
2 hours	*Pre-*	0.159 (0.109)	0.089 (0.082)	0.094 (0.059)	0.136 (0.103)
	Post-	0.168 (0.096)	0.134 (0.099)	0.142 (0.081)	0.198 (0.145) †
4 hours	*Pre-*	0.163 (0.128)	0.120 (0.198)	0.097 (0.061)	0.160 (0.137)
	Post-	0.156 (0.082)	0.108 (0.092)	0.136 (0.102)	0.193 (0.112)
6 hours	*Pre-*	0.178 (0.161)	0.054 (0.028)	0.066 (0.023)	0.145 (0.127)
	Post-	0.191 (0.156)	0.072 (0.069)	0.087 (0.082)	0.213 (0.166) †
8 hours	*Pre-*	0.112 (0.093)	0.065 (0.062)	0.049 (0.031)	0.099 (0.099)
	Post-	0.122 (0.066)	0.059 (0.066)	0.051 (0.042)	0.193 (0.107) †
TRL cholesterol AUC (mmol.h/L)	*Pre-*	1.259 (0.866)	0.684 (0.493)	0.725 (0.314)	0.949 (0.866)
	Post-	1.301 (0.580)	0.873 (0.593)	1.051 (0.560)	1.640 (1.009) †

Values reported represent mean (standard deviation) of study groups. Significance of difference during supplementary period investigated using repeated measures analysis of variance (ANOVA), with Least Significance Difference (LSD) post hoc testing and two-way ANOVA was used for summary variable (AUC) also with post hoc testing
† $P < 0.05$ - significant change from baseline

Table 3.3.8 (iv) a Postprandial TPL TAG response (mmol/l)

Dose fish oil		0.0g	0.3g	0.6g	0.9g
0 hours	*Pre-*	0.560 (0.174)	0.544 (0.151)	0.623 (0.193)	0.660 (0.427)
	Post-	0.605 (0.199)	0.569 (0.147)	0.613 (0.190)	0.617 (0.209)
2 hours	*Pre-*	0.559 (0.144)	0.543 (0.157)	0.625 (0.190)	0.696 (0.314)
	Post-	0.643 (0.142)	0.572 (0.165)	0.600 (0.180)	0.670 (0.269)
4 hours	*Pre-*	0.594 (0.205)	0.615 (0.173)	0.626 (0.215)	0.579 (0.312)
	Post-	0.656 (0.171)	0.611 (0.202)	0.626 (0.193)	0.609 (0.172)
6 hours	*Pre-*	0.504 (0.202)	0.546 (0.152)	0.626 (0.206)	0.643 (0.328)
	Post-	0.640 (0.173)	0.563 (0.193)	0.602 (0.203)	0.642 (0.272)
8 hours	*Pre-*	0.622 (0.232)	0.530 (0.205)	0.546 (0.194)	0.545 (0.273)
	Post-	0.566 (0.175)	0.504 (0.197)	0.583 (0.225)	0.588 (0.282)
TPL TAG AUC (mmol.h/L)	*Pre-*	4.468 (1.204)	4.388 (1.007)	4.845 (1.457)	4.815 (2.261)
	Post-	5.063 (0.990)	4.565 (1.336)	4.851 (1.457)	5.167 (1.665)

Values reported represent mean (standard deviation) of study groups. NSD between groups (ANOVA)

Table 3.3.8 (v) a Postprandial TPL cholesterol response (mmol/l)

Dose fish oil		0.0g	0.3g	0.6g	0.9g
0 hours	*Pre-*	5.125 (1.312)	4.943 (1.061)	5.219 (1.112)	5.212 (1.093)
	Post-	5.115 (1.398)	4.973 (0.849)	4.983 (1.127)	5.159 (0.654)
2 hours	*Pre-*	5.164 (1.253)	4.784 (0.967)	4.871 (1.083)	4.864 (1.139)
	Post-	5.130 (1.508)	4.908 (0.735)	4.790 (1.085)	4.789 (0.772)
4 hours	*Pre-*	5.112 (1.272)	4.997 (1.090)	4.895 (1.125)	4.808 (1.185)
	Post-	4.812 (1.195)	5.042 (0.824)	4.970 (1.115)	4.735 (0.683)
6 hours	*Pre-*	5.139 (1.713)	4.978 (0.972)	5.015 (1.208)	5.021 (0.969)
	Post-	4.935 (1.663)	5.005 (0.825)	5.054 (1.132)	4.964 (0.830)
8 hours	*Pre-*	5.168 (0.973)	5.045 (1.020)	5.168 (1.252)	4.808 (1.056)
	Post-	4.428 (0.887)	5.142 (0.874)	5.095 (1.084)	5.209 (1.604)
TPL cholesterol AUC (mmol.h/L)	*Pre-*	38.901 (7.706)	39.358 (7.022)	39.218 (8.009)	37.511 (7.527)
	Post-	37.963 (10.294)	40.026 (6.240)	39.707 (8.742)	39.699 (5.819)

Values reported represent mean (standard deviation) of study groups. NSD between groups (ANOVA)

3.3.8 b Plasma lipoprotein concentrations - HDL

Mean HDL concentrations of the study groups pre- and post-trial are presented in Tables 3.3.8(i) b. Table 3.3.8 (ii) b displays results from repeated measures analysis of variance of HDL fractions. A significant repeat effect was highlighted by repeated measures ANOVA of total plasma HDL cholesterol and HDL_2 cholesterol, reflecting a significant change at the end of the trial in these fractions. These changes were most apparent in the fish oil-supplemented groups where total plasma HDL cholesterol and HDL_2 cholesterol concentrations increased, although these increases were not statistically significant. No consistent significant change occurred in HDL_3 cholesterol in any group. A statistical difference was observed between treatment groups in HDL_3 cholesterol but this reflects the fact that groups had different levels at baseline rather than a difference as a result of intervention, as there was no significant treatment * repeat interaction.

Table 3.3.8 (ii) b Results of repeated measures analysis of total HDL, HDL_3 and HDL_2 cholesterol

Fatty Acid	Repeat Effect †	Treatment Effect ‡	Treatment*Repeat §
HDL Cholesterol	0.0176	0.0010	NSD
HDL_3 Cholesterol	NSD	0.0008	NSD
HDL_2 Cholesterol	0.0367	0.0283	NSD

† Significance of pre- versus post-trial changes in plasma lipoprotein concentrations;
‡ Significance of difference between treatment groups;
§ Significance of change following intervention between groups.

Table 3.3.8 (i) b Plasma HDL, HDL_3 and HDL_2 cholesterol concentrations pre- and post-trial

n-3 PUFA		0.0g	0.3g	0.6g	0.9g
HDL cholesterol	*Pre-*	1.538 (0.500)	1.071 (0.266)	1.182 (0.245)	1.396 (0.265)
	Post-	1.550 (0.578)	1.200 (0.321) †	1.207 (0.272)	1.585 (0.359)
HDL_3 cholesterol	*Pre-*	1.127 (0.482)	0.847 (0.220)	1.017 (0.220)	1.087 (0.244)
	Post-	1.200 (0.350)	0.838 (0.276)	0.942 (0.274)	1.272 (0.343)
HDL_2 cholesterol	*Pre-*	0.411 (0.313)	0.225 (0.174)	0.169 (0.140)	0.309 (0.178)
	Post-	0.350 (0.316)	0.365 (0.212)	0.265 (0.127)	0.353 (0.242)

Values reported represent mean (standard deviation). Significance of difference during supplementary period investigated using two-way ANOVA.

† Least Significant Difference (LSD) Post Hoc Tests

† $P < 0.01$ - significant change from baseline.

3.4 Discussion

Fish oil supplementation had no adverse effect on glycaemic control in our group of healthy volunteers, as demonstrated by no change in either glucose or insulin concentrations throughout the study period. Previous studies report both positive and negative effects of n-3 fatty acids on glycaemic control, particularly in diabetics (Friedberg *et al*, 1998). However, in this meta-analysis it was noted that low doses (< 3 g/d) would effectively lower plasma TAG concentrations without effecting glycaemic control. Slightly higher doses (3.65 g/d) have recently been shown to have no adverse effect on plasma glucose and insulin concentrations in patients with normal plasma concentrations at baseline (Mori *et al*, 1999), which reflects our findings.

Haemoglobin concentrations remained stable throughout the experiment, indicating that no adverse effects from blood sampling occurred. No significant change was observed in body weights during the study period, which was to be expected as volunteers were advised to maintain their usual dietary habits throughout. The extra caloric load provided by the amount of oil consumed (0.9 g/d) would provide an extra 8 kcals/d or a total of 896 kcals throughout the study period should not contribute to weight gain, especially in the light of evidence from other studies where small doses of fish oil were given for six and nine months which should have resulted in an increase in body weights by 2 kg and 1.3 kg respectively, however no change in body weight was observed (Leaf *et al*, 1994, Eritsland *et al*, 1994).

The postprandial rise in plasma TAG following ingestion of a fat-rich test meal has previously been shown to be largely due to an increase in particle number (71%) and to a much smaller extent due to an increase in the amount of TAG carried per particle (29%) (Poapst *et al*, 1985). The use of specific markers for intestinally derived lipoproteins in the present study is of crucial importance, as TAG concentrations in the TRL fraction can reflect both intestinally-derived and hepatically-derived TAG. Apo B-48 is a protein unique to intestinally derived TAG and therefore can be used as a specific marker for intestinally-derived TAG or chylomicrons (CM). The increase in particle number following a meal is due to an increase in both apo B-48-containing lipoproteins (intestinally-derived TRL) and also apo B-100-containing particles (hepatically-derived VLDL) (Cohn *et al*, 1988, Potts *et al*, 1991). In the present study, TAG concentrations

increased in all subjects both in plasma and in TRL following ingestion of the fat-rich test meal. TAG concentrations in both plasma and TRL fractions showed a mean peak time of 4 hours postprandially, and returned to baseline values by 8 hours. Apo B-48 concentrations increased significantly from baseline at 4 hours, reflecting this increase in particle number. Overall, a significant correlation was observed between plasma apo B-48 and plasma TAG (r = 0.202, P = 0.0002) and also with overall plasma apo B-48 and TRL TAG concentrations (r = 0.164, P = 0.0042), which again is reflective of the fact that the vast majority of any increase in TAG production is due to an increase in particles, as reflected by an increase in apo B-48 concentration, with a much smaller amount owing to an increase in the amount of TAG carried per particle.

Fish oil supplementation lead to an attenuated postprandial TAG response in the highest-dose fish oil group. Previously prospective evidence revealed that non-fasting TAG concentration is a strong independent predictor of future myocardial infarction (Stampfer *et al,* 1996). In the present study, paired t-Test demonstrated that the maximum plasma TAG concentrations were significantly reduced post intervention (P = 0.05), however maximum TRL TAG concentrations were not significantly lowered. Plasma apo B-48 concentrations remained unaffected by fish oil supplementation even in the high dose fish oil group, both fasting and at peak TAG concentrations 4 hours postprandially. As already discussed, the postprandial rise in TAG is due to an increase in particles containing both apo B-48 and apo B-100. Fasting plasma TAG correlated significantly with TRL TAG response (AUC) both before (r = 0.827, P = 0.0001) and after (r = 0.577, P = 0.0001) the study period in the whole study group. This correlation was previously noted (Agren *et al,* 1996) indicating that following chronic fish oil supplementation, TRL TAG response could be expected to decrease in relation to the decrease in fasting plasma TAG concentrations. This decrease in TRL is most likely to be due to a decrease in VLDL TAG as the fish-oil induced reduction in peak plasma TAG concentration observed in the highest-dose fish oil group is not paralleled by a reduction in peak apo B-48 concentration at the same time point. Therefore it must be due to a reduction in TAG derived from the liver (VLDL TAG). Experimental studies have examined this theory and found that EPA inhibits TAG synthesis/secretion in rat and in rabbit hepatocytes, in HepG2 cells and in perfused rat livers (Lang and Davis 1990, Rustan *et al,* 1988, Benner *et al,* 1990, Wong *et al,* 1989, Wong *et al,*

1984, Zhang *et al*, 1993). This phenomenon has also been observed in human studies (Harris *et al*, 1990, Phillipson *et al*, 1985), which is consistent with our observations.

For years investigations have examined the possible mechanisms of the TAG-lowering effect of fish oils. It had previously been shown that high fasting VLDL concentrations decrease chylomicron clearance rate due to competition for lipoprotein lipase (Grundy and Mok, 1976). It is now believed that n-3 fatty acids accelerate TRL lipid clearance mainly by causing a reduction in VLDL production, which leads to decreased competition for lipolysis by LPL, allowing accelerated hydrolysis of incoming and competing chylomicrons (Nestle *et al*, 1984, Rustan *et al*, 1988, Westphal *et al*, 2000).

The lower doses of fish oil used in this study (0.3 g and 0.6 g) had no effect on plasma or TRL TAG concentrations either fasting or following the fat-rich test meal. A comprehensive review examining the effects of fish oils on plasma TAG concentrations showed that in 73% of studies TAG concentrations were significantly reduced in hyperlipidaemic volunteers, but only 50% studies in normolipidaemic volunteers achieved the same result (Harris, 1996). Thus it is not uncommon for a decrease in TAG concentrations with fish oil supplementation not to reach statistical significance, particularly in normolipidaemic volunteers, as was observed in our study.

Volunteers displayed a mean monophasic response to the fat-rich test meal, which parallels responses to single meals after an overnight fast previously recorded (Zampelas *et al*, 1994, Agren *et al*, 1996). Biphasic and multiphasic responses have also been reported (Williams *et al*, 1992, Fielding *et al*, 1996, Evans *et al*, 1998) when a meal is fed within a few hours of a previous meal, reflecting the entry of TAG stored in the gut from the first meal. Biphasic responses have also been reported in response to a single, mixed, high-carbohydrate, high-fat meal (Shishehbor *et al*, 1998). However, the test meal used in the present study had a low carbohydrate content (18g), producing the standard monophasic response observed in other studies. The effect of test meal composition on postprandial TAG response is further discussed in Chapter 1.

Cholesterol concentrations were not altered postprandially by fish oil supplementation, which is consistent with previous findings (Sanders *et al*, 1989, Blonk *et al*, 1990, Harris *et al*, 1991). Even though the placebo group showed a reduction from baseline post intervention at some time points, fasting and AUC values were not affected. The slight reduction observed at these time points was probably due to the

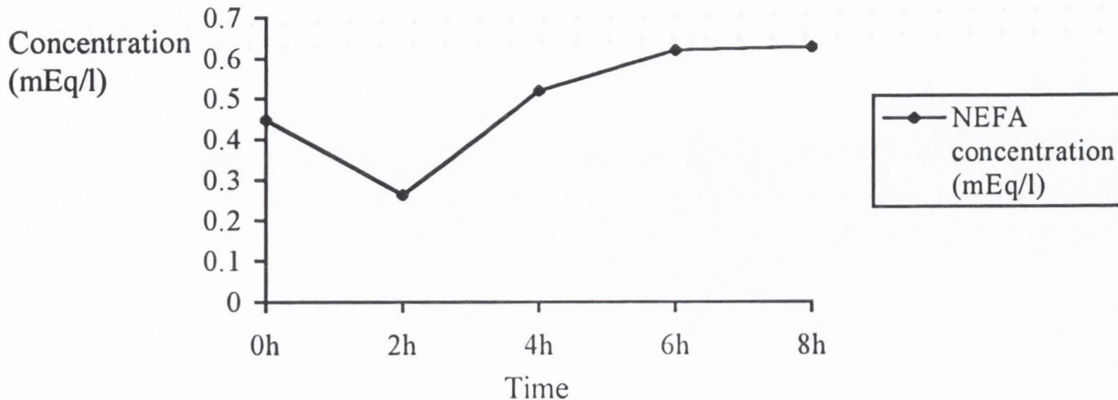
significant subject variability observed (P = 0.0001). In the past, many large epidemiological studies examining risk factors for CHD did not measure plasma TAG concentrations and concentrated on examining plasma cholesterol concentrations (Shekelle *et al,* 1981, Kromhout *et al,* 1985, Burr *et al,* 1989, Ascherio *et al,* 1995). In the early 1970's the first report was published linking fasting TAG concentrations and newly occurring incidents of CHD. Since then this relationship has been further extensively examined. Two recent meta-analyses of population-based prospective studies examining the magnitude of this association in the general population revealed that TAG is indeed a risk factor for cardiovascular disease (Hokanson and Austin, 1996, Austin *et al,* 1998), some reporting an even greater correlation than that reported between cholesterol and CHD. These studies revealed that this association is independent of HDL cholesterol concentrations. The Diet and Reinfarction Trial (DART) (Burr 1989) revealed that supplementation with fish or fish oil reduced the incidence of new CHD events and overall mortality; in that study TAG concentrations reduced as a result of supplementation but cholesterol concentrations remained unchanged, similar to the findings in our study. De Lorgeril *et al,* (1994) compared the effect of a Mediterranean alpha-linolenic acid-rich diet to the usual post-infarct diet in a prospective, single blind secondary trial in 605 patients. They again found that despite no significant changes in total cholesterol concentrations overall mortality reduced in the alpha-linolenic acid-rich group compared with usual-diet group. Large, prospective population-based studies in the Netherlands, Sweden and in the United States have shown an inverse relationship between fish consumption and risk of death from coronary heart disease in populations with a wide range of fish intakes (Kromhout *et al,* 1985, Norell *et al,* 1986, Shekelle *et al,* 1985), but the same relationship was not found in the Physicians' Health Study in the United States (Morris *et al,* 1995). The most plausible explanation for the negative findings from this study was that the average consumption of n-3 PUFA in the study group was all ready high, which would reduce the likelihood of a dose-response relationship being observed (Nestel, 2000). The positive studies have shown that as little as one to two fish dishes per week may be of preventative value in relation to coronary heart disease (Kromhout *et al,* 1985). Increasing fish intake to five to six dishes a week does not seem to incur any reduced risk of CHD in men who are initially free of cardiovascular disease (Ascherio *et al,* 1995). This study also showed that total cholesterol concentrations are unaffected by level of n-3 fatty acid intake, as no

difference was observed in serum concentrations when subjects were divided according to quintiles of n-3 fatty acid intake. Our results reflected these findings, as we found no difference in total cholesterol concentrations between groups following intervention.

Total HDL and HDL_2 concentrations increased in the highest-dose fish oil group, however this did not reach statistical significance. No significant changes were observed in any other group in plasma total and HDL cholesterol, HDL_2 or HDL_3 cholesterol, which is consistent with other findings using low doses of fish (Bronsgeest-Schoute *et al,* 1981, Fehily *et al,* 1983, Davidson and Gold 1988, Blonk *et al,* 1990). Even the Eskimo diet containing large amounts of n-3 fatty acids does not lead to extremely low cholesterol values (Bang and Dyerberg, 1972).

NEFA concentrations were unaffected by fish oil supplementation. The pattern of the postprandial NEFA response observed in the present study (Fig. 3.4.1) has been previously reported (Gibney and Daly, 1994, Frayn, 1998). NEFA concentrations sharply dropped postprandially, reaching minimum concentrations by 2 hours but gradually rose reaching baseline values by 8 hours. This is a well-recognised pattern of plasma NEFA concentrations. In the fasting state, hormone sensitive lipase (HSL) hydrolyses adipose tissue TAG to release free fatty acids (NEFA) into circulation. On ingestion of a meal, HSL activity is suppressed in response to increased insulin activity. NEFA are no longer released from adipose tissue into circulation to the same extent as happens in the fasting state. As insulin activity decreases postprandially, HSL activity increases and NEFA concentrations eventually return to baseline values. Also in this postprandial period, LPL acts on circulating TRL TAG releasing NEFA into circulation; it is estimated that up to half of hydrolysed TRL fatty acids are not taken directly into adipose tissue but enter circulation as NEFA (Binnert *et al,* 1996, Frayn *et al,* 1996).

Fig. 3.4.1 Postprandial NEFA response



In conclusion, the present data indicate that a small intake of fish oil has a lowering effect on fasting and postprandial TAG concentrations. Studies using similar doses of fish oil (Davidson and Gold, 1988, Leaf and Weber, 1988) did not find the same hypotriacylglycerolaemic effect. However, the longer duration of the present study lends a plausible reason for the more potent effect of the low dose of fish oils observed. Pre- and post- prandial lipoproteins have been implemented in a causal role in atherogenesis (Zilversmit, 1995). Supplementation with low and acceptable doses of fish oil for a sufficient length of time, as was used in the present study, leads to the attenuation of postprandial lipaemia in normolipidaemic volunteers. In the light of this evidence implicating the role of low doses of fish oils in reducing CHD risk, further intervention studies are warranted to elucidate the optimal dose and supplementation period which would benefit hypertriacylglycerolaemic volunteers in the reduction of CHD risk.

Habitual intakes of fish in this country are low, however prospective evidence from the DART trial has shown a protective effect of low doses of fish oil (0.34g n-3 PUFA/d) from death from CHD (Burr *et al,* 1989). As discussed in Chapter 1, the recommendations set out by FSAI, FAO/WHO, BNF, ISSFAL and SCF greatly exceed current estimated Irish intakes. As this and other studies have shown, the health benefits of low doses of fish oils, even lower than that recommended by scientific bodies, are particularly relevant in Ireland where cardiovascular disease including heart disease,

stroke and related diseases accounted for 43% all deaths in 1997 (Department of Health and Children, 1998). CHD resulted in the largest number of deaths accounting for almost one quarter of all deaths at all ages. Encouragement to eat oily fish, to take fish oil supplements and working with industry to produce acceptable functional foods are all strategies which should be taken on board to help increase the intake of n-3 PUFA in the country and improve the n-6: n-3 ratio of fatty acids in our diet, as recently recommended by several authors (Connor 2000, Sanders 2000, Simopoulos 1999).

Chapter Four

Biomarkers of Dietary Fat; Acute and Chronic

4.1 Introduction

In the acute setting, researchers have shown that the fatty acid composition of a test meal is reflected in the fatty acid composition of TRL in the postprandial period (Gibney and Daly 1994, Phan *et al,* 1999). The addition of even small amounts of fish oil to a fat-containing meal has been shown to attenuate the postprandial response when compared to the same meal in which the fish oil is replaced by olive oil (Yahia and Sanders, 1996). PUFA-rich background diets, particularly n-3 PUFA-rich diets, cause a reduction in residence time and in concentration of TRL particles in circulation in the postprandial period. Lipoprotein lipase (LPL) is the rate-limiting enzyme responsible for the removal of TAG from circulation and therefore can influence the magnitude and duration of the postprandial response. Animal studies have shown that background diets rich in MUFA increase post heparin LPL activity relative to background diets rich in SFA (Groot *et al,* 1988, van Heek and Zilversmit, 1990). Results from human studies are less clear, with little consensus between authors regarding the effect of background diet fatty acid composition on LPL activity. LPL activity is not the only factor which influences the rate of TAG clearance from circulation; substrate fatty acid composition is also important. PUFA-rich background diets produce larger CM, which are preferred substrates for LPL and are therefore cleared more quickly from circulation (Groot *et al,* 1988, Levy *et al,* 1990). Therefore it was decided to analyse the fatty acid composition of TRL before and after intervention in both the fasting and postprandial period, to examine the effects of supplementation with a low dose of n-3 PUFA on their fatty acid composition and to elucidate whether the composition of TRL affects their rate of clearance following the ingestion of a standard n-6 PUFA meal. A more detailed discussion of the effect of background diet on LPL activity and postprandial lipaemia in animal and human studies is available in Chapter 1.

The use of biomarkers which reflect dietary fat quality and quantity are frequently employed in intervention studies, either complimentary to, or instead of traditional dietary assessment methods. Change in fatty acid composition of a particle can also affect its function, for example, as already discussed, TRL enriched with n-3 PUFA are a better substrate for LPL and are therefore removed from more quickly from circulation. It is thought that the differential incorporation of EPA and DHA into cell membrane and

lipoprotein fractions may be related to their varying metabolic effects. For example, EPA and DHA-rich oils have been reported to be effective in reducing serum TAG concentrations, whilst only DHA is effective in increasing total HDL and HDL_2 (Sanders *et al,* 1989). Fatty acid alterations in phospholipids and cholesterol, which are structural components of cell membranes in the brain, may induce changes in neurotransmitter systems (Maes *et al,* 1996). Previous studies have reported a much smaller percentage of DHA in CE than phospholipids, indicating a preferential incorporation of DHA into phospholipids following supplementation with fish oil (Subbaiah *et al,* 1993). The same authors observed no difference in the incorporation of EPA into phospholipids and cholesteryl esters. The preferential incorporation of DHA into phospholipids relative to CE has also been reported in other studies (Holub *et al,* 1987, Rapp *et al,* 1991). DHA is thought to be a poor substrate for LCAT (EC 2.3.1.43) (Subbaiah *et al,* 1993), the enzyme involved in CE formation, which may explain the discrepancy between concentrations of this fatty acid between the two fractions. However CE fatty acid composition has been shown to reflect dietary fatty acid intake patterns (Nikkari *et al,* 1986, Simon *et al,* 1995).

The present study examines how supplementation with low doses of n-3 PUFA is reflected in the concentration of these fatty acids in TRL, platelet phospholipids and in cholesteryl esters, and appraises their use as biomarkers in the acute setting (TRL fatty acids) and as biomarkers for long-term n-3 PUFA intake (platelet phospholipids and cholesteryl esters).

4.2 Methods

4.2.1 Study design

This study was approved by The Ethics Committee of the Federated Dublin Voluntary Hospitals and was conducted in the Nutrition Laboratory, Department of Clinical Medicine, at the Trinity Centre Medical School, St. James' Hospital. Recruitment details, screening information and treatment group information pertaining to volunteers have previously been outlined in Chapter 3, section 3.2.1.

4.2.2 Collection and storage of samples

Details regarding the collection and storage of samples have previously been outlined in Chapter 3, section 3.2.2. Plasma for CE fatty acid analysis was collected from lithium heparin and stored as described in Chapter 3 (3.2.2). Blood was drawn into evacuated tubes containing 0.105M sodium citrate (v/v: 1:10) to investigate platelet phospholipid fatty acid composition. Isolation of TRL was performed within 24 h of collection and subsequent samples were stored at -70°C. A detailed description of all analytical methods is described in Chapter 2.

4.2.3 Biochemical parameters investigated

The following parameters were investigated pre- and post-trial in the fasting state:

- Platelet phospholipid fatty acid composition
- Plasma cholesteryl ester fatty acid composition
- Plasma TRL fatty acid composition

Postprandial Study Days

TRL fatty acid composition was investigated in placebo and highest dose groups following ingestion of a fat-rich test meal pre- and post-intervention at 4 hours. Details have all ready been outlined in Chapter 3, section 3.2.2.
A detailed description of all analytical methodologies is described in Chapter 2.

4.2.4 Statistical analysis

All statistical analyses were completed with the Apple Macintosh compatible statistical package Datadesk 5.0.1 (Data Description, Inc., Ithaca, N.Y., USA.). Results are presented as mean values with their standard deviations. All data was transformed to give the data a normal Gaussian distribution (Table 4.2.2).

Table 4.2.2 Data Transformations

Parameter	Transformation
Platelet Phospholipid fatty acids	Natural log (ln)
Cholesteryl ester fatty acids	Natural log (ln)
TRL fatty acids	Natural log (ln)

Statistical significance was accepted at a level of $P < 0.05$. The total study group was divided according to supplementation level. Repeated measures analysis of variance (ANOVA) with least significant difference contrasts was used to assess differences between groups, using group as the dependent variable. Two-way analysis of variance investigated any differences between parameters pre- and post-trial. 2-sample t-Test was used to describe differences between two fatty acid fractions post-trial.

4.3 Results

4.3.1 (i) Platelet phospholipid saturated and monounsaturated fatty acids

Platelet phospholipid saturated and monounsaturated fatty acid composition at weeks 0, 4, 8 and 16 of the study groups consuming 0.0g, 0.3g, 0.6g, 0.9g n-3 PUFA are presented in Tables 4.3.1 (i) a – 4.3.1 (i)d respectively. PUFA platelet phospholipid compositions are presented in Tables 4.3.1 (ii)a – 4.3.1 (ii)d.

Results of repeated measures analysis of variance for saturated and monounsaturated fatty acids are presented in Table 4.3.1 (i) e. Platelet phospholipid palmitic acid (C16:0) and stearic acid (C18:0) showed no significant repeat effect, confirming no increase throughout the study period. However, a significant difference was observed between groups as demonstrated by a significant treatment effect in these fatty acids. A significant treatment * repeat effect was also observed in palmitic and stearic acid levels, demonstrating the significance of change following intervention between groups.

Neither palmitoleic acid (C16: 1) nor oleic acid (C18: 1) showed a significant repeat effect during the study. However, there was a significant difference between treatment groups in both fatty acids, and C16: 1 levels displayed significant change following intervention between groups. No significant treatment * repeat effect was observed in platelet phospholipid oleic acid concentration, which reflects the fact that groups had different levels of this fatty acid at baseline rather than a difference as a result of intervention.

Table 4.3.1 (i) e **Results of Repeated Measures Analysis of Variance Saturated and Monounsaturated Platelet Phospholipid Fatty Acids**

Fatty Acid	Repeat Effect †	Treatment Effect ‡	Treatment * Repeat§
C16:0	NSD	0.0208	≤ 0.0001
C18:0	NSD	0.0282	≤ 0.0001
C16:1	NSD	0.0103	≤ 0.0001
C18:1	NSD	0.0017	NSD

† Significance of pre- versus post-trial changes in platelet phospholipid fatty acid composition; ‡ Significance of difference between treatment groups; § Significance of change following intervention between groups.

4.3.1 (ii) Platelet phospholipid polyunsaturated fatty acids

Results of repeated measures ANOVA are summarised in Table 4.3.1 (ii) e. Significant pre-versus post-trial changes were observed in levels of linoleic acid (C18:2 *n*-6), arachidonic acid (C20:4 n-6) and C22:3 n-6. There was also a significant difference between treatment groups in these fatty acids. A significant treatment * repeat effect was observed in C18:2 n-6 and C22:3 n-6 fatty acid levels, displaying a significant difference in these fatty acids between groups. The most marked change in linoleic acid levels from baseline was observed in the highest dose fish-oil group ($P \leq 0.0001$). However, levels of arachidonic acid, which is the most important n-6 PUFA, did not change significantly following intervention between groups. There was a significant difference between treatment groups in total n-6 fatty acid level, but no change pre- and post intervention or change following intervention between groups was observed.

Platelet phospholipid EPA (C20:5 *n*-3) levels were significantly altered as a result of fish oil supplementation, showing highly significant repeat effect, treatment effect and

treatment * repeat effect. The relationship between the dose of *n*-3 PUFA supplemented (*D*) and the level of EPA in platelet phospholipids (*PPE*) can be described using the regression equation:

$$D = 0.26 + 1.29\ (PPE)$$

In the highest dose fish-oil group a three-fold increase was observed in EPA levels by week 8 (from 0.778 to 2.313 w/w%), however this decreased slightly by week 16 to 2.199 w/w%. DHA (C22:6 n-3) and total *n*-3 fatty acid concentrations changed significantly pre- versus post-trial and also changed significantly following intervention between groups but the difference between treatment groups only approached significance. Again, in the highest dose fish-oil group the greatest change from baseline was observed in both DHA and total *n*-3 fatty acid concentrations, where significant increases from baseline were observed at weeks 4, 8 and 16 ($P \leq 0.0001$).

The relationship between dose of n-3 PUFA supplemented (*D*) and final DHA concentration in platelet phospholipids (*PPD*) can be described using the following:

$$D = -0.166 + 1.31\ (PPD)$$

Total n-6 concentrations changed significantly from baseline in the group consuming 0.9g fish oil only, showing a significant reduction by week 16 ($P \leq 0.001$). No difference was seen between treatment groups in the ratio of n-3: n-6 fatty acids, but significant pre- versus post-trial differences and also a significant change following intervention was observed in this fatty acid ratio, reflecting the highly significant increases in platelet phospholipid n-3 fatty acids at the expense of n-6 fatty acids, particularly in the highest-dose fish oil group.

Table 4.3.1 (ii) e Results of repeated measures analysis of polyunsaturated platelet phospholipid fatty acids

Fatty Acid	Repeat Effect †	Treatment Effect ‡	Treatment * Repeat§
C18:2 n-6	≤ 0.0001	≤ 0.0001	≤ 0.0001
C20:4 n-6	0.0222	≤ 0.0001	NSD
C20:5 n-3	≤ 0.0001	0.0002	≤ 0.0001
C22:3 n-6	0.0182	0.0005	0.0017
C22:6 n-3	≤ 0.0001	0.0611	≤ 0.0001
Total n-3	0.0004	0.0798	0.0234
Total n-6	NSD	0.0004	NSD
n-3 : n-6	0.0004	NSD	0.0121
PUFA			

† Significance of pre- versus post-trial changes in platelet phospholipid fatty acid composition; ‡ Significance of difference between treatment groups; § Significance of change following intervention between groups.

Table 4.3.1 (i) a Saturated and monounsaturated fatty acid composition of platelet phospholipids (w/w%) in placebo group

Fatty Acid	Week 0	Week 4	Week 8	Week 16
16:0	14.237 (2.154)	13.531 (1.786)	13.636 (1.959)	13.099 (1.664)
16:1	0.732 (0.247)	1.179 (0.700) †	0.579 (0.136)	0.617 (0.142)
18:0	19.131 (1.665)	18.234 (2.428)	19.875 (1.678)	21.469 (1.986) ‡
18:1	15.673 (2.070)	14.627 (1.917)	15.778 (1.416)	15.225 (1.829)

Values reported represent mean (standard deviation) platelet phospholipid fatty acid composition (w/w%). Significance of difference during supplementary period investigated using two-way ANOVA.
† ‡ The Least Significant Difference (LSD) Post Hoc Tests
† $P < 0.01$, ‡ $P < 0.001$ - significant change from baseline.

Table 4.3.1 (i) b Saturated and monounsaturated fatty acid composition of platelet phospholipids (w/w%) in group consuming 0.3 g fish oil

Fatty Acid	Week 0	Week 4	Week 8	Week 16
16:0	12.828 (1.284)	13.677 (2.949)	12.783 (1.267)	13.702 (1.530)
16:1	0.628 (0.178)	0.626 (0.256)	0.632 (0.223)	0.731 (0.117)
18:0	19.008 (1.465)	19.298 (2.588)	18.417 (1.435)	17.699 (1.497) †
18:1	14.092 (1.016)	13.847 (3.149)	14.168 (1.065)	14.430 (0.834)

Values reported represent mean (standard deviation) platelet phospholipid fatty acid composition (w/w%). Significance of difference during supplementary period investigated using two-way ANOVA.
† The Least Significant Difference (LSD) Post Hoc Tests
† $P < 0.01$ - significant change from baseline.

Table 4.3.1 (i) c **Fatty acid composition of platelet phospholipids (w/w%) in group consuming 0.6 g fish oil**

Fatty Acid	Week 0	Week 4	Week 8	Week 16
16:0	12.550 (1.466)	12.022 (1.825)	12.855 (1.217)	13.729 (1.497)
16:1	0.513 (0.179)	0.538 (0.265)	0.669 (0.172)	0.736 (0.133) †
18:0	19.288 (2.086)	19.181 (1.223)	18.408 (1.451)	18.219 (1.477)
18:1	13.811 (1.238)	13.828 (1.041)	14.356 (1.260)	14.618 (1.556)

Values reported represent mean (standard deviation) platelet phospholipid fatty acid composition (w/w%). Significance of difference during supplementary period investigated using two-way ANOVA.
† The Least Significant Difference (LSD) Post Hoc Tests
† $P < 0.01$ - significant change from baseline.

Table 4.3.1 (i) d Fatty acid composition of platelet phospholipids (w/w%) in group consuming 0.9 g fish oil

Fatty Acid	Week 0	Week 4	Week 8	Week 16
16:0	14.597 (1.662)	14.414 (1.275)	14.448 (0.948)	13.042 (1.718) †
16:1	0.712 (0.136)	1.006 (0.510)	0.566 (0.130)	0.604 (0.092)
18:0	19.197 (2.121)	19.071 (1.692)	18.464 (1.748)	21.807 (3.339) ‡
18:1	15.671 (1.209)	15.871 (1.376)	15.559 (1.188)	14.750 (1.732)

Values reported represent mean (standard deviation) platelet phospholipid fatty acid composition (w/w%). Significance of difference during supplementary period investigated using two-way ANOVA.
† ‡ Least Significant Difference (LSD) Post Hoc Tests
† $P < 0.01$, ‡ $P < 0.001$ - significant change from baseline.

Table 4.3.1 (ii) a Polyunsaturated fatty acid composition of platelet phospholipids (w/w%) in placebo group

Fatty Acid	Week 0	Week 4	Week 8	Week 16
18:2 n-6	6.450 (0.747)	6.862 (0.737)	6.286 (0.544)	5.919 (0.514) †
20:4 n-6	29.711 (3.858)	30.452 (2.963)	31.850 (2.946)	31.455 (4.217)
20:5 n-3	0.989 (0.487)	1.034 (0.336)	1.044 (0.390)	1.018 (0.290)
22:3 n-6	2.440 (0.675)	2.755 (0.856)	2.672 (0.588)	2.838 (0.659)
22:6 n-3	2.533 (0.568)	2.678 (0.691)	2.653 (0.530)	2.594 (0.612)
Total n-3	4.700 (1.063)	5.147 (0.873)	4.144 (0.974)	4.700 (1.286)
Total n-6	41.167 (4.681)	41.407 (3.443)	42.416 (3.257)	41.190 (4.502)
n-3 : n-6	0.103 (0.023)	0.127 (0.027)	0.099 (0.030)	0.123 (0.040)

Values reported represent mean (standard deviation) platelet phospholipid fatty acid composition (w/w%). Significance of difference during supplementary period investigated using two-way ANOVA.
† The Least Significant Difference (LSD) Post Hoc Tests
† $P < 0.01$ - significant change from baseline.

Table 4.3.1 (ii) b Polyunsaturated fatty acid composition of platelet phospholipids (w/w%) in 0.3 g fish oil group

Fatty Acid	Week 0	Week 4	Week 8	Week 16
18:2 n-6	5.111 (0.574)	5.420 (0.791) †	5.174 (0.598)	5.371 (0.716)
20:4 n-6	34.894 (2.037)	34.271 (3.485)	35.415 (2.102)	33.960 (1.504)
20:5 n-3	0.665 (0.240)	1.086 (0.243) §	1.142 (0.329) §	1.289 (0.441) §
22:3 n-6	3.239 (0.619)	2.984 (0.608)	3.166 (0.497)	3.075 (0.417)
22:6 n-3	2.660 (0.537)	2.840 (0.834)	2.995 (0.509) †	2.984 (0.564) †
Total n-3	3.905 (0.543)	4.574 (0.735) †	4.705 (0.636) ‡	4.980 (0.784) †
Total n-6	45.017 (1.930)	44.327 (3.380)	45.411 (2.225)	44.375 (1.430)
n-3 : n-6	0.087 (0.012)	0.101 (0.018) †	0.103 (0.015) †	0.112 (0.017) ‡

Values reported represent mean (standard deviation) platelet phospholipid fatty acid composition (w/w%). Significance of difference during supplementary period investigated using two-way ANOVA.
† ‡ § The Least Significant Difference (LSD) Post Hoc Tests
† $P < 0.05$, ‡ $P < 0.001$, § $P < 0.0001$ - significant change from baseline.

Table 4.3.1 (ii) c Polyunsaturated fatty acid composition of platelet phospholipids (w/w%) in 0.6 g fish oil group

Fatty Acid	Week 0	Week 4	Week 8	Week 16
18:2 n-6	5.263 (0.346)	5.255 (0.433)	5.337 (0.476)	5.328 (0.495)
20:4 n-6	34.209 (2.216)	33.783 (2.341)	34.163 (1.827)	33.325 (3.097)
20:5 n-3	0.958 (0.466)	1.431 (0.512) †	1.505 (0.681) †	1.553 (0.435) §
22:3 n-6	3.138 (0.630)	2.716 (0.505) †	2.741 (0.521) †	2.752 (0.657) †
22:6 n-3	3.088 (0.724)	3.062 (0.548)	3.264 (0.687) †	3.293 (0.571) †
Total n-3	4.742 (1.257)	4.758 (0.732)	5.511 (1.052)	5.409 (1.018) †
Total n-6	44.302 (2.483)	43.289 (2.619)	43.753 (1.894)	43.622 (2.852)
n-3 : n-6	0.109 (0.034)	0.110 (0.020) †	0.129 (0.029)	0.120 (0.024) †

Values reported represent mean (standard deviation) platelet phospholipid fatty acid composition (w/w%). Significance of difference during supplementary period investigated using two-way ANOVA.
† ‡ § The Least Significant Difference (LSD) Post Hoc Tests
† $P < 0.05$, ‡ $P < 0.001$, § $P < 0.0001$ - significant change from baseline.

Table 4.3.1 (ii) d Polyunsaturated fatty acid composition of platelet phospholipids (w/w%) in 0.9 g fish oil group

Fatty Acid	Week 0	Week 4	Week 8	Week 16
18:2 n-6	6.520	6.790	6.114	5.736
	(0.612)	(0.741)	(0.769) †	(0.592) §
20:4 n-6	30.594	29.537	32.004	31.148
	(2.649)	(2.822)	(2.716)	(4.262)
20:5 n-3	0.778	1.697	2.313	2.199
	(0.392)	(0.454) §	(0.534) §	(0.429) §
22:3 n-6	2.730	2.209	2.386	2.235
	(0.572)	(0.478) ‡	(0.493) †	(0.408) ‡
22:6 n-3	2.251	2.839	3.165	3.193
	(0.604)	(0.666) §	(0.708) §	(0.679) §
Total n-3	3.880	5.808	5.940	6.062
	(0.861)	(0.977) §	(1.164) §	(0.953) §
Total n-6	43.130	40.102	42.058	40.433
	(1.648)	(2.710) †	(2.548)	(4.573) †
n-3 : n-6	0.094	0.148	0.142	0.153
	(0.021)	(0.030) §	(0.030) §	(0.037) §

Values reported represent mean (standard deviation) platelet phospholipid fatty acid composition (w/w%). Significance of difference during supplementary period investigated using two-way ANOVA.
† ‡ § Least Significant Difference (LSD) Post Hoc Tests
† $P < 0.05$, ‡ $P < 0.001$, § $P < 0.0001$ - significant change from baseline.

4.3.2 Cholesteryl ester fatty acid composition

4.3.2 (i) Saturated, monounsaturated and polyunsaturated fatty acids

Cholesteryl ester fatty acid composition is presented in Tables 4.3.2 (i) and 4.3.2 (ii). There was no difference in saturated or monounsaturated fatty acids between groups, as displayed by repeated measures analysis of variance. A clear dose-response relationship was observed in cholesteryl ester EPA and DHA composition from supplementation with varying low doses of fish oil (Table 4.3.2 (ii) and Fig. 4.3.2 (i)). For CE EPA (CEE) concentrations, this relationship can be described using the equation

$$D = 0.36 + 0.56\ (CEE), \qquad \textit{where } D\textit{=dose}$$

and the relationship between amount of n-3 PUFA supplemented (D) and the level of DHA in CE (CED) can be described as follows:

$$D = 0.47 + 0.30\ (CED)$$

CE EPA concentrations were greater than placebo at all supplementation levels, and this reached statistical significance with supplementation doses of 0.6g and 0.9g fish oil, as demonstrated by repeated measures analysis of variance (ANOVA). DHA concentrations were also greater in all supplementation groups when compared with placebo (Fig. 4.3.2 (ii)). This difference was significant at all supplementation levels, again as demonstrated by ANOVA ($P < 0.05$).

Table 4.3.2 (i) **Fatty acid composition of cholesteryl esters following supplementation with varying doses of fish oils for 16 weeks - saturated and monounsaturated fatty acids**

Dose fish oil	0.0g	0.3g	0.6g	0.9g
16:0	13.77 (4.93)	16.95 (6.94)	11.94 (2.52)	14.83 (6.64)
16:1	5.93 (3.87)	6.60 (2.53)	6.55 (4.05)	6.87 (6.17)
18:0	3.59 (2.31)	4.54 (2.17)	3.94 (1.76)	3.46 (1.79)
18:1	15.01 (3.27)	11.70 (2.70)	14.29 (3.32)	13.44 (4.32)

Values reported represent mean (standard deviation) cholesteryl ester fatty acid composition (w/w%) following supplementation with fish oil for 16 weeks. Significance of difference between groups following supplementation was assessed using two-way ANOVA.
NSD between groups

Table 4.3.2 (ii) **Fatty acid composition of cholesteryl esters following supplementation with varying doses of fish oils for 16 weeks - polyunsaturated fatty acids**

Fatty Acid	0.0g	0.3g	0.6g	0.9g
18:2n-6	38.43 (8.82)	33.09 (9.34)	28.85 (11.53) *	37.63 (8.46)
18:3n-3	1.43 (1.22)	1.45 (1.11)	2.15 (2.15)	1.69 (1.76)
18:4n-3	1.87 (1.89)	2.54 (2.27)	2.04 (1.19)	2.10 (2.05)
20:4n-6	2.13 (1.24)	1.58 (0.96)	2.20 (0.82)	1.84 (1.15)
20:5n-3	1.10 (0.43)	1.76 (0.77)	1.97 (0.88) *	2.19 (0.80) *
22:6n-3	0.90 (0.75)	1.79 (0.93) *	2.02 (1.55) *	2.29 (1.67) *

Values reported represent mean (standard deviation) cholesteryl ester fatty acid composition (w/w%) following supplementation with fish oil for 16 weeks. Significance of difference between groups following supplementation was assessed using two-way ANOVA.

* $P < 0.05$ - significantly different from placebo

4.3.3 TRL fatty acid composition

4.3.3 (i) Saturated and monounsaturated fatty acids

TRL fatty acid composition is presented in Tables 4.3.3 (i) - 4.3.3 (iii). TRL palmitic acid (C16:0) concentrations decreased significantly from fasting following the PUFA-rich test meal at 4 hours postprandially in both groups. Concentrations of this fatty acid were not affected by intervention. TRL stearic acid (C18:0) concentrations showed significant subject variability on repeated measures analysis of variance ($P \leq 0.0001$) however concentrations of this fatty acid did not change significantly postprandially or as a result of intervention. Repeated measures ANOVA of TRL C16:1 revealed significant subject variability ($P \leq 0.0001$). Both pre- and post-trial concentrations of this fatty acid were significantly lower at 4 hours than fasting in both placebo and high-dose fish oil groups. Concentrations of TRL oleic acid (C18:1) were not affected by fish oil supplementation, however they did change significantly postprandially in both groups pre- and post-trial, showing a reduction from fasting at 4 hours, as demonstrated by repeated measures ANOVA.

4.3.3 (ii) Polyunsaturated fatty acids

TRL linoleic acid concentrations (C18:2:6) were examined using repeated measures ANOVA and showed significant subject variability ($P = 0.0199$). Concentrations of this fatty acid increased significantly in both groups 4 hours following the test meal both pre- and post trial. Concentrations of this fatty acid were not affected by supplementation, except at 4 hours in the placebo group which showed an increase from baseline ($P = 0.0318$). Using the same statistical model, significant subject variability was observed for TRL arachidonic acid (C20:4 n-6) ($P = 0.0199$), however concentrations of this fatty acid were not affected by the ingestion of the test meal or by intervention in either group.

TRL EPA concentrations showed significant between-person variability (P = 0.0029), but were not affected by the ingestion of the test meal. Concentrations of this fatty acid increased significantly as a result of intervention in the group consuming 0.9g fish oil, showing significant changes from baseline at 0 hours (P = 0.00008) and at 4 hours (P = 0.000021). TRL EPA concentrations were not affected by intervention in the placebo group. TRL DHA concentrations exhibited significant subject variability (P ≤ 0.0001) and also showed significant treatment * repeat interactions (P = 0.0009), displaying a significantly different change following intervention between groups. The group consuming 0.9g fish oil showed significant increases in this fatty acid from baseline at both 0 hours and 4 hours (P = 0.0093 and 0.0186 respectively).

Summary variables for TRL fatty acids are presented in Table 4.3.3(iii). Total n-3 TRL fatty acids showed significant subject variability (P ≤ 0.0001) and also revealed significant treatment * repeat interactions (P ≤ 0.0001), showing the significance of difference following intervention between groups. The fish oil group showed significant increases in TRL total n-3 fatty acids post-trial from baseline at both 0 hours and 4 hours (P = 0.00068 and P = 0.00021 respectively).

At baseline and following intervention repeated measures ANOVA revealed that total n-6 concentrations increased following ingestion of the test-meal (P ≤ 0.0001 for both groups). Total TRL n-6 concentrations were not otherwise affected by intervention.

TRL n-3: n-6 ratio showed significant between-subject variability (P ≤ 0.0001). This ratio showed a significant reduction 4 hours postprandially in response to the ingestion of the test meal in both groups both before and after intervention. A significant change following intervention between groups was observed (P ≤ 0.0001), which transpired to be as a result of a significant increase in TRL n-3: n-6 ratio from baseline values at both 0 hours and 4 hours. TRL n-3: n-6 ratio was unaffected in the placebo group as a result of intervention.

Table 4.3.3 (i) TRL saturated and monounsaturated fatty acid composition (w/w%)

Group		0.0g fish oil		0.9g fish oil	
		0 h	4 h	0 h	4 h
C16:0	*Pre-*	24.958 (2.896)	18.111 (5.946)	23.774 (2.140)	17.678 (5.631)
	Post-	23.332 (2.696)	17.593 (2.553)	23.727 (3.194)	19.29 (3.621)
C18:0	*Pre-*	4.963 (2.146)	5.505 (1.865)	5.029 (2.252)	5.542 (3.087)
	Post-	5.094 (0.736)	5.332 (0.84)	5.470 (1.364)	6.056 (1.210)
C16:1	*Pre-*	4.441 (1.131)	3.009 (0.784)	4.207 (1.053)	2.366 (1.090)
	Post-	3.454 (1.678)	2.185 (0.898)	3.459 (1.385)	2.046 (0.855)
C18:1	*Pre-*	30.450 (4.145)	26.975 (4.815)	32.221 (4.822)	26.094 (4.066)
	Post-	31.456 (4.904)	23.949 (2.399)	29.367 (4.436)	23.277 (3.204)

Values reported represent mean (standard deviation) of study groups. Significance of difference during supplementary period investigated using repeated measures analysis of variance (ANOVA), with Least Significance Difference (LSD) post hoc testing.
NSD between groups

Table 4.3.3 (ii) TRL polyunsaturated fatty acid composition (w/w%)

Group		0.0g fish oil		0.9g fish oil	
		0 h	4 h	0 h	4 h
C18:2 n-6	*Pre-*	21.429 (3.749)	35.229 (5.688)	23.44 (2.95)	38.375 (8.693)
	Post-	23.246 (3.77)	40.203 (6.398) †	23.509 (3.578)	37.810 (8.128)
C20:4 n-6	*Pre-*	4.166 (1.365)	3.847 (1.264)	3.49 (0.874)	3.935 (1.889)
	Post-	3.786 (1.604)	3.488 (1.302)	3.225 (1.219)	3.081 (0.804)
C20:5 n-3	*Pre-*	0.978 (0.668)	0.959 (0.422)	0.861 (0.501)	0.703 (0.453)
	Post-	0.939 (0.543)	0.783 (0.571)	1.995 (1.282) §	1.856 (1.082) §
C22:6 n-3	*Pre-*	2.340 (1.327)	2.197 (1.147)	2.172 (1.409)	1.955 (0.88)
	Post-	2.009 (1.104)	1.961 (1.131)	3.221 (1.825) †	2.818 (1.17) †

Values reported represent mean (standard deviation) of study groups. Significance of difference during supplementary period investigated using repeated measures analysis of variance (ANOVA), with Least Significance Difference (LSD) post hoc testing.
† $P \leq 0.05$, ‡ $P \leq 0.001$, § $P \leq 0.0001$ - significant change from baseline

Table 4.3.3 (iii) Summary variables for TRL fatty acid composition (w/w%)

Group		0.0g fish oil		0.9g fish oil	
		0 h	4 h	0 h	4 h
Total n-3	*Pre-*	3.318 (1.995)	3.156 (1.570)	3.033 (1.910)	2.659 (1.333)
	Post-	2.947 (1.647)	2.745 (1.702)	5.216 (3.107) ‡	4.674 (2.252) ‡
Total n-6	*Pre-*	26.776 (5.616)	40.320 (7.648)	28.155 (4.286)	43.425 (11.393)
	Post-	28.631 (6.604)	44.795 (8.103)	27.792 (5.222)	41.921 (9.321)
n-3 : n-6	*Pre-*	0.124 (0.355)	0.078 (0.205)	0.108 (0.446)	0.061 (0.117)
	Post-	0.103 (0.249)	0.061 (0.210)	0.188 (0.595) ‡	0.112 (0.242) †

Values reported represent mean (standard deviation) of study groups. Significance of difference during supplementary period investigated using two-way analysis of variance (ANOVA), with Least Significance Difference (LSD) post hoc testing.

† $P \leq 0.01$, ‡ $P \leq 0.001$, § $P \leq 0.0001$ - significant change from baseline

4.3.4 Comparison; biomarkers of chronic fat intake

Fig. 4.3.4 (i) and 4.3.4 (ii) show the difference between platelet phospholipid and CE EPA and DHA concentrations at the end of the supplementation period. There was no significant difference between EPA concentrations at any supplementation level between the two groups, as demonstrated by 2-sample t-Tests. The same statistical test however revealed significant differences in DHA concentrations between CE and platelet phospholipids at all supplementation levels, including the placebo ($P < 0.05$).

Fig. 4.3.4 (i) **Incorporation of Fish Oil Supplements into Cholesteryl Esters & Platelet Phospholipids Following 16 Weeks' Supplementation**

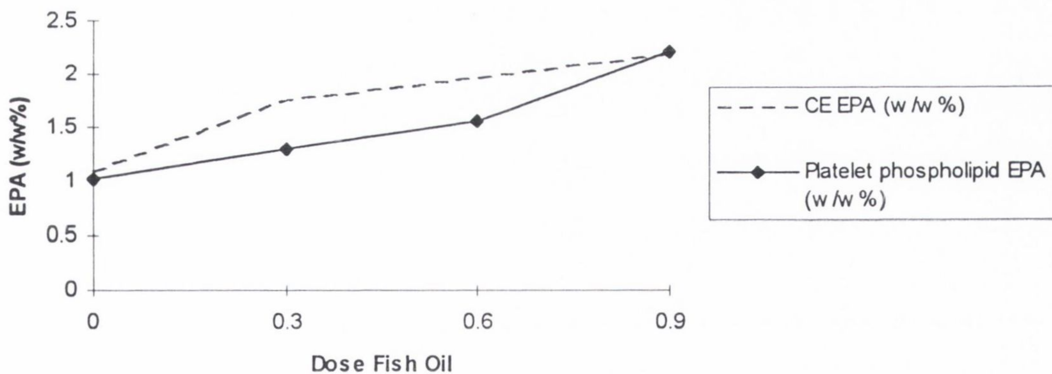
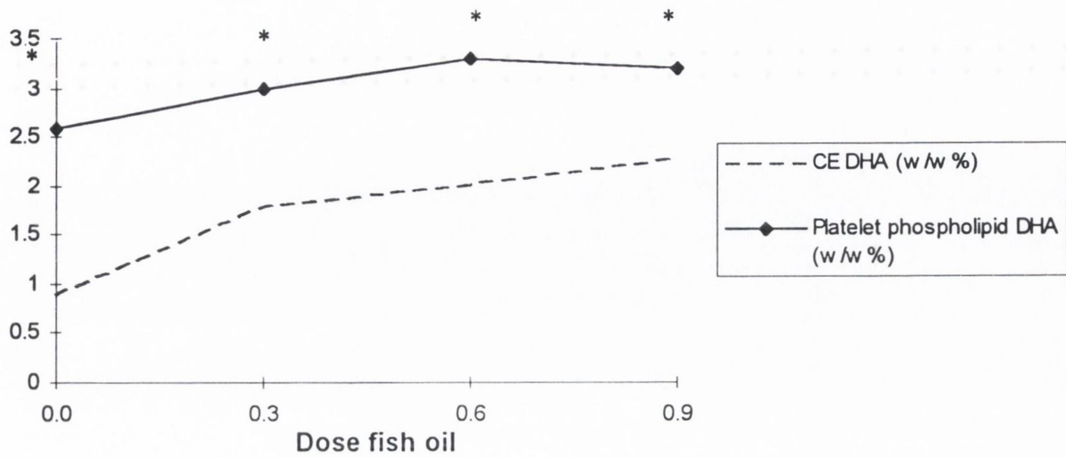


Fig. 4.3.4 (ii) **Incorporation of DHA from Fish Oils into Cholesteryl Esters & Platelet phospholipids Following 16 Weeks' Suplementation**



* P < 0.05, 2 sample t-Test

4.4 Discussion

4.4.1 Compliance

Reliable biomarkers of dietary intake have been commonly used as alternatives or complimentary to conventional dietary assessment methods (Subbaiah *et al,* 1993, Anttolainen *et al,* 1996, Andersen *et al,* 1996, Zock *et al,* 1997) or to validate these methods (Lemaitre *et al,* 1998). In the present study adherence to the supplementation program was assessed by measuring platelet phospholipid fatty acid composition at weeks 0, 4, 8 and 16 and by performing a capsule counts at 4-week intervals throughout the study, which also served to maintain motivation. There was no significant difference in compliance between groups and overall compliance was excellent (95 – 98%).

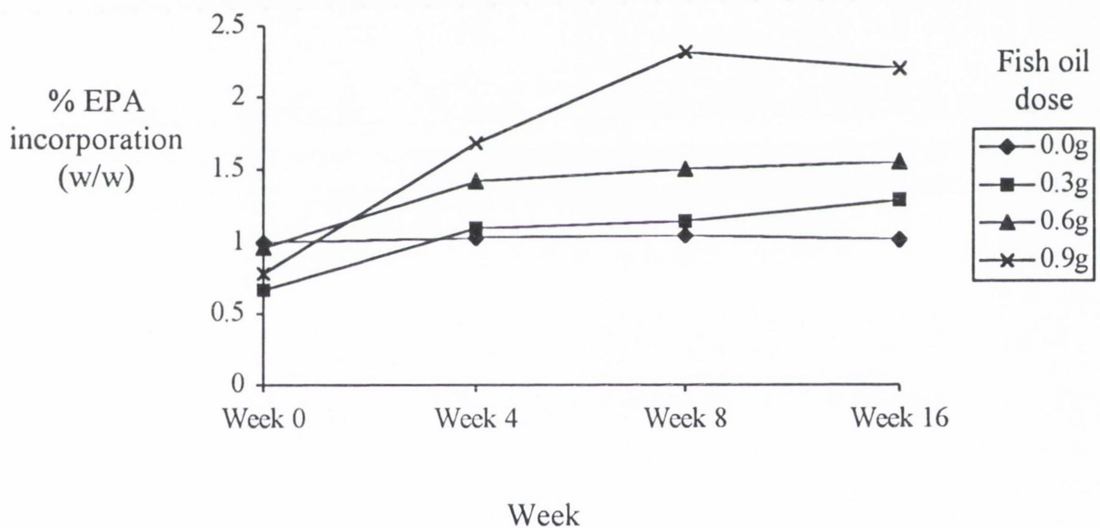
4.4.2 Platelet phospholipid fatty acid composition

The dose-response relationship between amount of fish oil ingested and degree of incorporation into platelet phospholipids has previously been shown with high doses of fish oil over short supplementary periods (Sanders and Roshanai, 1983, Agren *et al,* 1990, Li and Steiner, 1991, Roche and Gibney, 1996). In the present study n-3 fatty acids were incorporated into platelet phospholipids at the expense of n-6 fatty acids, which is in line with results from other studies (Sanders and Roshanai, 1983, Agren *et al,* 1990, Li and Steiner, 1991). An unchanged proportion of arachidonic acid was observed in all groups, which has also been previously reported (Nagakawa *et al,* 1983).

Figure 4.4.1 shows the dose-response EPA curve constructed in this study. As would be expected, the placebo group showed no increment in EPA platelet phospholipid concentration. Baseline EPA concentrations in the group consuming 0.3g fish oil were significantly lower than the group consuming 0.6g fish oil. At week 4 the group consuming 0.3g fish oil showed a statistically significant 63% increase from baseline platelet phospholipid EPA concentrations, rising to 72% increase from baseline by week 8 and 94% at week 16. The group consuming twice that dose of fish oil showed smaller, but still significant increases from baseline (50% at week 4, 57% at

week 8 and 62% at week 16), owing to their higher baseline values. Similarly, this group had significantly higher baseline DHA levels than either of the other 2 fish oil groups. Although by week 8 they showed a significant effect of supplementation, the percentage change from baseline was less than half that seen at week 8 in the lowest dose fish oil group and just over one eighth of that seen at week 8 in the highest dose fish oil group.

Fig 4.4.1 EPA incorporation into platelet phospholipids (w/w%)



In the present study, EPA platelet phospholipid concentrations in the 0.9g fish oil group fell slightly between weeks 8 and 16, suggesting a saturation point somewhere within this time frame. The same group showed a significant rise in DHA levels even after 4 weeks of supplementation, but again DHA concentrations stabilised between weeks 8 and 16, suggesting that 16 weeks supplementation is adequate to achieve a maximal n-3: n-6 ratio in platelet phospholipids.

EPA continued to be incorporated into platelet phospholipids in the lower dose fish oil groups (0.3g/d and 0.6g/d) even after 16 weeks supplementation, suggesting that at this dose 16 weeks is an inadequate length of time to achieve maximal n: 3 - n:6 ratio at these supplementation levels.

Of all the dose-response studies looking at the effect of fish oils on platelet phospholipids, the study by Agren *et al,* (1990) is the most comparable to the present study as it supplemented volunteers with similar doses of fish oil (0.25g, 0.5g, 0.6g and 1.1g) for 12 weeks. They observed a continuing increase of DHA at the end of the 12-week study period, even though a final increase of EPA was attained after 5 weeks in all except the lowest dose group. From this they proposed that 12 weeks is not an adequate length of time to see the beneficial effect of fish oil supplementation.

Looking closer at the results, the principal difference between our study and that by Agren *et al,* (1990) lies in baseline EPA and DHA concentrations. At the lower doses used in the present study (0.3g/d and 0.6g/d, 16 weeks) both EPA and DHA concentrations continued to rise significantly from baseline at the end of the study period – Agren *et al* observed a no-effect level of supplementation with 0.25g fish oil for 12 weeks. Using multiple regression analysis, it has been demonstrated that baseline EPA and DHA values are the most influential factor on degree of incorporation of these fatty acids into platelet phospholipids (Roche, 1995). Our baseline values were lower than those reported by Agren *et al,* (1990), which would explain why a significant effect of intervention was found in the present study even with very low doses of fish oil, whilst Agren *et al,* found no effect of intervention with similar doses.

Fig. 4.4.2

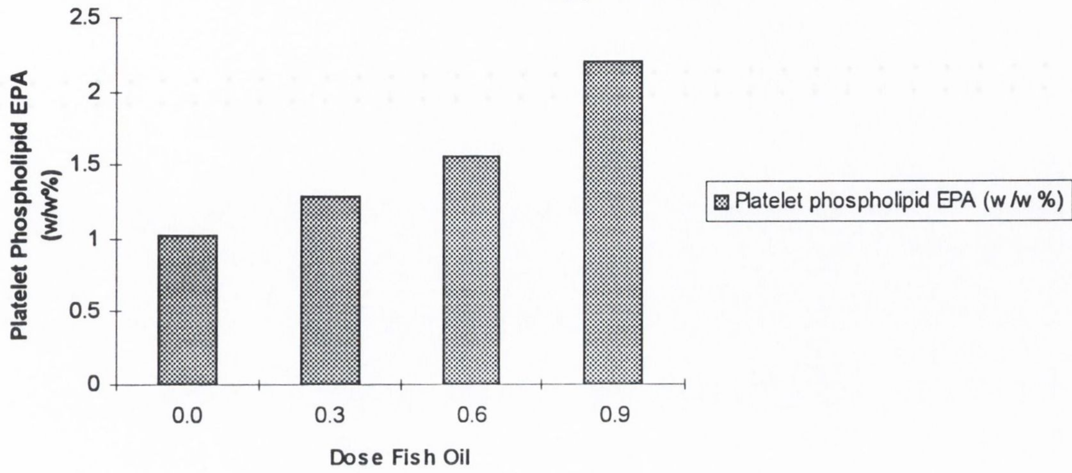
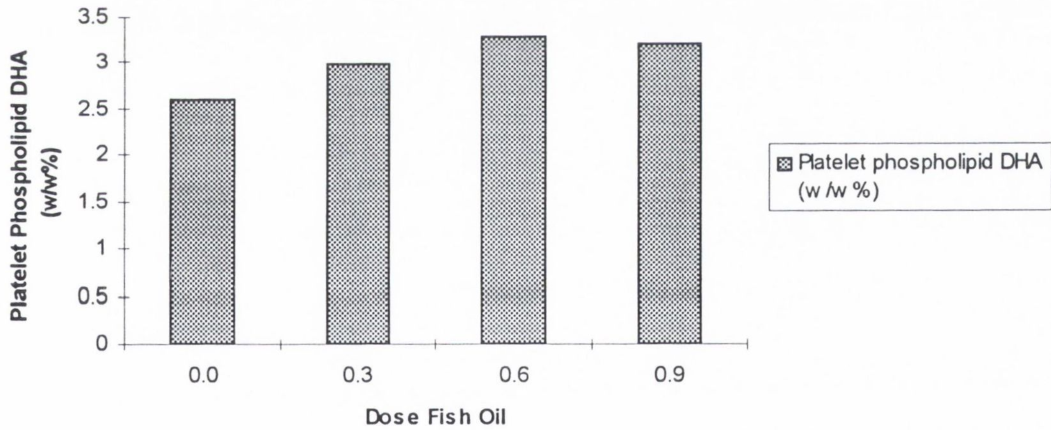


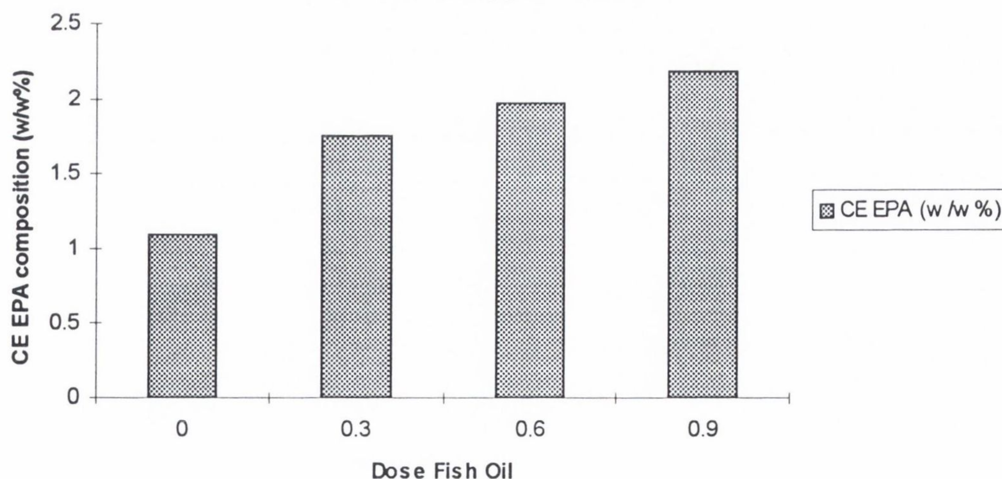
Fig. 4.4.3

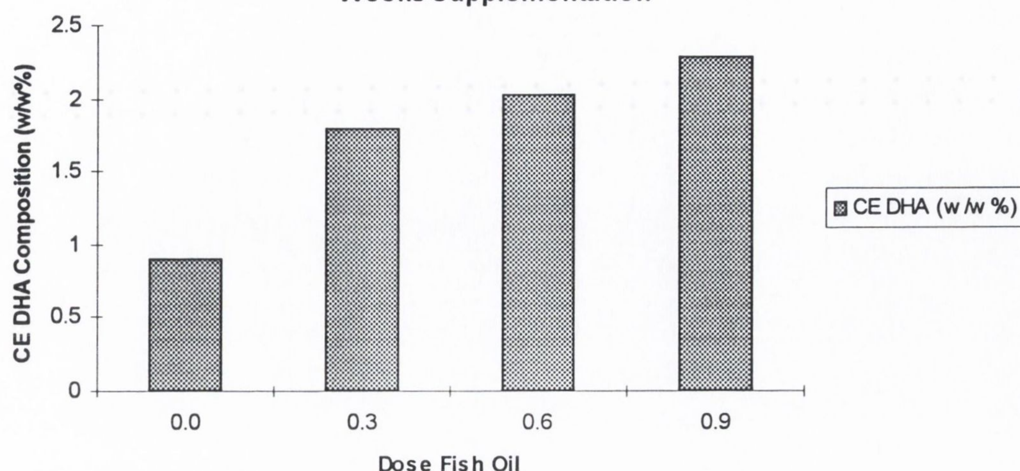


4.4.3 Cholesteryl ester fatty acids

A clear dose-response relationship was observed in cholesteryl ester EPA and DHA following supplementation with fish oils (Fig. 4.4.4 and 4.4.5). EPA concentrations increased by 60% from placebo through supplementation with 0.3g fish oil. Concentrations of this fatty acid rose significantly higher at supplementation levels of 0.6g and 0.9g fish oil, showing increases of 79% and 99% respectively from placebo. CE DHA concentrations showed an even more dramatic response to fish oil supplementation, rising significantly from placebo at all supplementation levels. The group supplemented with 0.3g fish oil demonstrated CE DHA concentrations that were 99% elevated from placebo; 0.6g group showing 124% greater CE DHA concentrations and the 0.9g fish oil group displaying a 154% increase from placebo concentrations. There was no significant difference between groups in any other fatty acid, as would be expected.

Fig. 4.4.4 Incorporation of EPA from Fish Oils into Cholesteryl Esters After 16 Weeks Supplementation





4.4.4 TRL fatty acid composition

This study has demonstrated the chronic effects of feeding a low dose of a n-3 PUFA oil on the fatty acid composition of TRL. At 4 hours postprandially, TRL fatty acid composition was found to contain a large proportion of linoleic acid in both groups, reflecting its presence as the main fatty acid in the test meal. The fatty acid compositions of TRL TAG and NEFA have previously been shown to reflect the fatty acid content of the test meal (Sakr *et al,* 1997). TRL fatty acid composition 0 hours and 4 hours after the test meal were very similar in both the placebo and the highest dose group's pre-trial. The main fatty acid in olive oil is C18: 1, however supplementation with 0.9g olive oil for 16 weeks did not affect the fatty acid composition of TRL in the placebo group at either time point examined. However, EPA and DHA concentrations changed dramatically in the group consuming 0.9g fish oil ($P \leq 0.05$) as a result of intervention. In this group, total n-3 concentrations and the n-3: n-6 ratio also increased significantly both fasting and 4 hours postprandially. Since TAG is the predominant class of fatty acid containing lipid in chylomicrons (90% by weight) (Redgrave, 1983) the changes in overall fatty acid composition will reflect much more the changes in TAG composition than those in other lipids. TRL size is mainly determined by the rapidity of TAG absorption as large TRL are produced when TAG levels increase (Sakr *et al,* 1997). Large chylomicrons are produced after ingestion of PUFA, relative to the size of

chylomicrons produced after ingestion of a SFA-rich test meal (Kalogeris and Story, 1992). In this study, TRL became significantly enriched with EPA and DHA following supplementation in both the fasting period and at peak TAG concentrations 4 hours postprandially. Previous studies have shown that large size and/or PUFA-rich chylomicrons are rapidly cleared from serum compared with SFA-rich chylomicrons (Groot *et al,* 1988, Levy *et al,* 1991) due to differences in susceptibility to lipolytic enzymes (Weintraub *et al,* 1988), therefore it can be assumed that the PUFA-enriched chylomicrons were more rapidly cleared from circulation than they were before the supplementation programme. The sampling periods only occurred every two hours thus even though no significant reduction in the time to maximum TRL TAG concentration was found, it is possible that the time to peak occurred at an earlier time after the supplementation programme.

4.4.5 Comparison; biomarkers of chronic fat intake

When the incorporation of EPA and DHA into CE and platelet phospholipids is compared, similar results are seen for EPA concentrations, but varying results for DHA concentrations (Fig. 4.3.4 (i) and 4.3.4 (ii)). There was no significant difference in EPA concentrations post-trial at any supplementation level between the two groups; a clear dose-response relationship was observed for both groups. The same however was not observed for DHA concentrations. Placebo group DHA concentrations were higher in platelet phospholipids than in cholesteryl esters. A clear dose-response relationship was observed for CE; the greater the dose the higher the DHA concentration in CE, however platelet phospholipid incorporation of DHA plateaued between 0.6g and 0.9g fish oil, suggesting a maximal capacity for DHA incorporation in this fraction. CE DHA concentrations observed are comparable with those observed by other authors (Cambien *et al,* 1988, Sarkkinen *et al,* 1994, Babin *et al,* 1999). Platelet phospholipid concentrations are also similar to those observed by other authors (Gibney and Hunter, 1993, Roche and Gibney, 1994). It is possible that DHA is preferentially incorporated into platelet phospholipids, which would account for the difference in DHA concentrations between the two fractions in the placebo group. It would seem that

platelet phospholipids either have a limited capacity for accumulating DHA or have increased utilisation of this fatty acid, as reflected in the plateau observed in concentrations of this fatty acid at two levels; firstly in the plateau between those supplemented with 0.6g and 0.9g fish oil at the end of the supplementation period, and secondly in the plateau observed between weeks 8 and 16 of the supplementation period for the 0.9g fish oil group. The latter would seem to be a more likely explanation as PUFA stored in phospholipids of cell membranes are substrates for the formation of eicosanoids formed through the cycloxygenase and lipoxygenase pathways. Eicosanoids formed from arachidonic acid (AA) are strongly pro-inflammatory, whereas those from EPA and DHA are weakly inflammatory. By increasing the ratio of n-3: n-6 PUFA in phospholipid membranes, as occurred in the present study, more n-3 PUFA may be used in the production of these eicosanoids, as n-3 PUFA are the preferred substrates for eicosanoid formation (Gurr, 1999). This would explain the limited net of DHA into platelet phospholipids compared to CE. CE DHA concentrations were significantly lower ($P < 0.05$) than platelet phospholipids at all supplementation levels (Fig 4.4.7), however if we examine CE DHA concentrations in their own right we find a clear dose-response relationship; the higher the supplementation level the greater the CE DHA concentration. Supplementation for a longer period would possibly reveal the maximal capacity not only for CE DHA but also for EPA incorporation into platelet phospholipids and CE.

The regression equations produced here are applicable for intakes of fish oils in the range used in the current study. They have enormous practical consequences for future study design and application. Through knowing a particular level of n-3 PUFA which would cause a particular rise in these fatty acids in cell membranes, particularly at low levels, we can apply this to a number of situations where low intakes of n-3 PUFA are linked with disease states. One such situation, that is the case of patients with Alzheimer's disease, is explored in the next chapter of this thesis.

Chapter Five

Fish Oils and Alzheimer's Disease

5.1 Introduction

In Ireland we have a growing number of people in the over-65 age category, rising from 11.4% in 1996 to 14.7% in 1998 (Minitel, 1999). The incidence of Alzheimer's disease is also on the increase and is currently estimated to be around 6 - 8% (Nourhashémi *et al,* 2000). Both causative and associative genes for Alzheimer's disease have been identified (Hendrie, 1998). The search for non-genetic risk factors remains an exciting area of research with much exploration yet to evolve. Any intervention strategy aimed at decreasing potentially modifiable risks or delaying onset of the disease will obviously have a marked influence on health care costs. Nutritional factors seem to play a large protective role in Alzheimer's disease. The correlation between cognitive skills and serum concentrations of folate, vitamin B12, vitamin B6 and homocysteine has become apparent in recent years (Nourhashémi *et al,* 2000). There is also a growing body of evidence suggesting a link between fish oil consumption and Alzheimer's disease, however evidence is currently scant but growing. Tables 5.1 and 5.2 summarise the relevant prospective and intervention studies in this area.

The first two prospective studies (Kalmijn *et al,* 1997a and 1997b) demonstrated a positive link between fish oils and AD incidence and severity, however no definitive biomarkers of fish oil intake were used, and detailed dietary analysis was only performed in one (Kalmijn *et al,* 1997b). More recently, a short report was published assessing fish oil intake, using PC-DHA as a biomarker, in a large group of elderly people (*n* 1188), and followed them up over 10 years (Kyle *et al,* 1999). There was a two-fold higher frequency of AD in subjects from the lower half of the DHA distribution (11 v 5), however this was not reported to be significant. These figures imply that only 1.35% of this elderly American population had AD (16 / 1188). This is much lower than the reported prevalence of dementia in subjects 65 years and older in North America, which is approximately 6 - 10%, with AD accounting for two-thirds of cases (Hendrie, 1998). Hendrie noted that if milder cases are included the prevalence rates double. It is possible that Kyle *et al* only chose people with severe AD when making their classification, however this was not referred to in the paper. Their follow-up revealed that subjects whose PC-DHA was in the lower half of the distribution but who did not

have AD at the beginning of the study period, had a significantly greater likelihood (67%) of developing the disease than those in the other half of the distribution.

Table 5.1 Prospective studies examining fish oil intake and incidence and severity of Alzheimer's disease.

Authors	**Assessment method**	*n*	**Follow-up (years)**	**Results**
Kalmijn *et al,* 1997a	semi-quantitative FFQ	5386	2.1	inverse relationship between fish intake & risk of AD
Kalmijn *et al,* 1997b	crosscheck dietary history + additional info on n-3 PUFA content of food	342	3	inverse relationship between fish intake & cognitive impairment & decline
Kyle *et al,* 1999	biomarker of fish oil: serum PC-DHA	1188	10	low levels of serum PC-DHA significant risk factor for presence & development of AD

Two pilot intervention studies have been published to date, again showing positive results for a therapeutic role for fish oils in the treatment and/or prevention of AD. This area certainly warrants more detailed research, possibly through the use of a biomarker of fish oil intake, whether from oily fish, supplements or functional foods or a combination of all, and/or detailed dietary assessment, which would precisely quantify EPA and DHA levels, thus confirming the protective role for these fatty acids in patients with, or at risk of, Alzheimer's disease.

Table 5.2 Intervention studies on the effects of fish oils on Alzheimer's disease

Authors	*n*	Dose	Follow-up	Results
Peers, 1990	1 patient	oily fish twice a week	'several months'	improvement in severity of disease
Yehuda *et al*,1996	60 patients 40 controls	2ml oil / d (Linoleic: linolenic acid 4:1)	4 weeks	improvement in severity of disease in 82% (*n* 49) patients
Terano *et al*, 1999	10 patients 10 controls	0.72g DHA/d (6 capsules)	1 year	improvement in severity of disease

Whilst evidence for a therapeutic role for fish oil in AD is somewhat tentative, there is a plausible biological explanation for the association. Firstly, fish may protect against dementia by reducing cardiovascular disease risk, as CVD has been shown to be associated with AD risk (Hofman *et al*, 1997) and numerous studies have proven the protective effect of fish oils on CVD risk (Chapter 1, review). Inflammatory processes are thought to be involved in AD, as inflammatory reactions are important contributors to neuronal loss (McGeer & McGeer, 1998). Fish oil supplementation causes a reduction in concentrations of the strongly pro-inflammatory eicosanoids produced from the n-6 PUFA arachidonic acid with the preferential formation of weakly inflammatory eicosanoids produced from EPA and DHA, as they are the preferred substrates for eicosanoid formation (Gurr, 1999). n-3 PUFA could possibly therefore contribute to decreasing risk of the disease. Further evidence supporting this view lies in the fact that many studies have shown a protective effect of NSAID's on severity of the disease (Jorm, 1997, Stewart *et al*, 1997). Finally, examinations of AD patients' brain tissue have revealed significantly lower concentrations of DHA when compared with controls (Prasad *et al*, 1998) and n-3 PUFA may be important in nerve cell regeneration

(Compston, 1994) thus their relative absence in the diet may cause nerve cell damage over a period of time.

The aim of the present study is to compare the fatty acid content of CE in patients stratified according to i) diagnosis, ii) MMSE, iii) Clinical Dementia Ratio (CDR) with healthy elderly controls to see if there is a discrepancy in n-3 fatty acid CE concentrations between diseased and control subjects. From this, and from the work in chapter 4, it would be possible to predict a supplementation level (if any necessary) which would maintain an adequate level of n-3 PUFA in the elderly population, which may elicit a protective effect from AD.

5.2 Methods

Patients were recruited from the Mercer Institute for Research on Aging (MIRA), St. James' Hospital as part of an on-going multi-factorial study on patients with AD. All patients attending the clinic were community based; none were institutionalised. A battery of examinations were performed prior to diagnosis, including cognitive assessment tests i.e. MMSE, CDR, Blessed Dementia Rating Procedure (BDRP), Hachinski Ischaemic Scale (HIS), Instrumental Activities of Daily Living Scale (IADL), Physical Self-Maintenance (PSMS), Behaviour Scale, and a Driving Questionnaire. A medical history was taken and general physical and neurological examinations were also performed. A number of biochemical indices including thyroid function were examined and a CT scan of the brain, MRI and SPECT scan were performed before diagnosis was confirmed. Non-fasting serum samples were then taken from patients, centrifuged, aliquoted and stored until subsequent analysis for CE fatty acid composition. Control samples were collected from volunteers attending Healthy Active Retirement Groups in the Dublin 8 area following screening using a medical history, weight and height measurement, blood pressure and MMSE. Exclusion criteria were as follows:

i) History of stroke

ii) Hypertension

iii) MMSE < 26

iv) Patient on warfarin.

Habitual dietary supplement use was also examined. Non-fasting serum samples were taken from eligible volunteers, and were centrifuged, aliquoted and stored at -20°C until analysis.

Lipid extractions were performed using the procedure of Folch *et al* (1957). Cholesteryl esters were isolated using thin layer chromatography on Silica 60 LKD 19 Lane TLC plates (Whatman, 9 Bridewell Pl., Clifton, New Jersey, U.S.A.) using a solvent system of petroleum ether: diethyl ether : formic acid (80:20:2 v/v) (Gibney and Bolton-Smith, 1988). Cholesteryl esters were hydrolysed using 0.5M NaOH in methanol and component fatty acids were methylated using BF_3 in methanol. Gas liquid chromatography (GLC) was used to identify fatty acid methyl esters of CE using a Shimadzu GC-14A Series Gas Chromatograph (Mason Technologies, Dublin, Ireland).

Specific fatty acid levels were expressed as a percentage of total fatty acids and fatty acid compositions of patients and controls were analysed concurrently.

The oldest sample in our patient pool was 6 years old, with the average sample age being 2.54 years. Control samples were 1 - 2 months in storage prior to analysis. The stability of frozen serum samples for CE fatty acid analysis has previously been confirmed in serum samples that were 7 - 10 years old (Simon *et al,* 1995). They analysed randomly selected samples for their malondialdehyde (Lee, 1980) and conjugated diene (Klein, 1970) content, and also examined Folch extractions for fluorescent degradation products, their vitamins A and E content, and fatty acid profiles (Bieri *et al,* 1979). Comparison of results (Simon *et al* 1995) with reference pools and with fresh serum confirmed that very little oxidative damage had occurred. Repeated measures analysis of variance (ANOVA) was used to describe differences between groups. Fatty acids were log transformed to give a normal distribution.

5.3 Results

5.3.1 Subject information

Complete information was available on 168 patients and 45 control volunteers. Due to the nature of patient recruitment i.e. community-based patients attending the MIRA clinic at St. James Hospital, Dublin, Ireland, none of the patients had a severe form of the disease and therefore were not institutionalised due to the severity of their disease. Table 1 shows the demographic data of patients divided according to quartiles of MMSE score and controls. There were no significant differences in the men/women ratio between healthy volunteers and each patient group. There were no significant differences in age between patient groups, however each group was significantly older than the control group.

5.3.2 Cholesteryl ester composition and Alzheimer's disease - The Mini-Mental Score

Table 5.1 shows the CE fatty acid composition of patients divided according to quartiles of MMSE score. Repeated measures analysis of variance demonstrated that all patient groups had significantly lower MMSE scores ($P < 0.001$) than the control group, and also had significantly higher CDR scores than controls ($P < 0.001$).

Concentrations of C16:0 were significantly higher in quartiles 1 and 2 of the patient groups ($P < 0.05$), which are the patients with the lowest scores from the MMSE. The percentage of EPA was significantly lower in all patient groups than the control group. DHA concentrations were also significantly lower in all patient groups than the control group, as demonstrated using ANOVA. ALA concentrations were higher (although not significantly) than control values in the first quartile MMSE, i.e. those who were most severely affected by AD (Fig. 5.1). Total n-3 fatty acid compositions were lower in all patient groups when compared to the control group, however this difference only reached significance in the 2nd, 3rd and 4th quartiles of patient groups, due to the higher ALA concentrations in the 1st quartile. Total n-6 fatty

acid concentrations were significantly lower (P < 0.05) in the most severely affected group (quartile 1) than the control group. The ratio of n-3 : n-6 fatty acids was significantly lower in quartiles 2, 3 and 4 than the control group (P < 0.05). No significant differences were observed between groups in the saturated fatty acids C16:1, C18:0 and C18:1, in the n-6 PUFA C18:2 n-6 and C20:4 n-6 concentrations and in the n-3 fatty acids C18:3 n-3 and C18:4 n-3.

Fig. 5.1 ALA concentrations (w/w%)

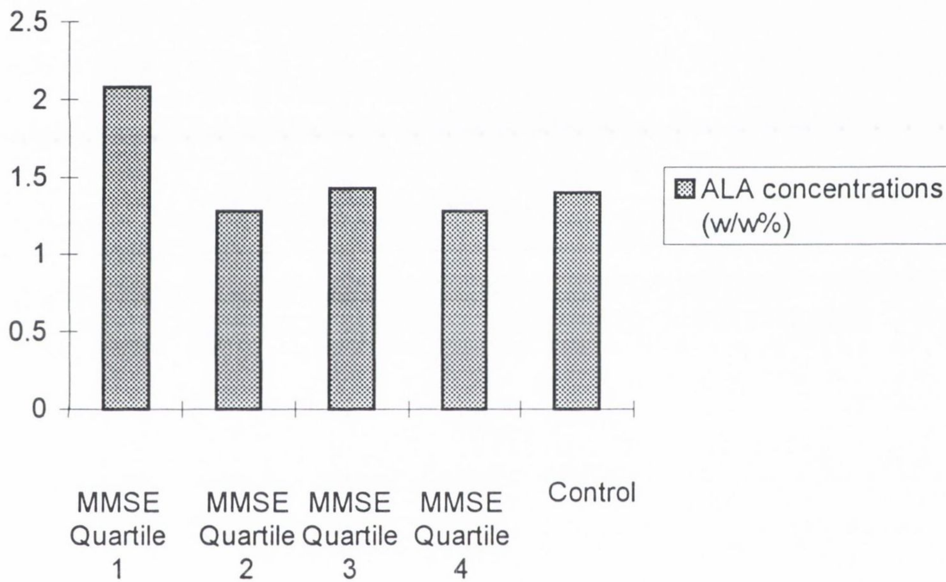


Table 5.1 Characteristics of Alzheimer's disease patients and control volunteers divided according to quartiles of MMSE

	1st quartile	2nd quartile	3rd quartile	4th quartile	Controls
n	42	42	42	42	45
F / M	33 / 9	33 / 9	34 / 8	31 / 11	36 / 9
MMSE score	12.88 (3.88) †	19.57 (0.94) †	22.12 (0.83) †	25.33 (1.54) †	28.91 (1.10)
CDR score	1.71 (0.64) †	1.29 (0.53) †	0.81 (0.33) †	0.71 (0.51) †	0.0 (0.0)
age	76.0 (9.06) †	77.38 (4.98) †	75.98 (5.59) †	74.68 (7.59) †	68.82 (6.01)
C16:0	12.03 (3.41)*	12.20 (3.16)*	11.01 (2.24)	11.64 (3.58)	10.64 (2.01)
C16:1	4.94 (1.95)	5.20 (1.84)	4.31 (1.49)	4.41 (1.35)	4.49 (1.44)
C18:0	4.64 (3.21)	4.52 (3.72)	4.65 (3.89)	4.46 (3.73)	4.55 (3.73)
C18:1	16.83 (5.13)	18.54 (4.76)	18.77 (4.51)	18.68 (6.09)	18.87 (4.71)
C18:2n-6	35.35(11.05)*	37.52(10.84)	37.74 (9.02)	38.95 (8.14)	39.53 (8.75)
C18:3n-3	2.08 (2.18)	1.28 (1.79)	1.43 (1.91)	1.28 (1.18)	1.40 (1.51)
C18:4n-3	2.05 (1.50)	1.63 (1.35)	1.79 (1.63)	1.40 (1.22)	2.11 (2.38)
C20:4n-6	2.33 (3.25)	1.64 (1.48)	1.48 (1.69)	1.29 (1.31)	2.18 (2.01)
C20:5n-3	1.04 (0.83)*	0.91 (0.83) †	0.94 (0.72) †	0.96 (1.09) †	1.58 (0.62)
C22:6n-3	0.58 (0.45) †	0.56 (0.41) †	0.54 (0.36) †	0.66 (0.65) †	1.15 (0.88)
Total n-3	5.40 (2.91)	4.20 (2.49) †	4.56 (2.98) †	3.89 (2.02) †	5.85 (2.80)
Total n-6	44.59 (8.05)*	45.67 (8.64)	45.87 (6.58)	46.08 (7.16)	48.11 (6.61)
n-3 : n-6	0.14 (0.11)	0.10 (0.08)*	0.11 (0.09)*	0.09 (0.06)*	0.12 (0.07)

* Significantly different from control, $P < 0.05$

† Significantly different from control, $P < 0.001$

5.3.3 Cholesteryl esters and Alzheimer's disease - Clinical Dementia Rating

Patients and controls were divided according to their CDR score (Table 5.2). Only four patients had a CDR of 3, which denoted severe dementia, thus these were omitted from the analysis due to inadequate numbers. Repeated measures ANOVA demonstrated that all patient groups had significantly lower scores from the MMSE than controls. Patients were significantly older than controls. Concentrations of the saturated fatty acid C16:0 were higher in all patient groups than control, however this only reached significance in patients with moderate AD. All patients with AD, regardless of the severity of the disease as defined by CDR, had significantly lower EPA and DHA levels ($P < 0.001$) than control volunteers. No differences were observed between groups in the saturated fatty acids C16:1, C18:0 or C18:1. Concentrations of the fatty acids C18:2 n-6 or C20:4n-6 were not altered as a result of the disease. Repeated measures ANOVA demonstrated that whilst concentrations of C18:4 n-3 were slightly lower in all patient groups in comparison with control, this only reached significance in the 0.5 CDR group ($P < 0.05$). Concentrations of ALA, the precursor of EPA and DHA, remained unaltered by the disease however the ratio of ALA to EPA was significantly higher in all patient groups when compared to controls. There was no difference in the ratio of arachidonic acid: ALA ratio between patients and controls.

Table 5.2 Characteristics of patients and controls divided according to Clinical Dementia Rating (CDR)

	Control	Mild AD	Moderate AD	Moderate - severe AD
CDR score	0.0	0.5	1.0	2.0
n	45	53	64	41
F / M	36 / 9	40 / 13	48 / 16	37 / 4
MMSE score	28.91 (1.10)	23.38 (3.40) †	20.13 (3.55) †	16.10 (4.48) †
age	68.82 (6.01)	74.68 (6.97) †	75.95 (5.30) †	78.66 (7.43) †
C16:0	10.64 (2.01)	11.59 (3.63)	11.91 (3.03)*	11.73 (2.82)
C16:1	4.49 (1.44)	4.25 (1.38)	4.93 (1.87)	4.90 (1.56)
C18:0	4.55 (4.52)	4.36 (3.76)	4.51 (3.74)	4.86 (3.61)
C18:1	18.87 (4.71)	18.80 (5.57)	17.99 (5.16)	17.48 (4.51)
C18:2n-6	39.52 (8.75)	39.11 (8.20)	37.56 (10.11)	35.05 (11.14)*
C18:3n-3	1.40 (1.51)	1.38 (1.65)	1.57 (1.97)	1.61 (1.76)
C18:4n-3	2.11 (2.38)	1.37 (1.26)*	1.78 (1.48)	2.03 (1.58)
C20:4n-6	2.19 (2.01)	1.46 (1.65)	1.34 (1.24)	2.62 (3.41)
C20:5n-3	1.58 (0.62)	0.80 (0.71)†	1.05 (0.95)†	1.09 (0.98)†
C22:6n-3	1.15 (0.88)	0.58 (0.42) †	0.51 (0.34) †	0.49 (0.35) †
C18:3n-3 : C20:5n-3	1.10 (1.39)	3.98 (8.14) *	4.80 (14.42) †	2.85 (5.64) *
C20:4n-6 : C20:5n-3	2.00 (2.24)	1.52 (2.25)	1.89 (4.61)	2.40 (3.48)

* Significantly different from control, $P < 0.05$

† Significantly different from control, $P < 0.001$

5.3.4 Cholesteryl ester composition and Alzheimer's disease - Clinical Diagnosis

Table 5.3 describes characteristics of volunteers divided according to their clinical diagnosis. By far the most common diagnosis of patients attending the clinic was Probable Alzheimer's disease (108 out of 168), with 16 patients being diagnosed as having Possible AD, 13 having Mixed AD and 11 patients having Vascular AD. Similar results from ANOVA were observed between most patient groups and controls as had been observed when patients were divided according to quartiles of MMSE or CDR i.e. Significantly higher C16:0 concentrations in patients, and significantly lower EPA and DHA concentrations in patients. The exception to this rule applied to the group with Mixed AD who showed a more similar fatty acid profile to controls than the other groups, however, they still had lower DHA concentrations in their CE ($P < 0.05$), but did not have significantly lower EPA concentrations.

Table 5.3 Characteristics of patients divided according to Clinical Diagnosis

	AD prob	AD poss	AD mixed	Vascular AD	Control
n	108	16	13	11	45
CDR score	1.23 (0.58) †	0.56 (0.17) †	1.69 (0.72) †	0.95 (0.69) †	0.0 (0.0)
F / M	86 / 22	14 / 2	8 / 5	11 / 0	36 / 9
MMSE score	19.49 (4.52) †	22.50 (3.03) †	14.69 (5.84) †	21.0 (3.58) †	28.91 (1.10)
age	76.26 (7.58) †	75.39 (4.41)*	77.6 (8.02) †	80.60 (5.31) †	68.82 (6.01)
C16:0	11.75 (2.90)*	10.28 (2.26)	11.96 (3.56)	13.66 (5.50)*	10.64 (2.01)
C16:1	4.84 (1.70)	3.65 (1.13)	5.35 (2.35)	4.92 (0.98)	4.49 (1.44)
C18:0	4.73 (3.79)	5.15 (5.46)	4.41 (2.0)	3.56 (1.79)	4.55 (4.52)
C18:1	18.32 (4.68)	20.15 (7.81)	17.60 (5.39)	16.24 (5.20)	18.87 (4.71)
C18:2n-6	37.0 (9.94)	41.82 (9.55)	37.11 (10.64)	34.56 (8.42)	39.52 (8.75)
C18:3n-3	1.55 (1.92)	0.81 (0.85)	1.12 (1.59)	1.73 (1.81)	1.40 (1.51)
C18:4n-3	1.73 (1.51)	1.44 (1.26)	2.35 (1.75)	1.88 (0.96)	2.11 (2.38)
C20:4n-6	1.75 (2.33)	2.10 (2.37)	1.39 (1.52)	1.53 (1.61)	2.19 (2.01)
C20:5n-3	0.98 (0.87) †	0.95 (0.87)*	1.49 (1.16)	0.62 (0.44) †	1.58 (0.62)
C22:6n-3	0.56 (0.41) †	0.54 (0.36) †	0.67 (0.66)*	0.49 (0.47) †	1.15 (0.88)

* Significantly different from control, $P < 0.05$

† Significantly different from control, $P < 0.001$

5.3.5 Cholesteryl ester composition and Alzheimer's disease; Age- and Sex- matched patients and controls

As age and gender have been reported to possibly affect the fatty acid composition of human serum lipids (Holman *et al*, 1979) patients and controls were age (± 3 years) and sex-matched our, resulting in 41 pairings. Four controls could not be paired as there was an inadequate number of patients to age-match in the sample group. Table 5.4 describes the characteristics of these volunteers. Results were similar to those reported from dividing patients according to MMSE, CDR or clinical diagnosis. Repeated measures ANOVA showed that patients had significantly lower MMSE scores and significantly higher CDR scores ($P < 0.001$ for both) than controls. C16:0 CE fatty acid composition was higher in the patient group, however this did not reach statistical significance. EPA and DHA concentrations were both significantly reduced in the patient group ($P < 0.001$). Patients had EPA concentrations almost a third lower than controls, and DHA concentrations in patients were dramatically reduced by almost two-thirds. Concentrations of the other saturated fatty acids, of n-6 and n-3 PUFA were not significantly different between groups.

5.3.6 Cholesteryl ester composition and Alzheimer's disease; Gender Differences

Patients were divided according to gender and these results can be seen in Table 5.5. Females out-numbered males by almost a ratio of 4:1 (132 / 36). There were no differences in age, MMSE, CDR or any of the fatty acid concentrations with the exception of DHA which was higher in male patients ($P < 0.05$, 0.74 *v* 0.54w/w%). Total n-3 fatty acid concentrations were also higher in males patients ($P < 0.05$), however the total n-3:n-6 ratio remained unaltered between the two groups.

Table 5.4 Characteristics of age and sex-matched patients and controls

	Patient	Control
n	41	41
CDR score	1.06 (0.66) †	0.0 (0.0)
F / M	32 / 9	32 / 9
MMSE score	19.78 (4.93) †	28.90 (1.14)
age	70.80 (4.63)	69.83 (5.04)
C16:0	11.74 (3.20)	10.54 (2.00)
C16:1	4.44 (1.73)	4.39 (1.45)
C18:0	3.82 (2.55)	4.66 (4.71)
C18:1	18.91 (5.62)	18.94 (4.69)
C18:2n-6	38.95 (8.81)	40.04 (8.76)
C18:3n-3	1.29 (1.79)	1.44 (1.53)
C18:4n-3	1.74 (1.65)	1.74 (1.65)
C20:4n-6	1.55 (1.75)	2.22 (2.01)
C20:5n-3	1.06 (1.04) †	1.55 (0.63)
C22:6n-3	0.44 (0.30) †	1.07 (0.83)

* Significantly different from control, $P < 0.05$

† Significantly different from control, $P < 0.001$

Table 5.5 Comparison of Male and Female Patients

	Female	Male
n	132	36
CDR score	1.17 (0.66)	1.0 (0.60)
MMSE score	19.86 (5.07)	19.91 (5.24)
age	76.45 (6.66)	74.44 (8.07)
C16:0	11.68 (3.36)	11.90 (2.22)
C16:1	4.76 (1.66)	4.56 (1.89)
C18:0	4.70 (3.92)	4.10 (2.12)
C18:1	18.33 (5.47)	17.71 (3.87)
C18:2n-6	37.57 (9.74)	36.64 (10.37)
C18:3n-3	1.41 (1.57)	1.93 (2.54)
C18:4n-3	1.69 (1.46)	1.84 (1.41)
C20:4n-6	1.81 (2.29)	1.33 (1.33)
C20:5n-3	0.92 (0.79)	1.15 (1.09)
C22:6n-3	0.54 (0.46)	0.74 (0.51)*
Total n-3	4.29 (2.54)	5.47 (3.03)*
Total n-6	45.61 (7.69)	45.29 (7.34)
n-3 : n-6	0.10 (0.08)	0.14 (0.11)

* Significant difference between two groups, $P < 0.05$

5.3.7 Cholesteryl ester composition and Alzheimer's disease; Age Differences

Table 5.6 shows characteristics of patients with Probable AD divided according to quartiles of age. Patients in quartiles 2, 3 and 4 were significantly older than those in the first quartile, however this was the only difference observed between groups. There was no difference in MMSE, CDR or fatty acid composition between groups, as demonstrated using repeated measures ANOVA.

Table 5.6 Characteristics of patients with diagnosis of probable Alzheimer's disease divided according to quartiles of age

	Quartile 1	Quartile 2	Quartile 3	Quartile 4
n	27	27	27	27
age	67.67 (5.64)	74.37 (0.88) †	78.44 (1.01) †	84.0 (3.22) †
CDR score	1.15 (0.68)	1.06 (0.45)	1.37 (0.55)	1.33 (0.59)
F / M	21 / 6	17 / 10	27 / 0	21 / 6
MMSE score	18.73 (6.19)	20.70 (3.27)	19.30 (3.67)	19.19 (4.48)
C16:0	11.73 (3.05)	11.48 (2.10)	11.82 (2.99)	11.95 (3.44)
C16:1	4.28 (1.39)	4.65 (1.51)	5.15 (1.80)	5.27 (1.97)
C18:0	3.43 (1.78)	3.95 (1.89)	6.07 (4.90)	5.47 (4.86)
C18:1	17.75 (3.38)	16.89 (3.90)	19.78 (5.10)	18.87 (5.72)
C18:2n-6	39.83 (8.86)	35.43 (10.67)	36.65 (9.45)	36.12 (10.61)
C18:3n-3	1.38 (1.77)	1.77 (1.65)	1.21 (1.67)	1.86 (2.48)
C18:4n-3	1.73 (2.01)	1.74 (1.21)	1.81 (1.44)	1.66 (1.32)
C20:4n-6	1.33 (1.27)	1.63 (1.33)	1.45 (1.18)	2.57 (4.11)
C20:5n-3	1.19 (1.07)	0.89 (0.67)	0.89 (0.72)	0.97 (0.98)
C22:6n-3	0.52 (0.32)	0.67 (0.46)	0.49 (0.31)	0.55 (0.50)

† Significantly different from quartile 1, $P < 0.001$

5.4 Discussion

There are four main reasons as to why patients with AD would have lower EPA and DHA intakes than control volunteers:

i) reduced intake of either ALA or EPA and DHA
ii) increased utilisation of EPA and DHA as a result of the disease process
iii) partitioning of EPA and DHA into other tissues as a result of the disease process
iv) reduction in very long chain PUFA elongation / desaturation enzyme activity

Reduced Intake

Cholesteryl ester fatty acid composition has been shown to be a robust indicator of fat quality intake as Nikkari (1986) reported a highly significant relationship between intake of PUFA and percentage in cholesteryl esters in almost 3000 Finnish people on their habitual diet. Theoretically there are only two ways to supply the body with EPA and DHA, that is, either by the provision of the precursor ALA or the direct intake of preformed EPA and DHA. From a practical point of view, neither of these mechanisms are particularly efficient as none of these fatty acids either in their precursor or preformed state are abundant in commonly eaten foods in Western society. EPA and DHA in their preformed state can be incorporated into the diet through the ingestion of oily fish, supplement use, or the ingestion of foods fortified with these n-3 fatty acids. Oily fish is not consumed on a large scale in the global population, with the exception of the Japanese and Greenland Eskimos. In Japan fish consumption rates are three times higher than those in the USA (67 *v* 22 kg per person per year, respectively) (Horrocks and Yeo, 1999a). In Ireland we do not have data on specific intakes of oily fish, however we know that overall seafood intakes are estimated to be 8 - 14 kg per person per year (Lee and Cunningham 1990) and it has been estimated that in the UK only a quarter of all seafood eaten in Britain is from oily sources (Gregory *et al,* 1990), which would make us compare abysmally to the Japanese with respect to oily fish intakes. Data on intakes of fish oil supplements are not yet available in this country, but we do know that in European terms the consumption of fish, particularly oily fish, declined

markedly in the twentieth century (Sanders, 2000). To date only two food types have been fortified with n-3 PUFA (Golden Vale Ireland), one spread and one bread, however neither are any longer available due to product unacceptability and poor sales.

Only certain oils contain significant amounts of ALA e.g. soybean, rapeseed and canola oil, and none of these would be considered common household oils in Ireland. In the commercial sector, they are partially hydrogenated for use in frying which reduces their ALA content. ALA is also found in ruminant meat, however in recent years certain practices such as changing the feed of the livestock to that with low ALA content and our reduction in consumption of lamb and beef all have lead to a decline in ALA intakes from animal sources. Again, data on specific intakes of ALA are not yet available in this country. Overall, the ratio of n-6 to n-3 fatty acids in the food supply has increased over the past 50 years. UK figures have shown that intakes of linoleic acid have increased by 50% from the late 1970's, from ~10g/d to ~15g/d in the 1990's (Sanders, 2000). High intakes of this n-6 fatty acid have been shown to inhibit the synthesis of DHA from ALA, which further reduces our supply of n-3 PUFA, due to increased elongation/desaturation enzyme competition.

The elongation and desaturation of ALA to EPA and DHA is quite an inefficient pathway in humans (Galli, 1999). A recent analysis of this area has shown that in short term human studies, 70mg/d of EPA was more effective than 3.7g/d of ALA and almost as effective as 15.4g/d of ALA in raising platelet EPA levels (Li *et al,* 1999). Theoretically, poor intakes of ALA could lead to low levels of EPA and DHA in CE however results from the present study do not support the view that our patients had lower intakes of ALA than controls as there was no difference between the two groups in CE concentrations of this fatty acid.

Increased Utilisation

There may be increased utilisation of n-3 PUFA as a result of the disease process, and therefore the disease may not manifest itself in those with a regular supply of these fatty acids over a long period of time. This argument is further strengthened by the evidence that AD patients whose diet was originally high in n-6 PUFA and almost devoid of n-3 PUFA show improvement in severity and rate of decline of the condition when supplemented with oily fish twice weekly (Peers, 1990, Yehuda, 1996, Terrano, 1999). Furthermore, the large, prospective study carried out by Kalmijn *et al* (1997a) revealed an inverse relation between fish intake and risk of dementia, which would indicate that whilst even small amounts of n-3 PUFA seem to be protective against the disease, the disease process may cause increased utilisation of these fatty acids, thus, in time, depleting already low stores contributing to the severity of the disease.

Inflammatory processes are thought to be involved in Alzheimer's disease. Strongly pro-inflammatory eicosanoids are formed from the n-6 PUFA arachidonic acid, however n-3 PUFA stored in phospholipid cell membranes are the preferred substrates for eicosanoids. The eicosanoids produced from these fatty acids are weakly inflammatory so it is possible that there is increased usage of these fatty acids in an attempt to reduce inflammatory processes in the disease process, thus depleting already low n-3 PUFA stores. This theory is in agreement with the work of Prasad *et al,* (1998) who examined membrane phospholipid fatty acid concentrations in Alzheimer's disease patients and controls. They found that DHA concentrations were significantly decreased in both phosphatidyl ethanolamine (PE) and phosphatidyl inositol (PI) fractions of Alzheimer's disease patients. Soderberg *et al,* (1991) have also shown a decrease in DHA in brain PE in Alzheimer's disease patients compared with controls, again supporting the 'increased utilisation' theory. For these reasons, cholesteryl esters would be a more robust indicator of fatty acid status, as it would seem that the disease process might effect phospholipid n-3 PUFA concentrations.

Partitioning into Other Tissues

Nikkari (1986) also found that most CE fatty acids had highly significant correlations with those in other serum lipid fractions, adipose tissue and platelets, and they had good 'tracking' for up to four years. The population used in this study ranged in age from 1 - 85, and the correlations remained strong at older ages so it is unlikely that EPA and DHA are partitioned into fractions other than CE with aging. Whether this occurs with AD remains unknown, however there is no evidence available to support this theory, especially in the light of the suggestion that an age-related accumulation of long chain fatty acids is likely to occur in CE resulting from the age-related decrease in peroxisomal β-oxiditation (Périchon *et al,* 1998).

It cannot be concluded that a person with chronically poor intakes of ALA and/or EPA and DHA will automatically develop AD in later life, as, if that were the case the majority of elderly Irish people would suffer from the disease. Neither can we presume that all elderly Irish people with AD have chronically low intakes of n-3 PUFA, nor can we assume that all elderly Irish people who are free from the disease are habitual consumers of oily fish, fish oils and ALA-rich oils, especially in the light of evidence from Broe *et al* (1990) who found no association between absence of fish in the diet and AD in a large group of elderly volunteers in a case-control study. It is possible however those higher intakes of oily fish have a protective effect on AD incidence and/or severity as more recent dietary studies have shown that the prevalence of AD correlates negatively with fish consumption (Kalmijn *et al,* 1997a, Kalmijn *et al*, 1997b).

Elongation / Desaturation Enzyme Activity

One of the main influences of membrane structure and function is its fatty acid composition, which is kept constant through an elite synthesis/degradation system (Périchon and Bouree, 1996). DHA is a major component of membranes, particularly of the brain and retina. Modifications of membrane composition can cause changes to membrane functions. The aging process has been shown to cause alterations in membrane fatty acid composition (Zs-Nagy, 1979, Hegner, 1980) and it has been hypothesised that these modifications may contribute to the onset of age-related illnesses such as AD (Périchon and Bourre, 1996) as they may be implicated in the impaired signal transduction found in AD (Roth *et al,* 1995). However, there is little unanimity between authors in explaining this phenomenon as alterations in peroxisomal β-oxiditation, Δ6-desaturase and Δ5-desaturase have all been implicated in causing these alterations

Very long chain fatty acids are essentially degraded by peroxisomes by a specific β-oxiditation pathway and an accumulation of these fatty acids has been observed in those with peroxisome deficiencies (Périchon and Bourre, 1996). Peroxisomal β-oxidation is also required for the synthesis of DHA from ALA and a marked reduction in DHA has been observed in brain, retina, liver and kidney patients with peroxisomal disorders (Martinez, 1992). Alteration in peroxisomal β-oxiditation has been linked with the aging process which would theoretically contribute to the age-related changes in membrane composition. Babin *et al,* (1999) surmised that peroxisomal β-oxiditation deficiency could be a factor in the reduction of DHA observed in elderly volunteers when compared to younger controls, as they found little evidence to support theory of a reduction in Δ6-desaturase activity.

In adult animals, synthesis and oxiditation rates operate efficiently to maintain a balanced fatty acid composition in liver and other organs such as the brain and retina which have a high requirement for DHA (Périchon and Bourre, 1996). During aging and aging-related diseases such as AD they believe that synthesis and oxiditation rates decline and therefore the metabolic capacity of the synthesis/degradation system is reduced. From their mouse studies they have shown an aging-related decrease in peroxisomal fatty acid oxiditation for very long chain PUFA (specifically 20:4n-6 and

22:6n-3) that potentially holds two health implications. The synthesis/degradation system is down-regulated, resulting in the body being less capable of maintaining the fine balance between fatty acid synthesis and degradation resulting firstly in altered membrane fatty acid composition and secondly higher concentrations of very long chain saturated and monounsaturated fatty acids as they can no longer be efficiently degraded into their metabolites. Results of the present study reflected both of these theories as a significant reduction in CE DHA in patients was found compared with controls, so alteration of peroxisomal function could be implicated. Also, when the results were divided according to quartiles of MMSE, it was found that those with the lowest MMSE scores, i.e. those most severely affected by the disease of our study group, had higher ALA concentrations than controls, which again would implicate a defect in retroconversion, Δ5- or Δ6-desaturase activities. When the patients were divided according to CDR, their ALA: EPA ratio was significantly greater in any patient group when compared to controls. EPA concentrations were also reduced which would also suggest reductions in Δ5- or Δ6-desaturase activities. Secondly, an accumulation of very long chain monounsaturated and saturated fatty acids in plasma is characteristic of patients affected by an inborn error of peroxisomal β-oxiditation and is associated with multiple abnormalities, especially neurological (Wanders *et al,* 1990). In agreement with this, the present findings revealed higher C16:0 concentrations in patient CE compared with controls. Simon *et al* (1995) have shown that increased concentrations of C16:0 in cholesteryl esters are directly associated with CHD risk, even after controlling for serum lipid levels. A large population-based study has shown that as degree of cardiovascular disease increases so does degree of AD, and this association between the two diseases is particularly strong in those with the apo-E4 phenotype (Hofman *et al,* 1997). High saturated fat intakes have also been reported from a large prospective population-based study to be associated with the incidence of AD (Kalmijn *et al* 1997a) so the increased C16:0 and reduced EPA and DHA concentrations from our findings could have been due to either higher intakes of C16:0 or as a result of decreased peroxisomal oxiditation activity.

Earlier studies proposed that the activity of Δ6-desaturase is reduced with aging in mice (Bourré *et al,* 1990) and in patients with AD (Nakada *et al,* 1990, Kyle *et al,* 1999). The results of the present study support the view that the activity of the enzyme

may be altered in AD patients as indicated by the lower EPA and DHA concentrations in our patients. Nakada *et al* (1990) found an elevation of 18:2n-6 in brain membrane phospholipids taken from autopsied AD patients compared to age-matched controls, with the associated reduction in 20:4n-6 and 22:4n-6. A decrease in 22:6n-3 was also observed in the patient group, indicating a possible abnormality in Δ6-desaturase activities or a peroxisomal defect. They also hypothesised that alteration in PUFA desaturation/elongation processes and resultant membrane abnormalities may play a key role in the pathogenesis of AD. Kyle *et al* in their 10 year follow-up study of over 1000 elderly Americans found that low levels of PC-DHA was a significant risk factor for low MMSE score and the development of AD. They hypothesised that the disease process caused a reduction in the Δ6-desaturase-enzyme activity, leading to a reduction in DHA production which would account for the patients inability to maintain a high or 'normal' level of PC-DHA, which concurs with our results. An increase in ALA concentrations would also be expected as a result of a reduction in Δ6-desaturase activities, however neither of the above groups reported such a change. The patient group most severely affected by the disease in the present study showed an accumulation of this fatty acid when compared with controls, but our more mildly affected patients did not reflect these changes. All patient groups showed higher ALA: EPA ratios than controls, which would again imply an accumulation of ALA relative to EPA. All of our patients had mild - moderate forms of the disease, so it is possible that a further increase in ALA concentrations and reduction in DHA could occur in these patients as the disease progresses to a more severe stage.

Data from recent human studies has indicated that the capacity of Δ6-desaturase is unchanged by the aging process (Babin *et al,* 1999, Vermunt *et al,* 1999). Babin *et al* analysed the fatty acid composition of CE, total lipids, TAG and erythrocytes in a cohort of 200 elderly women living at home and that of a control group of 50 young female volunteers. They found no difference in 18:3n-6 / 18:2n-6 ratio between the two groups, which would imply no change in Δ6-desaturase with age. Vermunt *et al* (1999) also proposed that activity of these enzymes are not influenced by age and added that the conversion of ALA to EPA and DHA is simply influenced by dietary intake of these fatty acids in their preformed state. In their study 15 elderly volunteers (age > 60 years) received diets rich in either oleic acid, ALA or EPA/DHA over a 6-week period.

Five younger volunteers (age < 35 years) received an ALA-rich diet for the same period of time after which they were given 45 mg uniformly labelled ^{13}C ALA as methyl ester dissolved in olive oil and further blood samples were taken at intervals up to one week later. Their results showed that the conversion of ALA into EPA / DHA was similar for both older and younger groups, except in those whose diet was supplemented with these fatty acids in their preformed state where the conversion of ALA to these fatty acids seemed to be inhibited. This implies that the body tries to maintain equilibrium in membrane fatty acid composition, which is not influenced by the age of the individual.

None of the aforementioned studies have actually measured the enzyme activity *per se* but a rat study has shown that the affinity of Δ6-desaturase for ALA is not greatly influenced by the aging process, however, it seems to lose its affinity for the *n*-6 fatty acid substrate with aging (Hrelia *et al,* 1990). If, as results from these studies has shown, the activity of Δ6-desaturase is not significantly altered by aging, then the theory that its activity is altered by AD is not simply a function of the aging process but by the disease process itself. No human study has measured the activity of this enzyme in patients with AD.

Some impairment in Δ5-desaturation has been implicated in the aging process (Babin *et al,* 1999) as a lower 20:4n-6 / 20:3n-6 ratio was observed in elderly women when compared to younger controls. In this study arachidonic acid levels alone were not different between the two groups, which would question the reduction in Δ5-desaturase theory. The authors commented that high meat intakes in the elderly volunteers could have contributed to an increase in 20:4n-6 levels in CE and phospholipids in elderly women which would buffer the alleged reduction in this fatty acid with aging, however no figures or specific details of dietary analysis are given. Our results revealed a higher (although not significant) AA: EPA ratio in moderate-severely affected patients than controls which would imply that there may be increased competition from the n-6 PUFA family for elongation / desaturation process.

In conclusion, the prevalence of AD has been shown to correlate positively with high fat and total calorie consumption and negatively with fish consumption (Horrocks and Yeo, 1999a) which is in agreement with the reductions in the very long chain PUFA, EPA and DHA, and the increase in C16:0 observed in our patients with AD. DHA has been shown to play an important role in the survival of neuronal cells, and it

has been postulated that it may be a modulator of phosphatidyl serine biosynthesis, which in turn can effect neuronal signalling (Kin and Edsall, 1999). Decreases in DHA in the brain have been shown to be associated with the onset of sporadic AD (Horrocks and Yeo, 1999a). In the absence of dietary data it is not possible to make any definitive conclusions, however the results of the present study indicate a possible reduction in Δ6- or Δ5-desaturase activities or a defect in peroxisomal β-oxiditation, reflected in the significant reductions in DHA concentrations and an increase in ALA concentrations in those most severely affected by the disease. DHA is produced by the β-oxiditation of 24:6 n-3 (Fig 1.2.2), thus this mechanism alone cannot be implicated as we also observed a reduction in CE EPA concentrations. Increased utilisation of EPA and DHA as a result of the disease process may also play a role. Whatever the mechanism, the degree of reduction in these fatty acids does not seem to be related to the severity of the disease; patients with high MMSE scores and low CDR scores still have significantly lower EPA and DHA concentrations than controls. However, it was only the most severely affected of our patients which showed an accumulation of ALA when compared with controls.

DHA therapy is strongly recommended for those with generalised peroxisomal disorders who are characterised by low DHA concentrations in all tissues and blood (Martinez *et al*, 2000), and therapy with fish oils has been shown to improve the severity of AD in patients already affected by the disease (Peers, 1990, Yehuda *et al*, 1996, Terano *et al*, 1999). Whilst results from this preliminary study seem promising, and other authors have noted that the preventive effects of n-3 PUFA against AD are worthy of study (Connor, 2000), further research should be focused on measuring the activities of Δ6- and Δ5-desaturase enzymes in patients with AD, as well as carrying out a large-scale double blind placebo-controlled study on fish oil supplementation in the elderly in order to further evaluate the beneficial effects of fish oil supplementation on the incidence and severity of this disease.

Chapter Six

General Discussion

The studies outlined in this thesis were designed to establish a dose-response effect of supplementation with low doses of fish oils into platelet phospholipids and cholesteryl esters, and to use this established relationship to predict habitual intake of these fatty acids in health and disease situations. It was only at the higher dose of fish oil used in this work that a beneficial effect on lipid profiles was seen, however this does not mean that the lower doses supplemented over a longer period of time would not be effective in producing a more favourable lipid profile. Levels of these fatty acids were continuing to rise in platelet phospholipids at the end of the study period in these groups supplemented with very low doses of fish oils, and they had not yet achieved that level observed in the highest dose group.

The balance between intakes of n-6 and n-3 fatty acids has been suggested to be of enormous importance in health and disease, however changing dietary patterns have resulted in a substantial rise in n-6 fatty acids with a relative lowering in n-3 fatty acids. In the food industry, traditional sources of fat, such as butter and lard, have been replaced with vegetable oils high in n-6 fatty acids. Animal feeding practices have also changed, now favouring n-6-rich feeds. Meat intake over the years has also fallen, which again contributes to a change in the ratio of n-6 to n-3 fatty acids in our food chain. The development of functional foods rich in n-3 PUFA serves to counteract this imbalance which has been created, however, from a food technologists point of view, it is only possible to incorporate low doses of fish oils into foods. This research has shown that even low doses of these substances are potent; they produce more favourable lipid profiles and can cause an increase in n-3 PUFA in cell membranes which could potentially benefit a whole host of disease states, particularly those of an inflammatory nature. There is further scope for the development of foods containing ALA along with EPA and DHA, which would beneficially affect the ratio of n-6 to n-3 fatty acids in our diet.

"Brain Foods", or functional foods containing n-3 PUFA, have increased enormously in popularity in Japan. "Edison Eggs" and "Einstein Milk" have resulted from the manipulation of animal feed to produce foods with a relatively high level of n-3 PUFA. The practicality of these foods in our food chain is of enormous importance, particularly in one of the fastest growing sectors in our population, the elderly. The percentage of elderly in Ireland is increasing and age-related diseases have become high priority as a research topic in the EU 5th Framework Programme. The incidence of one important age-related disease, Alzheimer's disease, is on the increase in the EU and it is

estimated that there are 2000 new cases of AD every day in EU countries. Any measure which would decrease potentially modifiable risks or delay disease onset will obviously have huge health care cost implications. The final study presented in this thesis revealed a substantially lower level of n-3 PUFA in patients with AD when compared with elderly controls. Whilst there are many biological explanations as to why this is the case, this work really raises more questions than it answers. It is unclear, particularly in the absence of diet history data whether the disease causes a reduction in n-3 PUFA levels, or whether low n-3 PUFA concentrations increase susceptibility to the disease in the elderly. If the disease is causing a lowering in these levels, it is obvious that the biochemical changes occur early in the disease state, as all patients examined in this study had mild-moderate forms of the disease and were all free living in the community, none requiring institutionalization. The research potential from this preliminary work is enormous. A large, prospective, population-based study assessing baseline and annual cognitive function, EPA/DHA levels in cholesteryl esters, and n-3 PUFA intakes, with a long follow-up period in the elderly would give us more information, as well as a comprehensive assessment of activities of enzymes involved in ALA and EPA/DHA metabolism in patients and controls. Whatever the mechanism, it seems that maintaining a particular level of n-3 PUFA in cells, as can be achieved by increasing intake of n-3 PUFA, is protective against the disease, but it will take many years of research to elucidate the mechanisms involved.

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Appendix

PLEASE COMPLETE ALL SECTIONS. ALL INFORMATION GIVEN IS TREATED WITH COMPLETE CONFIDENTIALITY.

CODE ______________

DATE ______________

NAME ____________________________

ADDRESS ____________________________

____

____

____

TELEPHONE NUMBER ____________________

WEIGHT __________________

HEIGHT __________________

BODY MASS INDEX ________________

DATE OF BIRTH ________________

AGE (ON SCREENING) ________________

DO YOU SMOKE? _________________

HOW MUCH ALCOHOL DO YOU DRINK IN ONE WEEK?

Beer Pints ______

Bottles ______

Cans ______

Spirits ______

Wine (glasses) _____

EXERCISE

Frequency		Type	Time taken
Once per week	_____		
1-2 times per week	_____		
2-3 times per week	_____		
> 4 times per week	_____		

DIETARY INFORMATION

FOOD FREQUENCY QUESTIONNAIRE

1) FISH QUANTITY

a) Salmon (fresh, tinned or frozen)

21 14 7 6 5 4 3 2 1 F M R___

b) Mackerel (fresh, tinned or frozen)

21 14 7 6 5 4 3 2 1 F M R___

c) Herring

21 14 7 6 5 4 3 2 1 F M R___

d) Tuna (fresh)

21 14 7 6 5 4 3 2 1 F M R___

e) Tuna (tinned)

21 14 7 6 5 4 3 2 1 F M R___

f) Kippers

21 14 7 6 5 4 3 2 1 F M R___

2) SPREAD/COOKING OIL

WHAT KIND OF SPREAD DO YOU USE? ________________

HOW MUCH OF THIS SPREAD DO YOU USE?

(per day, equivalent to pate of butter) ________________

WHAT KIND OF COOKING OIL DO YOU USE? ________________

HOW MUCH COOKING OIL DO YOU USE?

(per day, tablespoons) ________________

3) NUTRITIONAL SUPPLEMENTS QUANTITY

a) fish oils

21 14 7 6 5 4 3 2 1 F M R___

b) multivitamin and mineral

21 14 7 6 5 4 3 2 1 F M R___

c) vitamin C

21 14 7 6 5 4 3 2 1 F M R___

d) brewers yeast

21 14 7 6 5 4 3 2 1 F M R___

e) ginseng

21 14 7 6 5 4 3 2 1 F M R___

f) garlic supplements

21 14 7 6 5 4 3 2 1 F M R___

g) other (please specify) ______________________

21 14 7 6 5 4 3 2 1 F M R___

4) BREAD

a) Live (white)

21 14 7 6 5 4 3 2 1 F M R___

b) Live (wholemeal)

21 14 7 6 5 4 3 2 1 F M R___

c) other (please specify) ______________________

21 14 7 6 5 4 3 2 1 F M R___