The importance of alpha-linolenic acid as a source of n-3 polyunsaturated fatty acids and its influence on risk factors of cardiovascular disease

By

Paul Anthony Wilkinson BSc (Hons) SRD

This thesis has been submitted for the degree of

Doctor of Philosophy

At the

University of Surrey, School of Biomedical and Life Sciences,
Centre for Nutrition & Food Safety

June 2004
© Paul Wilkinson 2004
Abstract

Dietary long chain n-3 fatty acids in fish-oil have proven efficacy in reducing cardiovascular risk associated with an atherogenic lipoprotein phenotype (ALP) and in reducing CHD mortality. However, the acquisition of these health benefits is seriously limited by low habitual intakes of oily fish. Since the shorter chain fatty acid alpha-linolenic acid (ALA) can be converted, to a variable extent, in vivo to its longer chain counterparts, in theory, it should have the capacity to exert fish oil like effects on cardiovascular risk. To test this hypothesis, a pilot study was designed to assess the practical issues of delivering 16g of ALA per day to a group of normal healthy (n=9) volunteers. Outcomes were used in a larger study designed to examine the relative effects of diets enriched with ALA in flaxseed oil, and fish oil on plasma lipids, lipoproteins and selected haemostatic variables in subjects with an ALP. Normal, healthy male subjects (n=57) with an ALP were randomly assigned to one of three diets for 12 weeks; a diet enriched with flaxseed oil (high ALA n=21), a "control" diet enriched with sunflower oil (high linolenic acid n-17) and the "control" diet supplemented with fish oil capsules (3g EPA+DHA n=19). Evidence for dietary compliance was provided by 7-day records of food intakes and increases in the concentration of n-3 PUFA in erythrocyte membrane phospholipids. The pilot study provided valuable information on the delivery of ALA into the study diet, which improved accuracy of dietary dose, portability and stability of the oil and aided dietary compliance in the principal study. The flaxseed, fish oil and "control" diets achieved intake ratios of n-6:n-3 of 0.4, 5.2 and 30.0 respectively. There was no overall difference in any measured variable between the 3 diets (6 & 12 week post diet) or between the flaxseed and fish-oil groups compared to control. Total plasma cholesterol decreased relative to baseline values, within all 3 test diets (pre versus post-diet). Plasma TAG was significantly decreased after the fish oil diet, relative to baseline (-23%. P<0.001). The change in plasma TAG was inversely associated with the level of DHA (C22:6 n-3) in erythrocyte membrane fatty acids at 12 weeks (r2 = 48% p=0.001). LDL subclasses showed a significant reduction towards larger, lighter particles after fish-oil (small, dense LDL-3 -22% p=0.003). There was no change in the concentrations of plasma fibrinogen, factor VII, or in the plasma activity of PAI-1 on any diet or endothelial function as measured by flow-mediated dilatation on a subset on each diet. In conclusion, the fish-oil diet induced predictable changes in plasma lipids and lipoproteins that are associated with lower CHD risk. The flaxseed-oil diet did not reproduce these effects even in the presence of low intakes of dietary n-6 fatty acids.
Acknowledgments

Firstly I would like to thank my Supervisor Bruce Griffin for giving me the opportunity to undertake this project and in particular his help in the last few months of its completion. We have, over the last few years, both wondered whether I would ever complete it, but thanks to the help and support of all of those who follow I made it!

Thank you to Claire for all her love and support during this project, not only outside of the lab, but also in the last year of the project when she helped practically and academically in more ways than I can mention. Thank you to Yvonne Jeanes, Ruth Horner, Jo Sheppard, Jenny Hunniset, Caroline Emery Rosine de Tessierés and Margaret Griffin for all their help practically and dietetically during my time at Surrey. Thank you to Jaana Nurmi for all her help and support with the statistics and generally pushing me to finish! Thanks to the study nurse Amanda Dinsmore and her amazing venepuncture skills, she just never missed those veins and also to acknowledge all of those who volunteered to take part, without whom we could have achieved nothing.

I am very grateful to those who carried out many of the analyses used in this thesis, in particular Nahed Hussein and Eric Ah-Sing for all their work on the erythrocyte fatty acid analysis and GC work. Thank you to Bruce Griffin and Ian Davies for all the blood they donated during developmental work! Thank you to Sue Thornton for giving me the flexibility I so desperately needed at work to compete this write up and to all my work colleagues who covered for me during the final stages of write up.

Finally thank you to my family, for all their help and encouragement over the years. I wouldn't be where I am today if it wasn't for them. Cheers!
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>Arachadonic Acid (20:4n-6)</td>
</tr>
<tr>
<td>ALA</td>
<td>Alpha-linolenic acid (18:3n-3)</td>
</tr>
<tr>
<td>ALP</td>
<td>Atherogenic lipoprotein phenotype</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BHT</td>
<td>Butylatedhydroxytoluene</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
</tr>
<tr>
<td>CMR</td>
<td>Chylomicron remnants</td>
</tr>
<tr>
<td>CE</td>
<td>Cholesterol Ester</td>
</tr>
<tr>
<td>DGLA</td>
<td>Dihomogamma-Linolenic Acid (20:3n-6)</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic Acid (22:6n-3)</td>
</tr>
<tr>
<td>DPA</td>
<td>Docosapentaenoic Acid (22:5n-3)</td>
</tr>
<tr>
<td>EFA</td>
<td>Essential Fatty Acid</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic Acid (20:5n-3)</td>
</tr>
<tr>
<td>FMD</td>
<td>Flow mediated dilatory</td>
</tr>
<tr>
<td>FSA</td>
<td>Food standard agency</td>
</tr>
<tr>
<td>GTN</td>
<td>Glycerol Tri-nitrate</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>HDL-C</td>
<td>High density lipoprotein cholesterol</td>
</tr>
<tr>
<td>HDL-DGUC</td>
<td>High Density Lipoprotein Density Gradient Ultracentrifugation</td>
</tr>
<tr>
<td>HL</td>
<td>Hepatic Lipase</td>
</tr>
<tr>
<td>Hr.</td>
<td>Hours</td>
</tr>
<tr>
<td>IDL</td>
<td>Intermediate density lipoprotein</td>
</tr>
<tr>
<td>Kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>LA</td>
<td>Linoleic Acid (18:2n-6)</td>
</tr>
<tr>
<td>LCAT</td>
<td>Lecithin cholesterol acyl transferase</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
</tr>
<tr>
<td>LDL-C</td>
<td>Low density lipoprotein cholesterol</td>
</tr>
<tr>
<td>LDL-DGUC</td>
<td>Low Density Lipoprotein Density Gradient Ultracentrifugation</td>
</tr>
</tbody>
</table>
LPL: Lipoprotein Lipase
mg: milligram
MJ: Megajoule
MI: Myocardial Infarction
min. minute
ml: millilitre
mmol/l millimoles per litre
mmHg Millimetres of Mercury
MUFA: Monounsaturated Fatty Acids
ng: nanogram
NS Not significant
P/S: Polyunsaturated/saturated
PAI-1: Plasminogen Activator Inhibitor Type 1
PC: Phosphatidyl-Choline
PUFA: Polyunsaturated Fatty Acids
RBC: Red blood cells
SD: Standard deviations
SFA: Saturated Fatty Acids
TC: Total cholesterol
TAG: Triacylglycerol
TX: Thromboxanes
VLDL: Very-low density lipoprotein
Vs. Versus
wk: Week
μg: micro gram
μl: micro litter
# Table of contents

1 CHAPTER 1 GENERAL INTRODUCTION .................................................. 7
   1.1 Coronary Heart Disease...................................................................... 7
   1.1.1 Attributable risk versus absolute risk ........................................ 8
   1.2 Overview of lipoprotein metabolism.................................................. 9
   1.2.1 Triacylglycerol and CHD risk ...................................................... 10
   1.3 The Atherogenic Lipoprotein Phenotype (ALP) ................................... 11
   1.4 Small dense LDL and CHD risk ....................................................... 13
   1.4.1 HDL particle size and CHD risk .................................................... 15
   1.5 Diet and CHD ................................................................................. 15
   1.5.1 Role of dietary fatty acids in CHD ................................................ 18
   1.5.2 Long chain n-3 PUFA and CHD .................................................... 20
   1.5.3 Epidemiological studies on long chain n-3 PUFA ............................ 20
   1.5.4 Effects of dietary fish oil on plasma TAG ....................................... 21
   1.5.5 Effects of fish oils on lipoproteins and lipoprotein heterogeneity ....... 21
   1.6 Fish oils and diet-gene interaction .................................................... 23
   1.7 Effects of fish oils on haemostasis and vascular function ..................... 23
   1.8 Dietary sources of long chain n-3 PUFA in the UK diet .......................... 27
   1.9 Short chain n-3 PUFA and CHD ....................................................... 28
   1.10 Aims of investigations ................................................................. 38
       1.10.1 Study Objectives ................................................................... 38
2 CHAPTER 2: GENERAL MATERIALS AND METHODS ................................. 40
   2.1 Blood sample collection and storage ................................................. 40
   2.2 Preparation of red blood cells for erythrocyte membrane phospholipid fatty
       acid analysis ................................................................................. 40
   2.3 Analysis of plasma lipids, lipoproteins and glucose ................................ 41
   2.4 Determination of LDL subclasses by density gradient ultracentrifugation . 42
       2.4.1 Determination of HDL Subclasses by density gradient ultracentrifugation
       ....................................................................................................... 43
   2.5 Determination of erythrocyte membrane fatty acid phospholipid composition
       ....................................................................................................... 44
   2.6 Comparison of HDL subclasses as isolated by HDL-DGUC and
       electrophoretic separation ............................................................... 45
       2.6.1 A new gel rod electrophoresis system for the determination of HDL
           subclasses: HDL Lipoprint system ................................................ 45
           2.6.1.1 Method ............................................................................. 46
   2.7 Validation of HDL subclasses using HDL-DGUC and the Lipoprint HDL
       system ........................................................................................... 48
       2.7.1 Aims ....................................................................................... 48
       2.7.2 Study Design ........................................................................... 48
       2.7.2.1 Subjects: ............................................................................ 49
       2.7.3 Results: .................................................................................. 49
       2.7.4 Comparison of HDL-DGUC and gel rod electrophoresis in the separation
           of HDL subclasses in 3 subjects .................................................... 51
# Table of figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Serum cholesterol as an inadequate indicator of CHD risk (Fruchart &amp; Packard 1997)</td>
<td>9</td>
</tr>
<tr>
<td>1.2</td>
<td>The effects of increased TAG on the incidence of CHD as identified by the PROCAM study (Assman et al 1997)</td>
<td>11</td>
</tr>
<tr>
<td>1.3</td>
<td>Neutral lipid exchange between TAG rich particles and LDL. (Adapted from Griffin 2001)</td>
<td>13</td>
</tr>
<tr>
<td>1.4</td>
<td>Density Gradient (top) and GGE (bottom) profiles illustrating distinct subclasses within LDL</td>
<td>14</td>
</tr>
<tr>
<td>1.5</td>
<td>The chemical structure of saturated, mono, n-3 and n-6 polyunsaturated fatty acids</td>
<td>16</td>
</tr>
<tr>
<td>1.6</td>
<td>Effect of substituting 5% carbohydrate energy with SFA, MUFA or PUFA on lipoproteins and plasma triacylglycerol (Adapted from Mensink &amp; Katan 1992)</td>
<td>18</td>
</tr>
<tr>
<td>1.7</td>
<td>The predicted effects of 3 dietary therapeutic approaches (Step 1: 30% fat, Step 2: 20% fat and the Mediterranean diet: 38% fat) on plasma TAG and HDL-C compared to a typical Northern European intake: 38% fat.</td>
<td>19</td>
</tr>
<tr>
<td>1.8</td>
<td>Classical omega-3 and omega-6 fatty acid synthesis pathways and the role of omega-3 fatty acid in regulating health/disease markers.</td>
<td>24</td>
</tr>
<tr>
<td>1.9</td>
<td>Metabolic pathways of the conversion of linoleic acid and alpha linolenic acid to their respective longer chain PUFA.</td>
<td>30</td>
</tr>
<tr>
<td>1.10</td>
<td>The fatty acid composition of functional pools can be affected by the dynamic relationship between dietary intake and subsequent storage and transport of fatty acids.</td>
<td>36</td>
</tr>
<tr>
<td>2.1</td>
<td>Separation of plasma LDL subclasses by discontinuous density gradient ultracentrifugation</td>
<td>42</td>
</tr>
<tr>
<td>2.2</td>
<td>Diagram of the Beckman Fraction recovery system</td>
<td>43</td>
</tr>
<tr>
<td>2.3</td>
<td>HDL Lipoprint loading and preparation rack.</td>
<td>46</td>
</tr>
<tr>
<td>2.4</td>
<td>Diagram of electrophoresis gel tube from HDL Lipoprint kit illustrating the Rf bands for HDL₂ and HDL₃ subfractions.</td>
<td>47</td>
</tr>
<tr>
<td>2.5</td>
<td>HDL profile generated from HDL-Lipoprint System showing VLDL, HDL₂ and HDL₃ subclasses. Rf values calculated from VLDL to Albumin Front (Alb)</td>
<td>48</td>
</tr>
<tr>
<td>2.6</td>
<td>AUC (%) values for HDL₂ PAGE compared tp HDL₂ DGUC, from the same sample.</td>
<td>50</td>
</tr>
<tr>
<td>2.7</td>
<td>AUC (%) values for HDL₃ PAGE compared to HDL₃ DGUC, from the same sample.</td>
<td>50</td>
</tr>
<tr>
<td>2.8</td>
<td>HDL profiles generated using DGUC (Grey = Female, Red = Male)</td>
<td>50</td>
</tr>
<tr>
<td>3.1</td>
<td>Percentage change in dietary intake between baseline and intervention of fats and observed changes in erythrocyte fatty acid membrane composition.</td>
<td>59</td>
</tr>
<tr>
<td>3.2</td>
<td>LDL subclass profiles for all 9 volunteers, pre and post diet.</td>
<td>61</td>
</tr>
<tr>
<td>4.1</td>
<td>Questionnaire responses to general impressions of the study</td>
<td>72</td>
</tr>
</tbody>
</table>
Figure 5.1: Intakes of MUFA, n-6 and n-3 PUFA and the n-6:n-3 ratio of all 3 dietary groups on their respective study diets..................................................87
Figure 5.2: Erythrocyte membrane phospholipid fatty acids: relative abundance (SD) at baseline (0 weeks), 6 and 12 weeks in all three dietary treatment groups........................................................................................................89
Figure 5.3: Flax oil group: change in percentage of n-3 PUFA intake (habitual vs study diet) against the abundance of 18:3 n-3 in erythrocyte membrane phospholipids.....................................................90
Figure 5.4: Plasma triacylglycerol, total cholesterol, HDL-C and LDL-C on all 3 diets at baseline, 6 and 12 weeks (SEM)................................................................................................................93
Figure 5.5: Changes in TAG expressed as percentages compared to baseline on all three diets at weeks 6 and 12.................................................................................................................94
Figure 5.6: Fasting TAG versus % LDL-3 in all subjects at baseline (p=0.015, \( r=0.32 \) \( r^2=0.10 \)).............................................................................................................95
Figure 5.7: Percentage change in LDL subclasses from baseline at 6 & 12 weeks .........................................................................................................................................................................96
Figure 5.8: HDL subclasses at baseline and 12 weeks ........................................................................................................................97
Figure 5.9: Haemostatic results at baseline, week 6 and week 12 for the flax, sunflower and fish oil diets..............................................................................................................................99
Figure 5.10: Absolute change in plasma TAG in the fish oil group (pre-post diet) verus abundance of DHA in erythrocyte membrane phospholipids \( r = 0.69 \) \( r^2 = 0.48 \) p<0.001 ..................................................................................................................100
Figure 5.11: Vascular endothelial function measured by flow-mediated dilation at baseline and 12 weeks.............................................................................................................................................101
Figure 5.12: Relationship in all subjects at baseline between the abundance of DHA in erythrocyte membrane phospholipids and the TC:HDL-C ratio \( r=0.53 \) \( r^2=0.28 \) p<0.001................................................................................................................102
Figure 6.1: Power values to detect a medium size difference in TAG, between the three treatment groups............................................................................................................................119
Table of Tables

Table 1.1: Predisposing factors for the metabolic syndrome ............................................8
Table 1.2: Origin, principle function and role in CHD of the main lipoproteins. ......10
Table 1.3: Summary of interventions aimed at reducing CHD risk using differing dietary approaches (adapted from Hu & Willett 2002) .................................................................17
Table 1.4: Percentage of fatty acids in lipid fractions of Inuit and Danes (adapted from Salen et al 1999) ..................................................................................................................................25
Table 1.5: Effect of the fatty acids AA and EPA on prostaglandin and leukotriene production and their subsequent effects on haemostasis ..........................26
Table 1.6: Function and role in CHD risk of Factor VII, PAI-1 and Fibrinogen ....27
Table 1.7: Fatty acid compositions of fish per 100g commonly consumed in the UK diet .........................................................................................................................27
Table 1.8: Fatty acid composition of oils detailing P/S ratio and n-6:n-3 ratio ....31
Table 1.9: Summary of alpha-lionlenic acid supplementation studies and their effect on total and LDL cholesterol, triacylglycerol, EPA and DHA ........33
Table 2.1: mean values for AUC for the page and density gradient methods ....49
Table 2.2: Correlation between PAGE and DGUC methods ................................. 49
Table 2.3:: Results of %AUC for HDL Lipoprint at 0 and 12 weeks ..................51
Table 2.4 Results of %AUC for DGUC at 0 and 12 weeks ............................. 51
Table 2.5: Calculation of mmol values of HDL$_2$ and HDL$_3$ using the HDL Lipoprint system and DGUC methods .......................................................52
Table 3.1: Unit System for determining ALA intake in differing food products ...55
Table 3.2: Habitual and study diet information from the pilot study .................58
Table 3.3: Biochemical parameters and weights of all subjects at weeks 0,2 and 4 .................................................................................................................................58
Table 3.4: Fatty acids in erythrocyte membrane phospholipids at week 0 and week 4 .........................................................................................................................59
Table 3.5: Mean distribution of LDL subclasses at week 0 and week 4 ..........60
Table 4.1: Factors that may positively or adversely affect compliance ..........66
Table 4.2: Strategies used to determine compliance by volunteers ..................68
Table 4.3: Responses from volunteer questionnaire – General Impressions ....70
Table 4.4: Responses from volunteer questionnaire – assessing compliance ....70
Table 4.5: Responses from volunteer questionnaire – assessing compliance ...71
Table 4.6: Responses from volunteer questionnaire – food diaries ...............71
Table 4.7: Responses from volunteer questionnaire – overall compliance with instructions ..............................................................................................................71
Table 4.8: Responses from volunteer questionnaire – overall study rating ......71
Table 5.1: Initial selection criteria for potential volunteers ..............................79
Table 5.2: Entry criteria for the study after screening blood samples .............80
Table 5.3: Numbers of volunteers completing each diet ................................80
Table 5.4: Nutritional content of the margarines, oils and fish oil capsules supplied to volunteers .............................................................................................................82
Table 5.5: Intakes of Macronutrients on Habitual and Study diets .................86
Table 5.6: Anthropometric data, glucose, ApoB and TC:HDL ratio at baseline, 6 weeks and 12 weeks .............................................................................................................92
CHAPTER 1 GENERAL INTRODUCTION

1.1 Coronary Heart Disease

Coronary Heart Disease (CHD) accounted for 22% of all deaths in males under the age of 75 in the UK in 2001 (Office for National Statistics 2002), nevertheless, death from CHD in males has been declining, in 1971 375 per 100,000 deaths were attributable to cardiovascular disease, by 1990, that figure was 300. Between 1989 and 1999, there was a 43% fall in deaths from CHD in males aged 45-54 years, and a 34% decrease in those aged 65 to 74 years (BHF 2001). While advances in primary and secondary health care have undoubtedly contributed to this decline in CHD mortality, CHD morbidity figures have changed little. On the other hand the prevalence of obesity in the UK is increasing; in 2001, 24% cent of women and 21% of men were obese (Health Survey for England, 2001) compared to 8% of women and 6% of men in 1980 (ONS 1980). This is due primarily to inappropriate diet and reductions in physical activity. An increase in the prevalence of Type II diabetes has also immerged almost certainly due to decreases in physical activity and worsening dietary intake (UKPDS 1988). Obesity contributes to a range of diseases including cardiovascular disease. The National Audit Office estimated that obesity reduces life expectancy on average by 9 years (NAO 2001). In diabetics, the incidence of CHD risk is increased 2 to 3 fold compared with non-diabetics. In a large prospective study, CHD risk in people with diabetes, but without overt CHD, was similar to that in non-diabetics with established CHD (Haffner et al 1998).

The metabolic syndrome has been described as a ‘clustering’ of several risk factors for CHD, including central obesity, insulin resistance, dislipidaemia and hypertension (Table 1.1). In the UK, it has been suggested that as many as 25% of the population show clear signs of the metabolic syndrome (Tonkin et al. 2003). The Kuopio Ischaemic Heart Disease Risk Factor Study was a population based prospective study that found a 2-fold increase in CHD and all cause mortality in men with the metabolic syndrome compared to those without (Lakka 2001).
et al 2002). These data alone suggest that CHD risk may have already reached epidemic proportions in the UK.

Table 1.1: Predisposing factors for the metabolic syndrome

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Defining Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abdominal Obesity (Waist Circumference)</td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>&gt;102 cm (&gt;40 inches)</td>
</tr>
<tr>
<td>Women</td>
<td>&gt;88 cm (&gt;35 in)</td>
</tr>
<tr>
<td>TAG</td>
<td>≥1.7 mmol/l</td>
</tr>
<tr>
<td>HDL-C</td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Women</td>
<td>&lt;1.3</td>
</tr>
<tr>
<td>Blood Pressure</td>
<td>≥130/≥85 mmHg</td>
</tr>
<tr>
<td>Fasting Glucose</td>
<td>≥6.0 mmol/l</td>
</tr>
</tbody>
</table>

1.1.1 Attributable risk versus absolute risk

Until recently, most clinical investigations of diet and CHD have been cholesterol-centric in their approach. Large prospective epidemiological studies established a continuous relationship between TC levels and death from CHD. The MRFIT trial (1986) associated increasing TC with increasing mortality, and found a significant reduction of first major CHD events with dietary interventions to lower TC. The Framingham study found a direct relationship between TC levels and CHD mortality (Anderson et al 1987). The cross-cultural seven countries study, found that a 0.5mmol/l increase in TC was associated with 12% increase in CHD mortality (Verschuren et al 1995). Focusing only on TC is an inadequate predictor of CHD risk in a population such as the UK. Fruchart & Packard (1997) plotted the frequency of distribution of serum cholesterol levels in both the diseased and non-diseased population and found the absolute risk of CHD mortality with serum cholesterol of 7.5mmol/l was about 90%, however only 3% of those with CHD had this level, making the attributable risk just 2.7%. On the other hand, serum cholesterol levels of 5.2mmol/l were associated with a CHD risk around 20%, but 45% of those with CHD also had this level; the attributable risk therefore is greater at 9%. Figure 1.1 illustrates the overlapping distributions between CHD free subjects and those with established CHD. There exists a “grey area” within which the TC levels do not discriminate CHD from
health. In the principal dietary investigation we examined the effects of diet on a range of CHD risk factors, not solely TC.

Figure 1.1: Serum cholesterol as an inadequate indicator of CHD risk (Fruchart & Packard 1997)

1.2 Overview of lipoprotein metabolism

The TC value is a representation of all the lipoproteins in the circulation that contain free cholesterol or cholesterol esters. Cholesterol and TAG are transported around the body via lipoproteins, which are subdivided into those of exogenous (from the gut) and endogenous (from the liver) origin. Table 1.2 summaries the main lipoproteins their origin, structure, role in the body and in CHD risk.
### Table 1.2: Origin, principle function and role in CHD of the main lipoproteins.

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Origin &amp; Structure</th>
<th>Principal Function</th>
<th>Risk in CHD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chylomicrons (CM)</td>
<td>Formed in epithelial cells in lower intestine after consumption of a meal containing fat</td>
<td>Transport of TAG from the gut to the adipose and muscle cells. Liver processes what is left.</td>
<td>contributes to raised TAG in postprandial state. Remnant particles are proinflammatory and proatherogenic.</td>
</tr>
<tr>
<td>Very Low Density Lipoprotein (VLDL)</td>
<td>Liver: 10% Protein, mainly containing TAG</td>
<td>Transport of TAG formed in liver: mainly to adipose tissue</td>
<td>In IR and ALP may contribute in process of neutral lipid exchange in formation of more atherogenic LDL particles</td>
</tr>
<tr>
<td>Low Density Lipoprotein (LDL)</td>
<td>Mainly derived from delipidation of VLDL: Highest in CE as % of weight</td>
<td>Carry majority of cholesterol in the blood. Transport of CE to peripheral cells for membrane structure and hormone production</td>
<td>High levels &gt;4.0mmol are considered atherogenic, higher residency time increases likelihood of proinflammatory and proatherogenic effects.</td>
</tr>
<tr>
<td>High Density Lipoprotein (HDL)</td>
<td>Liver: Highest in density due to high protein/lipid ratio</td>
<td>Transport of CE from peripheral cells to the liver (Reverse Cholesterol Transport Pathway)</td>
<td>Considered anti-atherogenic levels &gt;1.1mmol/l desirable.</td>
</tr>
</tbody>
</table>

### 1.2.1 Triacylglycerol and CHD risk.

The increased use of statins and fibrates continue to reduce many CHD risk factors, but the media and to a certain extent health professionals seem to focus their attention on cholesterol as the key player in CHD. It is widely accepted that CHD is associated with not only cholesterol but also a variety of other factors including gender, genetics, environmental and dietary factors. While there is still intense interest in cholesterol as an independent indicator on CHD, epidemiological evidence has emerged that has also identified TAG. TAG was initially downgraded as risk factor for CHD because it was statistically associated with other variables in particular HDL-C. The PROCAM study (Assman et al 1997) suggested that a high HDL-C level is more protective than a low level of LDL-C, but also that for any given LDL-C level, CHD events were higher in those with TAG levels above 2.3mmol/l compared to those with <2.3mmol/l (Figure 1.2). Meta-analysis of 46,413 man and 10,864 women showed that for every 1
mmol/l increase in TAG there was an increase risk of CHD by 32% in men and 76% in women (Hokanson and Austin 1996). The findings of the Copenhagen Male Study reported a clear gradient of CHD risk with increasing TAG levels (Brewer 1999). Intervention with the TC and TAG lowering drug Gemfibrozil by the Helsinki Heart Study (Tenkanen et al 1994) reported a 70% fall in the incidence of CHD in those with initial fasting TAG >2.3 mmol/l. The Framingham Study (Castelli 1992) also found a strong inverse relationship between TAG and HDL-C. The highest incidence of CHD occurred in the group with the lowest HDL and highest TAG.

Figure 1.2: The effects of increased TAG on the incidence of CHD as identified by the PROCAM study (Assman et al 1997).

1.3 The Atherogenic Lipoprotein Phenotype (ALP)

An ALP is a collection of abnormalities in plasma lipoproteins, which increases the risk of heart disease in apparently healthy individuals. An ALP is associated with moderately raised serum TAG (>1.5mmol/l) with reduced HDL levels (<1.0mmol/l) and a predominance of small, dense LDL particles (Austin & Edwards 1996). The prevalence of an ALP in the free living population is not known, numbers are likely to parallel that of mild insulin resistance in middle aged males and post-menopausal women, with around 25% being affected
Insulin resistance (IR) describes a state where the ability of insulin to stimulate uptake and disposal of glucose in muscle is impaired. It is commonly found in overweight individuals and those with type-II diabetes. Ruderman et al (1981) described "metabolically obese" individuals who were a normal weight, but exhibited IR similar to those who were obese. IR does not only effect glucose uptake it also affects TAG uptake in adipose tissue and muscle. Lipoprotein Lipase (LPL) in adipose tissue is stimulated by insulin, and as such a decrease in LPL activity will affect the rate at which larger TAG rich lipoproteins are dilapidated and metabolised further. The net effect of this is not only prolonged postprandial lipaemia, but also greater fasting TAG levels. Raised fasting TAG in IR individuals may also be caused by a reduction of peripheral blood flow blunting the delivery of TAG rich lipoproteins to peripheral muscle (Summers et al 1990). IR is also associated with raised levels of circulating free fatty acids (FFA); these are the major substrate for hepatic VLDL synthesis, and as such there is an associated rise in TAG rich VLDL, even in the fasting state.

Prolonged postprandial lipaemia and raised fasting TAG levels increase the likelihood of potentially atherogenic lipoprotein particles being formed, primarily through the action of neutral lipid exchange. In this manner, a reduction in plasma TAG levels may confer some benefit in reducing potentially atherogenic structural changes occurring to lipoproteins, in particular LDL-C. Low-density lipoprotein can be remodelled in a manner that makes it more atherogenic, this
process is known as neutral lipid exchange (Figure 1.3). The effects are not just limited to LDL, but also affect HDL ultimately altering their size and composition. Changes in their heterogeneity have profound effects in terms of their atherogenicity and preponderance in the circulation. Prospective trials have shown that small LDL particles are strong predictors of future coronary events, similar to that of hypertension and smoking (Stampfer et al 1996, Gardner et al 1996).

Figure 1.3: Neutral lipid exchange between TAG rich particles and LDL. *(Adapted from Griffin 2001)*

The mechanisms by which LDL undergoes its structural changes are primarily through neutral lipid exchange. Cholesterol esters can be exchanged between VLDL and LDL in exchange for TAG (Nichols & Smith 1965). This results in a cholesterol ester (CE) rich VLDL and a TAG rich LDL particle. The effect of HL acting on the TAG rich LDL results in a small, dense cholesterol and protein rich particle or LDL-3. An increase in fasting TAG above 1.5 mmol/l is associated with an increase in the number of small, dense LDL particles in the circulation (Griffin 2001). This can be explained in part by the process of neutral lipid exchange. When fasting TAG levels are below 1.5 mmol/l there exists an equimolar exchange of lipids and CE between donor particles (Packard & Shepherd 1997). When fasting TAG increases above 1.5 – 1.6 mmol/l, this equimolar exchange is replaced by a net transfer of TAG into the LDL particle. This process generates TAG rich, CE poor LDL, which undergoes lipolysis by HL; resulting in a small, dense LDL particle.

### 1.4 Small dense LDL and CHD risk

LDL is a heterogeneous particle. Gradient gel electrophoresis (GGE) and more recently density gradient centrifugation have allowed isolation of distinct LDL
subclasses (Griffin 1990). Heterogeneity can be caused by factors such as differing lipid and cholesterol content. Density gradient ultracentrifugation resolves 3 distinct LDL subclasses, categorised by their respective densities within the gradient. The largest and most buoyant “LDL-1” lies between 1.025g/ml and 1.034g/ml, the intermediate size “LDL-2” between densities 1.034g/ml and 1.044g/ml, and the small, dense “LDL-3” between 1.044g/ml and 1.063g/ml. In some instances in individuals with overt hypotriacylglycerolaemic, a small, dense LDL particle “LDL-4” is measured. The largest LDL-1 subspecies is relatively TAG rich, the intermediate size LDL-2 is less TAG rich and has a higher protein content. The LDL-3 particle contains very little TAG is protein rich and smaller in size than either of its progenitors. Figure 1.4 illustrates the LDL subclasses generated using GGE and Density Gradient ultracentrifugation methods.

**Figure 1.4: Density Gradient (top) and GGE (bottom) profiles illustrating distinct subclasses within LDL**

Both methods quantify subclasses on their percentage relative to the total area under the curve. The distribution shown in Figure 1.4 is consistent with the pattern A phenotype as first described by Austin and co workers (1990). If over 40% of the LDL is LDL-3, the subject is said to have a pattern “B” phenotype. Pattern “B” describes a preponderance of small, dense LDL particles. Pattern “A” phenotype describes a low number of LDL-3 particles with most being found in
the densities 1.019 g/ml to 1.044 g/ml. Unlike GGE, density gradient centrifugation also reveals a pattern "I" phenotype (or intermediate) in which there is an almost equal preponderance of LDL-2 and LDL-3.

1.4.1 HDL particle size and CHD risk

HDL is often described as two distinct lipoproteins: a larger HDL\textsubscript{2} particle containing free cholesterol, CE, some TAG and a smaller HDL\textsubscript{3} particle, containing less free cholesterol, CE and 50% as much TAG as HDL\textsubscript{2} (Eisenburg 1984). The structural changes that occur in HDL are described by Tailleux and Fruchart (1996). The transition between HDL\textsubscript{2} and HDL\textsubscript{3} is a continuous one, and as such HDL-C should be viewed as a heterogeneous group of particles (Nicoll et al 1980). Two subclasses can be isolated and quantified by density gradient ultracentrifugation. As with LDL, area under the protein curve is used to determine the relative percentages of each sub fraction. Changes in the distribution of the two subclasses can be affected by other lipoproteins.

1.5 Diet and CHD

Figure 1.5 illustrates the chemical structure of saturated, mono and n-3 and n-6 polyunsaturated fatty acids. The prefixes n-3 and n-6 determine which family of polyunsaturates the fat originates from. Those with the prefix n-3 (or omega-3) have the first double bond on the third carbon atom from the carboxyl end. Those with the prefix n-6 have the first double bond on sixth carbon. While both families fall under the heading PUFA, they have different effects metabolically in humans, which are discussed later.
Figure 1.5: The chemical structure of saturated, mono, n-3 and n-6 polyunsaturated fatty acids

The work of Keys et al. (1965) was of particular importance in illustrating that a reduction in dietary energy from saturated fat (SFA) was associated with a reduction in TC. Following this discovery, ways in which SFA within the diet could be replaced were examined. In the 1970's and 80's the use of margarines to replace butter and lard became more widespread. The use of oils from seeds such as sunflowers became cheaper and hence more a cost-effective way to make margarine and oils; as such, energy derived from saturated fatty acids began to fall alongside increases in PUFA. At that time, a majority of dietary PUFA was n-6 in origin; the other types of PUFA (n-3 PUFAs) derived from marine and plant origin were not increased. Intakes of n-3 fats, primarily from oily fish and some plants failed to increase or fell slightly whilst those from n-6 continued to increase. Consequently, the ratio of n-6 PUFA to n-3 PUFA increased dramatically from around 1:1 at the beginning of the 20th Century, to around 10:1 in the UK in 1990 (Gregory 1990). Intakes of oily fish in the UK are generally low, due to taste, cultural and financial reasons. While there is convincing evidence of the beneficial effects of n-3 PUFA on CHD, particularly
from oily fish, attention was still firmly fixed on TC. The result of this was continued use of n-6 PUFA as a replacement for SFA rather than n-3 PUFA.

Over the last 30 years, the effects of altering total energy from fat and differing fatty acids on CHD risk have been examined. Table 1.3 summarises some of the larger dietary intervention studies conducted and their outcomes in terms of reducing CHD risk and the effect on TC.

**Table 1.3: Summary of interventions aimed at reducing CHD risk using differing dietary approaches (adapted from Hu & Willett 2002).**

<table>
<thead>
<tr>
<th>Trial</th>
<th>Patients (n=)</th>
<th>Dietary Intervention</th>
<th>% Energy from fat</th>
<th>Duration (Years)</th>
<th>Change in TC (%)</th>
<th>Change in CHD (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Low-Fat Approach</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MRC (low fat)</td>
<td>123 Male MI</td>
<td>Reduce total fat</td>
<td>22</td>
<td>3</td>
<td>-5</td>
<td>+4</td>
</tr>
<tr>
<td>DART</td>
<td>1015 Male MI</td>
<td>Reduce total fat</td>
<td>32</td>
<td>2</td>
<td>-4</td>
<td>-9</td>
</tr>
<tr>
<td><strong>High Polyunsaturated Fat Approach</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Finnish Mental Hospital Study</td>
<td>676 men with no CHD</td>
<td>Reduce SFA, increase PUFA</td>
<td>35</td>
<td>6</td>
<td>-15</td>
<td>-44†</td>
</tr>
<tr>
<td>MRC (soy oil)</td>
<td>206 Male MI</td>
<td>Reduce SFA, increase PUFA</td>
<td>39</td>
<td>5</td>
<td>-14</td>
<td>-25</td>
</tr>
<tr>
<td>Minnesota Coronary Survey</td>
<td>4393 men &amp; 4664 women</td>
<td>Reduce SFA, increase PUFA</td>
<td>38</td>
<td>1</td>
<td>-14</td>
<td>0</td>
</tr>
<tr>
<td><strong>Increase Omega-3 Fatty Acid</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DART</td>
<td>1015 MI Patients</td>
<td>Fish twice week or fish oil 1.5g</td>
<td>NR</td>
<td>2</td>
<td>NR</td>
<td>-16</td>
</tr>
<tr>
<td>GISSI</td>
<td>5666 Men MI</td>
<td>Fish oil 1g EPA+DHA</td>
<td>NR</td>
<td>3.5</td>
<td>0</td>
<td>-30†</td>
</tr>
<tr>
<td><strong>Whole-Diet Approach</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lyon Diet Heart Study</td>
<td>203 Men MI</td>
<td>High ALA &amp; Mediterranean Diet</td>
<td>31</td>
<td>3.8</td>
<td>0</td>
<td>-72†</td>
</tr>
</tbody>
</table>

† p<0.05
* Change to cholesterol refers to the percentage change is serum cholesterol level in the treatment group compared with the control group.
NR = Not Recorded

References

Changing the amount of fat and the type of fat consumed not only affects TC levels but also lipoprotein metabolism in humans. Mensink & Katan (1992) predicted the effect of replacing 5% of carbohydrate energy with saturated,
monounsaturated and polyunsaturated fatty acids on TAG, HDL and LDL, and the LDL:HDL ratio. Predicted effects are shown in Figure 1.6

**Figure 1.6: Effect of substituting 5% carbohydrate energy with SFA, MUFA or PUFA on lipoproteins and plasma triacylglycerol (Adapted from Mensink & Katan 1992)**

<table>
<thead>
<tr>
<th>Saturated</th>
<th>Monounsaturated</th>
<th>Polyunsaturated</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HDL-C</strong></td>
<td><strong>LDL-C</strong></td>
<td><strong>TAG</strong></td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>-2</td>
</tr>
<tr>
<td>-2</td>
<td>-4</td>
<td>-8</td>
</tr>
<tr>
<td>-8</td>
<td>-10</td>
<td>-8</td>
</tr>
</tbody>
</table>

**1.5.1 Role of dietary fatty acids in CHD**

Our understanding of the pathophysiological role of dietary TAG in atherosclerosis has changed over the last few decades. In terms of reducing CHD risk the main message advocated was to decrease the intake of saturated and trans-unsaturated fatty acids, primarily to reduce LDL-C concentration (AHA 2000). The effects of altering dietary fatty acids are not limited to LDL-C, but may also affect HDL-C and plasma TAG. Substituting 10% total daily energy intake from saturated fat with polyunsaturated, monounsaturated or carbohydrate decreases LDL by 0.46mmol/l, 0.38mmol/l and 0.33mmol/l respectively (Mensink & Katan 1992). The impact on LDL-C is favourable in terms of reducing CHD risk, with PUFA being the most effective, however while carbohydrate was associated with a decrease in LDL-C it also lowered HDL-C, effectively leaving the LDL-HDL ratio unchanged. The effect of PUFA on LDL-C is much greater than HDL with greater reductions in the former occurring, effectively decreasing the LDL-HDL ratio (Mensink & Katan 1992).
The effects of substituting dietary fats and/or carbohydrate on fasting and non-fasting TAG should also be considered. Substituting dietary fat for carbohydrate does not reduce fasting or postprandial TAG levels (Sacks & Katan 2002) as high carbohydrate intakes increase endogenous TAG production, primarily through VLDL secretion. Mensink & Katan (1992) predicted the effects of 3 dietary therapeutic approaches on TC, LDL-C, HDL-C and plasma TAG. They compared a typical Northern European intake with the Step 1, Step 2 diets and Mediterranean diet. Decreasing energy from fat to 20% (Step 2 diet) is associated with a 20% rise in plasma TAG, and a 20% reduction in HDL-C. On the other hand, keeping fat intake at 38% of total energy but decreasing SFA and increasing both MUFA and PUFA (Mediterranean Diet) is associated with no changes in plasma TAG and greater reductions in both TC and LDL-C compared to HDL-C, clearly a more favourable outcome. Figure 1.7 summarises the predicted effects of the 3 diets on plasma TAG and HDL-C.

Figure 1.7: The predicted effects of 3 dietary therapeutic approaches (Step 1: 30% fat, Step 2: 20% fat and the Mediterranean diet: 38% fat) on plasma TAG and HDL-C compared to a typical Northern European intake: 38% fat.
1.5.2 Long chain n-3 PUFA and CHD.

The potential benefits on reducing CHD risk by dietary n-3 PUFA, in particular EPA and DHA from fish oils was first identified by Dyberg (1975). Since then these effects have been studied more closely.

1.5.3 Epidemiological studies on long chain n-3 PUFA.

Epidemiological studies have shown that men who eat at least some oily fish have a lower incidence of CHD mortality than those who eat none (Kris-Etherton et al 2002). Kromhout and co workers (1985) examined fish consumption and 20-year mortality from CHD and found that as little as 35g of oily fish per day could reduce CHD mortality by 50%. Holob (1988) examined the intakes of EPA in Inuit Indians living in Greenland and found they averaged 4g per day; this compared to 0.1g per day in the UK population at around that time (DOH 1991); there were striking differences between the incidence of CHD between the Inuit and western society (Leaf & Weber 1988). More recently, the 30-year follow up of the Chicago Western Electric Study reported favourable effects of fish consumption on CHD mortality (Davilglus et al 1997). In 1999, Zhang and co-workers reported that fish consumption, across 36 countries, was associated with a reduction in CHD risk. Such epidemiological evidence has led to studies examining the effects of consuming fish or fish oil in both primary and secondary prevention of CHD. The GISSI Prevenzione Study (1999) is the largest prospective intervention study to date examining the effects of fish oil supplementation on secondary prevention of CHD; there was a 20% reduction in all cause-mortality after 3.5 years of 850mg of EPA and DHA per day. Previously, Singh and co-workers (1997) supplemented suspected MI patients with 1.8g of EPA and DHA per day and compared them to placebo; after 1 year there was a significant decrease in cardiac events in the fish oil group (p<0.01). While these studies have shown many favourable effects on reducing CHD risk, other studies looked more specifically at the mechanisms behind these effects.
1.5.4 Effects of dietary fish oil on plasma TAG

One of the most potent effects of fish oils is their ability to decrease both fasting and postprandial TAG levels (Harris & Connor 1980, Harris et al 1984, Demacker et al 1991). As little as 3g (1% of energy) can reduce TAG by as much as 30% (Harris 1997). Such a decrease may be associated with a reduction in CHD risk (Hokanson and Austin 1996). The mechanisms behind the hypotriacylglycerolaemic properties of fish oils are multifactorial. One of the main TAG carrying lipoproteins is VLDL. Diets enriched with n-3 PUFA are associated with decreases in the rate of VLDL synthesis in the liver (Nestel et al 1984), possibly due to diversion of fatty acids for phospholipid formation. The rate of VLDL flux from the liver is further suppressed by greater intakes of n-3 PUFA in a dose dependant manner (Nestel et al 1984, Simons et al 1985). Fish oils have been associated with a reduction in plasma free fatty acids (Singer 1992), and smaller VLDL particles. Smaller VLDL may also be due to the down regulation of acetyl CoA carboxylase (Schoonjans et al 1996), resulting in decreased lipogenesis, and consequently lower levels of free fatty acids for VLDL formation. The hypertriacylglycerolaemic state induced by a high carbohydrate diet can be reversed by dietary n-3 PUFA, primarily through its effect on the acetyl CoA carboxylase enzyme (Harris et al 1984). Abbey et al (1990) reported a significant fall in VLDL when 3.8g of fish oil were consumed per day. However, a strong correlation \( r = -0.84 \) between fall in VLDL cholesterol and increase in LDL-C was found.

1.5.5 Effects of fish oils on lipoproteins and lipoprotein heterogeneity.

The results of studies examining the effects of fish and fish oils on lipoproteins have been equivocal. Significant decreases (Illingworth et al 1984) and significant increases (Sullivan et al 1985, Abbey et al 1990, Harris 1997) in LDL-C have been measured on increasing intakes of EPA and DHA. The effects on HDL-C are generally minimal. A meta-analysis conducted by Harris (1996) showed a 3% increase in HDL-C in normolipidaemic subjects but little or no
change in those with raised triacylglycerol (>2.0mmol/l). Any reduction in TC without a decrease in HDL-C would improve the HDL:TC ratio; any rise in LDL-C or TC could be considered unfavourable in terms of reducing CHD risk. Some of the suggested reasons for differences observed in LDL-C are differing doses of fish oils and length of intervention (Rystan & Drevon 1989, Goh et al 1997). The effects of long chain n-3 PUFA on lipoprotein heterogeneity have also been investigated, in particular that of LDL and HDL.

Increased intakes of n-3 fatty acids affect cholesterol ester transfer protein (CETP) activity. Abbey and co-workers (1990) reported a 23% reduction in CETP activity on a fish oil diet. Since CETP facilitates the exchange of CE for TAG in lipoproteins, a reduction in activity should manifest itself in a number of ways: not only LDL but also VLDL and HDL would be affected. There is now good evidence that in the presence of moderate hypercholesterolemia (predominantly raised LDL cholesterol), LDL particle size is affected by the hypotriacylglycerolaemic properties (fasting and postprandial) of fish oil, rather than the long chain PUFA themselves (Griffin & Zampelas 1995). Decreasing fasting TAG below 1.5 mmol/l may be associated with a reduction in the number of small, dense LDL particles in the circulation (Griffin 1994, Griffin 2001), facilitated by a changes in neutral lipid exchange (Figure 1.3).

The benefit that fish oils could confer to those with moderately raised TAG but not overt hypertriacylglycerolaemia is a reduction in the number of potentially atherogenic particles in the circulation. Prospective trials have shown that small LDL particles are strong predictors of future coronary events, similar to that of hypertension and smoking (Stampfer et al 1996, Gardner et al 1996). Despite a possible increase in LDL-C, there is evidence that larger LDL (LDL-2) is more preferentially cleared from circulation compared to smaller, dense LDL thus reducing the time frame in which neutral lipid exchange can take place (Abbey et al 1990). The hypotriacylglycerolaemic affects of fish oils and associated effects on CETP in the transfer of cholesterol esters from HDL to LDL and VLDL, may
explain why increases in the HDL₂: HDL₃ sub fraction ratios are often observed (Abbey et al. 1990). It is likely that HDL₂ retains its free cholesterol and CE and consequently remains relatively large. This effect may be further accentuated by the reduction in CE acceptor particles, such as VLDL and possibly LDL in the circulation.

1.6 Fish oils and diet-gene interaction

More recently, the effects of fish oils on a genetic level have been investigated. Khan et al. (2002) showed that the attenuation of postprandial lipoaemia was associated with marked increases in the concentration of lipoprotein lipase (LPL) mRNA in adipose tissue. This is a direct example of fish oils working at a genetic level, or so-called diet-gene interaction. Minihane and co-workers (2000) showed that responses to fish oils differ depending on an individual's apolipoprotein E polymorphism. In individuals carrying the ApoE4 genotype, cholesterol levels tended to increase, and high-density lipoprotein cholesterol (HDL-C) decrease when supplemented with EPA and DHA. Only now is more evidence coming to light on the effects of fish oils on lipids and lipoproteins. Diet-gene interaction is clearly an area that warrants further research but is outside the scope of this review.

1.7 Effects of fish oils on haemostasis and vascular function

Improvements in CHD risk are not limited to lipids and lipoproteins. Long chain n-3 PUFA are the precursors to metabolically active eicosanoids. Eicosanoids are involved in multiple physiological processes, on the vascular wall regulating endothelial function and in platelets, affecting haemostasis (Thiemermann 1993). Eicosanoids, particularly those derived from western type diets are formed from Arachadonic Acid (AA) metabolically elongated from dietary LA. Eicosanoids can also be synthesised from the n-3 PUFA Eicosapentaenoic acid (EPA) (Fischer 1993). Dyerberg (1978) suggested that some of the anti-atherogenic properties of n-3 PUFA might be due to their involvement in eicosanoid production. Figure
1.8 illustrates the classical omega-3 and omega-6 fatty acid synthesis pathways and the role of omega-3 fatty acid in regulating health/disease markers.

Figure 1.8: Classical omega-3 and omega-6 fatty acid synthesis pathways and the role of omega-3 fatty acid in regulating health/disease markers.
The UK diet, like much of the Westernised world, has a greater intake of n-6 PUFA than n-3 PUFA. Consequently, metabolic conversion of LA results in a high level of AA present in plasma lipids. In populations with a high n-3 PUFA intake such as the Inuit, less AA is present in plasma lipids than those with a more Westernised diet such as the Danes (Table 1.4). Greater levels of EPA increase competition between AA for incorporation into cell membranes and subsequent conversion into eicosanoids.

Table 1.4: Percentage of fatty acids in lipid fractions of Inuit and Danes (adapted from Salen et al 1999)

<table>
<thead>
<tr>
<th></th>
<th>AA (20:4)</th>
<th>EPA (20:5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P.L.</td>
<td>C.E.</td>
</tr>
<tr>
<td>Danes</td>
<td>8.0</td>
<td>4.4</td>
</tr>
<tr>
<td>Inuit</td>
<td>0.8</td>
<td>0.0</td>
</tr>
</tbody>
</table>

P.L.= Phospholipids  C.E. = Cholesterol Esters  TAG= Triacylglycerol

Examination of the Inuit population showed a low incidence of myocardial infarction and a prolonged bleeding time (Goodnight 1981). Plasma lipids of the Inuit were found to contain small amounts of AA but high levels of EPA due to high intakes of n-3 PUFA in the diet (Goodnight 1982). A shift in haemostasis towards improved endothelial function and a less pro-inflammatory state is therefore mediated through the formation of eicosanoids of n-3 PUFA origin (Goodfellow 2000). Table 1.5 summarises the effects of AA and EPA on the formation of prostaglandins and leukotrienes and their effects on haemostasis.
**Table 1.5: Effect of the fatty acids AA and EPA on prostaglandin and leukotriene production and their subsequent effects on haemostasis.**

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Prostaglandins</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>TXA₂</td>
<td>TXA₂ strong platelet aggregator and vasoconstrictor more procoaguable state</td>
</tr>
<tr>
<td>EPA</td>
<td>TXA₂ ↓</td>
<td>TXA₃ ↑ TXA₃ physiologically inactive, competes with TXA₂ reducing effects results in less thrombotic state</td>
</tr>
<tr>
<td>AA</td>
<td>PGI₂ ↑</td>
<td>PGI₂ Involved in endothelial function promoting vasodilatory state.</td>
</tr>
<tr>
<td>EPA</td>
<td>-</td>
<td>PGI₃ ↑ PGI₃ complements the actions of PGI₂ promoting a more vasodilatory state.</td>
</tr>
</tbody>
</table>

**Leukotrienes**

| Fatty Acid | B₄          | B₅ ↑ EPA competes with 5-lipoxygenase enzyme for formation of B₄. Small amounts of B₅ compete with B₄ for receptors. Improvement in many anti-inflammatory effects. |

The PRIME study (Scarabin et al 1998) showed that fibrinogen, factor VII and PAI-1 explained 8%, 9% and 26% of the total variance in cardiovascular risk factors respectively. Fat rich diets are accompanied by greater hypercoagulable states and these seem to be mediated more by longer chain saturated fats (Miller et al 1997). Plasma factor VII activity (factor VIIc) is considered one of the independent risk factors for coronary artery disease and is controlled by both genetic and environmental factors (Saha et al 1994). Factor VII activity increases with body mass index, triglycerides, HDL and LDL-cholesterol (Scarabin P et al 1998). Nelson et al (1997b) showed no observable changes in blood coagulation and thrombotic tendencies when arachadonic acid (AA) was increased to 1.5g per day above normal intake, nor were any statistical changes in blood coagulation measured following the addition of 6g of DHA a day for 90 days to a typical westernised diet (Nelson et al 1997a). Long chain n-3 PUFA may be associated with impaired fibrinolysis mediated through an increase in PAI-1 levels (Mahrabian et al 1990, Boberg et al 1992). Spannagl et al (1991)
investigated fish oil supplementation in type I diabetics and healthy controls, observing significant increases in PAI-1 in both groups. In contrast to this, Mehta et al (1998) supplemented post MI patients and healthy volunteers with n-3 PUFA and measured a 21% and 22% decrease in PAI-1 levels respectively. While many studies show haemostasis is responsive to changes in the composition of dietary fats, a large number of the diets prescribed are not appropriate for long-term general dietary advice. Table 1.6 summaries the function of Factor VII, PAI-1 and Fibrinogen and their roles in CHD risk.

<table>
<thead>
<tr>
<th>Function</th>
<th>Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor VII</td>
<td>↑ in activity associated with greater procoaguable state and increased CHD risk</td>
</tr>
<tr>
<td>PAI-1</td>
<td>↑ levels correlated with development of thrombotic disease</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>↑ concentrations associated with increased risk of CHD and stroke.</td>
</tr>
</tbody>
</table>

Table 1.6: Function and role in CHD risk of Factor VII, PAI-1 and Fibrinogen.

1.8 Dietary sources of long chain n-3 PUFA in the UK diet

In the UK, intakes of cod and canned tuna are greater than oily fish such as mackerel and herring. As Table 1.7 illustrates, the metabolically active long chain n-3 PUFAs EPA and DHA are much lower in cod and tinned tuna than other oily fish.

Table 1.7: Fatty acid compositions of fish per 100g commonly consumed in the UK diet.

<table>
<thead>
<tr>
<th>FISH</th>
<th>Total fat (g)</th>
<th>EPA (20:5) (g)</th>
<th>DHA (22:6) (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cod</td>
<td>0.67</td>
<td>0.10</td>
<td>0.20</td>
</tr>
<tr>
<td>Herring</td>
<td>9.04</td>
<td>0.70</td>
<td>0.90</td>
</tr>
<tr>
<td>Salmon – farmed</td>
<td>7.67</td>
<td>0.39</td>
<td>0.82</td>
</tr>
<tr>
<td>Tuna – canned brine</td>
<td>0.50</td>
<td>0.04</td>
<td>0.07</td>
</tr>
<tr>
<td>Mackerel</td>
<td>13.87</td>
<td>0.90</td>
<td>1.40</td>
</tr>
</tbody>
</table>
Where fish oils may be have a role is in those individuals who are not considered at risk of CHD after routine health screening. While an individual may have total cholesterol level below 6.5mmol/l they may also have moderately raised TAG (>2.3mmol/l). These values may not exceed the action points set by many hospitals and GPs, and so would not trigger intervention. The hypotriacylglycerolaemic effects of fish oils draws attention to the role lower circulating levels of TAG may have on structural changes of LDL and HDL. These individuals who may not be identified as “at risk” by health screening, may possess an ALP. Now that the beneficial effects of even mild intakes of EPA and DHA are known, it raises the question of whether an increase in intake of these, through consumption of oily fish is feasible. Even if the cost, and palatability issues could be addressed, it raises concerns over the sustainability of oily fish (Institute of Biology 2003) were the population of the UK suddenly to increase their intake. Alpha-linolenic acid (ALA) the shorter chain (18:3n-3) progenitor of EPA and DHA as a dietary precursor may provide a suitable dietary alternative to oily fish.

1.9 Short chain n-3 PUFA and CHD.

ALA is present in small quantities in the habitual diet of most western countries and is found in most plant based foods including soyabean oil (3%), and rapeseed oil (9%). By far the highest concentrations are found in flaxseed oil (50-60%). ALA can undergo desaturation and elongation though the same metabolic pathway as linolenic acid (LA 18:2 n-6), and indeed shares some of the same enzymes as this pathway (Gerster 1988) (Figure 1.9) If the intake of ALA could be increased, in the absence of EPA and DHA in the diet, could adequate conversion of ALA to its longer chain fatty acids show similar effects to fish oil supplementation without the need for long chain PUFA consumption? If this were possible, it would not only benefit the consumer who did not like fish, but also put less strain on a potentially non-sustainable resource.
The conversion of ALA to EPA and possibly DHA is dependent on a number of factors. Cunnane and co-workers (1991) suggested that a large proportion of ALA undergoes beta-oxidation while more recent findings suggest the extent to which ALA is oxidised is not as great as previously thought (Burge 2004). In vegans, who consume no long chain n-3 PUFA, EPA and DHA are found in plasma lipids convincing evidence that not all ALA undergoes β-oxidation. However, there is still concern that intakes of ALA in vegans is low, compared to LA (Davis & Kris-Etherton 2003) due to the generally low fat intake of this group.

Holman and co-workers (1982) fed ALA to subjects with low levels of EPA and DHA in their plasma phospholipids; increases in EPA and DHA were observed. Other factors, such as the abundance of EPA and DHA already in cell membranes, and the amount of n-6 PUFA in the diet may also affect the conversion of ALA to EPA and DHA. The latter is of some importance as both 18 carbon n-3 and n-6 fatty acids use the same desaturase enzyme before elongation to 20 carbons. Figure 1.9 outlines the metabolic pathways of ALA and LA to their longer chain metabolites.
The dietary n-6:n-3 ratio in simple terms describes the ratio of LA and AA to ALA, EPA and DHA consumed. The two parent fatty acids LA and ALA are essential fatty acids in the diet. The ratio of n-6 PUFA to n-3 PUFA is in the order of 10:1 in the UK. Ten times the amount of LA to ALA in the diet may reduce the
amount of ALA effectively converted to EPA. Trying to consume a diet low in n-6 PUFA and high in n-3 PUFA is not as straightforward as it seems. Table 1.8 outlines some of the oils available in the UK. Flax oil is included in the table to illustrate its high ALA content compared to other oils.

Table 1.8: Fatty acid composition of oils detailing P/S ratio and n-6:n-3 ratio.

<table>
<thead>
<tr>
<th>Oil</th>
<th>18:0% Stearic</th>
<th>18:1% Oleic</th>
<th>18:2% Linoleic</th>
<th>18:3% a-linolenic</th>
<th>P/S ratio</th>
<th>n-6:n-3 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canola</td>
<td>1.8</td>
<td>56.1</td>
<td>20.3</td>
<td>9.30</td>
<td>4.2:1</td>
<td>2.2:1</td>
</tr>
<tr>
<td>Rapeseed</td>
<td>1.6</td>
<td>53.8</td>
<td>22.1</td>
<td>11.1</td>
<td>4.9:1</td>
<td>2:1</td>
</tr>
<tr>
<td>Sunflower</td>
<td>4.5</td>
<td>19.5</td>
<td>65.7</td>
<td>0.0</td>
<td>6.4:1</td>
<td>No n-3</td>
</tr>
<tr>
<td>Olive</td>
<td>2.2</td>
<td>72.5</td>
<td>7.9</td>
<td>0.6</td>
<td>0.6:1</td>
<td>13:1</td>
</tr>
<tr>
<td>Corn</td>
<td>1.8</td>
<td>24.2</td>
<td>58.0</td>
<td>0.0</td>
<td>4.6:1</td>
<td>No n-3</td>
</tr>
<tr>
<td>Flax</td>
<td>9.83</td>
<td>31.0</td>
<td>14.8</td>
<td>56.0</td>
<td>11:1</td>
<td>0.3:1</td>
</tr>
<tr>
<td>Butter</td>
<td>20.4</td>
<td>1.8</td>
<td>1.2</td>
<td></td>
<td>0.1:1</td>
<td>1.5:1</td>
</tr>
</tbody>
</table>

Several approaches have been used to examine what affects the conversion of ALA to its longer chain metabolites. Firstly, increasing the intake of ALA alone, with little change in other dietary factors; this would effectively begin to lower the n6:n3 ratio and provide more ALA for the Δ6-desaturase enzyme to convert (Boudreau et al 1992). Secondly, to not only increase ALA consumption, but to decrease LA intake. This would effectively lower the dietary n-6:n-3 ratio to 1:1 or below, reducing competition by 18:2 n-6 for the Δ6-desaturase enzyme (Allman et al 1995).

Table 1.9 lists some of the studies conducted using ALA in the last two decades. Outcomes, if measured, include effects on lipids and significant changes in EPA and/or DHA. The gender, health and numbers of volunteers, doses of ALA and duration of the studies vary widely, as does the source of ALA supplementation and methods of determining increases in EPA and DHA. Effects on lipoproteins and TAG are equivocal, with a greater number of studies showing no effect. A larger number of studies measure significant increases in EPA following intervention; none listed show a significant effect on DHA.
To determine if ALA was as effective as EPA or DHA at lowering lipids, flaxseed oil was fed at 35mg/kg per day for 12 weeks (Layne et al. 1996). No effects on lipids were measured in the ALA subjects. For the average 70kg male, that represented an intake of 2.4g of ALA per day. Supplementing with 3.3g of ALA per day for 3 years was associated with some anti-thrombotic effects and a significant increase in EPA in plasma lipids and platelets (Renaud et al. 1986). Increases in dietary EPA, even when very small, were considered significant in the context of thrombosis (Renaud & Norday 1983).

Using rapeseed oil as a mode of delivery, 5.9g per day of ALA supplemented for 6 weeks was associated with a non significant decrease in EPA and DHA (0.4% and 0.1% total fatty acids respectively) in cholesterol esters (Valsta & Jauhiainen 1995). The n-6:n-3 ratio of the diet was 3:1, and despite an increase in ALA intake, the diet still contained more LA than ALA, primarily as the main source of ALA was rapeseed oil. The fall in EPA and DHA compared to baseline was much less than that observed on the high LA diet, which was run in parallel. This may suggest that some, limited elongation of ALA is still taking place. When the n-6:n-3 ratio was lowered to 0.3:1 and subjects were supplemented with 7.4g of ALA per day for 4 weeks, significant increases in ALA, EPA and DPA were observed (Freese & Mutanen 1997). Increasing the intake of ALA to 9g per day in mildly hypercholesterolaemic men was associated with significant increases in EPA in plasma fatty acids (Abbey et al. 1990). The n-6:n-3 ratio was similar to that of Freese & Mutanen (1997) at 0.5:1. Increasing the intake of ALA from 7.4g to 9g per day, and maintaining a similar n-6:n-3 ratio did not alter the outcome significantly, despite several differences in study design.
Table 1.9: Summary of alpha-lionlenic acid supplementation studies and their effect on total and LDL cholesterol, triacylglycerol, EPA and DHA

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Source of ALA</th>
<th>N=</th>
<th>ALA dose per day</th>
<th>Group</th>
<th>Length of intervention</th>
<th>TC</th>
<th>LDL-C</th>
<th>TAG</th>
<th>EPA &amp; DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renaud et al</td>
<td>1986</td>
<td>Not known</td>
<td>-</td>
<td>3.3g</td>
<td>Free living population</td>
<td>3 years</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>EPA significantly increased</td>
</tr>
<tr>
<td>Adam et al</td>
<td>1986</td>
<td>Linseed</td>
<td>6</td>
<td>9.7-38.8g</td>
<td>Healthy Subjects (Females)</td>
<td>2 weeks</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>No significant changes</td>
</tr>
<tr>
<td>Singer et al</td>
<td>1986</td>
<td>Flax Oil</td>
<td>-</td>
<td>35g</td>
<td>Normal, hypertensive &amp; hyperlipidaemic subjects</td>
<td>2 weeks</td>
<td>Sig fall</td>
<td>Sig fall</td>
<td>Sig fall</td>
<td>N/A</td>
</tr>
<tr>
<td>Abbey et al</td>
<td>1990</td>
<td>Flax Oil</td>
<td>11</td>
<td>8.9g</td>
<td>Moderately raised TC</td>
<td>6 weeks</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>N/A</td>
</tr>
<tr>
<td>Bierenbaum et al</td>
<td>1993</td>
<td>Flaxseeds</td>
<td>15</td>
<td>4.6g</td>
<td>Moderately raised TC &amp; TAG</td>
<td>3 months</td>
<td>Sig fall</td>
<td>Sig fall</td>
<td>NO</td>
<td>N/A</td>
</tr>
<tr>
<td>Mentzioris et al</td>
<td>1994</td>
<td>Flax Oil</td>
<td>30</td>
<td>13.7g</td>
<td>Healthy</td>
<td>4 weeks</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>EPA significantly increased</td>
</tr>
<tr>
<td>Nydahle et al</td>
<td>1994</td>
<td>Rapeseed oil</td>
<td>101</td>
<td>N/A</td>
<td>Healthy</td>
<td>3 weeks</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>EPA significantly increased</td>
</tr>
<tr>
<td>Allman et al</td>
<td>1995</td>
<td>Flax Oil</td>
<td>11</td>
<td>20g</td>
<td>Healthy</td>
<td>23 days</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>EPA significantly increased</td>
</tr>
<tr>
<td>Valsta et al</td>
<td>1995</td>
<td>Rapeseed oil</td>
<td>40</td>
<td>5.9g</td>
<td>Healthy</td>
<td>6 weeks</td>
<td>Sig fall</td>
<td>Sig fall</td>
<td>Sig fall</td>
<td>No Significant Changes</td>
</tr>
<tr>
<td>Layne et al</td>
<td>1996</td>
<td>Flax oil</td>
<td>26</td>
<td>35mg/kg</td>
<td>Healthy</td>
<td>3 months</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No Significant Changes</td>
</tr>
<tr>
<td>Goh et al</td>
<td>1997</td>
<td>Flax oil</td>
<td>28</td>
<td>35mg/kg</td>
<td>Non-insulin dependant</td>
<td>3 months</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No Significant Changes</td>
</tr>
<tr>
<td>Freese et al</td>
<td>1997</td>
<td>Flax Oil</td>
<td>46</td>
<td>5.9g</td>
<td>Healthy</td>
<td>4 weeks</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No Significant Changes</td>
</tr>
<tr>
<td>Li et al</td>
<td>1999</td>
<td>Flax Oil</td>
<td>17</td>
<td>15.4g</td>
<td>Healthy</td>
<td>4 weeks</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>EPA significantly increased</td>
</tr>
<tr>
<td>Allman-Farinelli</td>
<td>1999</td>
<td>Flax Oil</td>
<td>30</td>
<td>11.8</td>
<td>Healthy</td>
<td>6 weeks</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>EPA significantly increased</td>
</tr>
<tr>
<td>Finnegan et al</td>
<td>2003a</td>
<td>Flax Oil</td>
<td>150</td>
<td>4.5 &amp; 9.5g</td>
<td>Moderate hyperlipidaemia</td>
<td>6 months</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>EPA significantly increased at both doses</td>
</tr>
</tbody>
</table>
Adam and co-workers (1986) compared the effects of increasing energy from flaxseed oil whilst keeping energy from LA constant at 4%. The study took part in a metabolic kitchen; all other long chain n-3 fatty acids (EPA, DPA and DHA) were excluded from the diet. Only the 16% energy from ALA diet caused a significant increase in platelet lipid ALA; this was achieved through 38.8g of ALA per day for 2 weeks. Intakes of this magnitude would not be feasible in a free-living population.

An effect on platelet aggregation to ADP and thrombin after 6 weeks was observed by Freese et al (1994) when comparing an n-6:n-3 ratio of 28:1 (high sunflower oil) to 2.8:1 (high rapeseed oil) the latter containing 6.8g of ALA. Haemostatic changes similar to those of Adams and co-workers (1986) were seen. Freese and co-workers also reported a decrease in DHA and EPA, the latter of which was significant. The decrease in longer chain n-3 fatty acids supports the hypothesis that LA competes for elongation with ALA and as such a n-6:n-3 ratio of 2.8:1 may not be sufficiently low enough to increase conversion of ALA to long chain n-3 PUFA. In another 6 weeks study (Allman-Farinelli et al (1999) no changes in any haemostatic factors were seen following 11.6g of ALA per day (n6:n3 ratio 0.8:1) despite a significant rise in platelet EPA. Similar findings were also reported by Li et al (1999) in vegetarian men. Finnegan et al (2003a) found no improvement in blood coagulation or fibrinolytic factors after 6 months on 4.5g or 9.5g of ALA per day, neither were there any effects on lipids and lipoproteins. Both doses were associated with significant increases in EPA in platelet phospholipids.

Mantzioris et al (1994) compared n-6:n-3 ratios of 18:1 to 0.6:1, an 8-fold increase in ALA and a 2.5-fold increase in EPA in plasma lipid fractions was reported after 4 weeks on the diet but no changes in lipoprotein or TAG levels. The incorporation of ALA into plasma lipids seemed to be at the expense of LA as well as oleic acid unlike Adams et al (1990) who observed displacement of oleic acid only. Further analysis of the data found a linear relationship between ALA intake (in the presence of a reduced LA diet) and EPA concentrations in the phospholipids of plasma (r=0.86) and platelets.
(Mantzioris 1995). This relationship may well facilitate practical dietary strategies for increasing EPA concentrations in tissues.

While there is good evidence that the elongation and desaturation of ALA takes place, this process is believed to be relatively slow (Singer 1992), and probably dependant on the n-6:n-3 ratio (Boudreau et al 1992). A two-week intervention cycle may be insufficient to show significant changes, particularly if the diets before the intervention were high in n-6 PUFA. Singer & Berger (1986) found that with just 2 weeks on a high ALA diet (35g ALA per day), a significant rise in plasma lipid ALA occurred, but no corresponding increase in EPA or decrease in AA. When 20g of ALA was supplemented for 23 days significant increases in EPA in platelets were measured, as well as reduced aggregation to collagen (Allman et al 1995). Intakes of this magnitude of 35g per day would not be feasible in the long term, nevertheless decreases in TC and TAG were observed suggesting the potential hypocholesterolaemic and hypotriacylglycerolaemic effects of ALA may be independent of its formation of EPA (Singer 1992).

Studies with increased length of intervention may produce more evidence for the rate and amount of conversion of shorter chain n-3 fatty acids to EPA and DHA. Perhaps the best indicator of this was the 3-year French farmers study (Renaud et al 1986), where significant increases in EPA were measured with intakes of 3.3g per day of ALA. A majority of studies with similar intakes of ALA, but shorter duration have failed to observe significant increases in EPA (Valsta et al 1995, Layne et al 1996, Goh et al 1997, Freese et al 1997). It is reasonable to speculate that the extended duration of this study may have affected adipose tissue composition, which in turn affected the FA composition of the metabolic transport and functional pools. Figure 1.10 illustrates the dynamic relationship between the dietary, transport, functional and storage pools.
It is important to recognise the role of the various metabolic pools shown in Figure 1.10. The composition of these pools is affected by dietary intakes; while the FA composition of transport pools such as TAG and CE may reflect recent intakes (Judd et al. 1989, Ma et al. 1995) they are also likely to be affected by longer-term dietary intake (Lands 1995). Western diets are typically high in n-6 PUFA, and there is now evidence that adipose tissue is strongly related to longer-term fatty acid intake (Tjønneland et al. 1993). In Western populations as much as 10 to 15% of adipose tissue can comprise of LA (Wood et al. 1987), thus adipose tissue may be providing a supply of LA to the metabolic transport pools during dietary restriction of LA. A typical 70kg man with 20% body fat (14kg) could be carrying as much as 2kg of LA. Turnover of these body stores such as LA may continue long after dietary restrictions have been instigated. Functional pools such as erythrocytes or platelets are often used as a measure of outcome or dietary compliance, unfortunately the effects of adipose tissue on the FA composition of these pools is not often considered.
A recent prospective trial concluded that 10-year risk of CHD was not associated with ALA intake (Oomen et al 2001). Other case control studies have also been inconclusive (Guallar et al 1999, Pedersen et al 2000). A number of other studies have suggested that ALA is beneficial to cardiovascular health (Hu et al 1999, Longeril et al 1999). There have been many studies conducted examining different doses of ALA, from different sources and over varying lengths of time however, these are far less in number than those studies examining the effects of fish oils or long chain n-3 PUFA on cardiovascular health.

In most cases ALA supplementation did not alter significantly TC, LDL-C or TAG. In some studies significant increases in the metabolic and transport pools of EPA was measured. The numbers of studies that have reported significant increases in DHA are very few. Different studies have used different transport or functional pools to determine ALA conversion such as cholesterol esters, platelets and erythrocytes. Duration of intervention may affect the results due to confounding factors such as adipose tissue composition; in particular LA content. The gender and general health of the subjects participating in the intervention will also affect the results; significant changes in lipids and lipoproteins are less likely to be measured in healthy volunteers, evidence is also emerging that males and females metabolise ALA differently (Burge 2004).

It is unclear whether ALA may be useful in terms of reducing CHD risk in a population expressing an ALP. Previous studies have suggested that reducing the n-6:n-3 ratio may improve conversion of ALA to EPA and DHA, if this can be achieved, it is unclear whether the increases in long chain n-3 PUFA will be sufficient to modify CHD risk in this population. It remains unclear whether 12 weeks is sufficient time to observe an effect; most studies under 2 weeks failed to see increases in the conversion of ALA to longer chain n-3 PUFA. The effect of LA in adipose tissue on the conversion of ALA remains unclear even in the presence of restricted dietary LA intake.
Our hypothesis is that increased intake of ALA and decreased intakes of LA will maximise the potential for metabolic elongation of ALA to EPA and DHA through reduced competition for the delta-6 desaturase enzyme. The effects of the diet on fasting TAG, haemostasis and vascular function will be measured alongside changes in lipoproteins and lipoprotein heterogeneity; any changes in CHD risk can then be elucidated.

1.10 Aims of investigations

The investigations aimed to examine the practical feasibility of delivering dietary ALA using flaxseed oil without the use of manufactured food items. To increase the likelihood of elongation in vivo of ALA to EPA and DHA the study aimed to carefully control the dietary intakes of both n-3 and n-6 fatty acids. It examined the effect of ALA supplementation on biomarkers of CHD in individuals expressing an ALP comparing them with dietary fish oil supplementation to determine if ALA was as effective in reducing risk factors for CHD. It aimed to examine the issue of dietary compliance and strategies that may improve it. It was targeted at middle-aged males as they have been identified as more at risk of CHD than pre-menopausal women.

1.10.1 Study Objectives

The pilot study was designed to look at issues surrounding delivery of dietary flaxseed oil to volunteers. Its objective was to identify any problems or useful strategies that may improve the running and compliance of the principal study. The principal study involved 2 diets run in parallel with a high ALA diet; these were both high in LA (sunflower oil), one of which was further supplemented with 3g of EPA and DHA per day (Harris 1997). The latter was used as a "control" to determine the effect of long chain n-3 PUFA in the study population, in particular the effects on plasma TAG and small, dense LDL. The high ALA diet was achieved using flaxseed oil, and was also low in dietary n-6 fats. If n-3 PUFA from ALA were as effective as fish oils in reducing CHD risk factors, the effects of the diet would compare favourably with the "control". The high LA diet represented the UK's high dietary n-6 fat
intakes. If successful the study would highlight a suitable alternative to oily fish and fish oils in the general population, benefiting not only cardiovascular health, but potentially affecting a greater proportion of the population who do not regularly consume long chain n-3 fatty acids.
CHAPTER 2: GENERAL MATERIALS AND METHODS

All practical work detailed in this Chapter, Chapters 3, 4 and 5 were carried out by myself, except where stated otherwise.

2.1 Blood sample collection and storage.

Bloods were taken after an overnight fast. Subjects were instructed to abstain from alcohol and avoid vigorous exercise 24 hours prior to venepuncture. Blood was drawn from the antecubital vein into Vacutainers (Beckton-Dickenson) by a qualified nurse. Blood was drawn into EDTA (final concentration 1mg/ml) for lipid and lipoprotein analysis, red blood cell fatty acid composition and LDL oxidation, into sodium citrate for the measurement of PAI-1, Factor VII and Fibrinogen and into fluoride oxalate for glucose determination. All blood, except Factor VII and Fibrinogen, were kept at 4°C for < 1 hour before being centrifuged at 3000g for 10 mins at 4°C (Megafuge 1.0 Heraeus Sepatech). The remaining samples were centrifuged at 3000g for 10 mins at room temperature. All plasma samples, except those for LDL subclass determination, were frozen to −18°C within 1 hour of centrifugation and then stored at −80°C until analysis. Plasma samples for LDL subclass determination were stored at 4°C and run within 24 hours of venepuncture.

2.2 Preparation of red blood cells for erythrocyte membrane phospholipid fatty acid analysis.

The composition of erythrocytes membrane fatty acids were used as a measure of dietary compliance, as well as an indirect marker for the conversion of shorter chain n-3 PUFA to longer chain n-3 PUFA. Approximately 15 mls of red blood cells (RBC), separated from plasma as described above, were washed three times with 0.9% saline solution at 4°C. Two-hundred and fifty microlitres of 0.1% butylated hydroxytoluene in propran-2-ol was added to FA prevent oxidation and samples stored at −80°C until analysis.
2.3 Analysis of plasma lipids, lipoproteins and glucose.

Plasma cholesterol, TAG, HDL-C, LDL-C, ApoB and glucose were all measured on the Cobas Mira Plus autoanalyzer (Roche Diagnostics Ltd, UK). Aliquots of frozen plasma were thawed at room temperature immediately prior to analysis.

Total plasma cholesterol was determined using the cholesterol enzymatic colourimetric assay endpoint method (Randox, UK). Total cholesterol is determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed and measured at 500nm. Plasma triacylglycerol was determined using the GPO-PAP method (Randox, UK). Plasma TAG is determined after enzymatic hydrolysis with lipases resulting in the formation of the indicator quinoneimine this is measured at 500nm and is directly proportional to the TAG concentration. High-density lipoprotein cholesterol was determined using the Direct HDL cholesterol enzymatic assay kit (Randox, UK). The assay consists of two distinct reaction steps, the first eliminates chylomicrons, VLDL and LDL-C by cholesterol esterase, cholesterol oxidase and subsequently catalase. HDL-C is released by detergents and the absorption of the quinoneimine dye produced is directly proportional to the cholesterol content, which is measured at 600nm. Coefficient of variation within runs is 1.2% and between batches 0.9%.

Low-density lipoprotein cholesterol was determined using the Direct LDL cholesterol enzymatic assay kit (Randox, UK). The assay consists of two distinct reaction steps, the first eliminates chylomicrons, VLDL and HDL-C by cholesterol esterase, cholesterol oxidase and subsequently catalase. LDL-C is released by detergents in the step to and the intensity of the quinoneimine dye produced is directly proportional to the cholesterol content, which is measured at 600nm. Glucose was determined using the Uni-kit II (Roche). This is an enzymatic test with hexokinase. The concentration of NADH formed is determined by monitoring absorbance at 340nm. Coefficient of variation within runs is 0.5% and between batches 1.0%. Apo B concentration was
determined using the apolipoprotein B immunoturbidimetric immunoassay (Randox UK). The principle is based on the reaction of a sample containing human apoB and a specific antiserum to form an insoluble complex, which can be measured turbidimetrically at 340nm. Co-efficient of variation within runs is 1.8% and between batches 4.2%.

2.4 Determination of LDL subclasses by density gradient ultracentrifugation.

LDL subclasses were separated according to the method of Griffin et al (1990). 3mls of plasma was adjusted to a density of 1.09g/ml by the addition of 0.38g of solid KBr. A salt gradient was prepared as shown in (Figure 2.1) using a multi-channelled peristaltic pump (Watson-Marlow, UK).

Figure 2.1: Separation of plasma LDL subclasses by discontinuous density gradient ultracentrifugation

The density of each solution was checked by an electronic density meter (Parr Scientific) before addition to the gradient. The gradient was centrifuged in a Beckman Optima XL100 ultracentrifuge at 40,000 rpm (200,000g) for 23 hours at 23°C in a 40Ti swing out rotor (Max acceleration, no brake) Upon completion the tubes were placed in a Beckman fraction recovery system (Figure 2.2). Maxidens (Nycomed, Norway) a dense hydrophobic fluid (Maxidens) density 1.9g/ml was used to displace the gradient by introduction under the plasma layer. The gradient was upwardly displaced through a
microflow cell by means of an infusion pump, running at 42ml/hr, and monitored continuously at 280nm in a spectrophotometer (Beckman DU650).

The resulting LDL profile provides a graphical representation of the different LDL subclasses. Each subclass' percentage area is integrated by dropping perpendiculars to a flat baseline. These percentage areas are corrected to lipoprotein mass equivalence by multiplying by specific extinction coefficients (for LDL-1 this coefficient is 2.63, for LDL-2 2.94 and for LDL-3 1.92) as previously determined (Griffin et al 1990).

2.4.1 Determination of HDL Subclasses by density gradient ultracentrifugation

2mls of plasma was adjusted to a density of 1.3g/ml by the addition of 0.98g of KBr. The adjusted plasma was placed at the bottom of a 40Ti tube (Beckman-Coulter). Three layers of differing densities were added on top of the plasma layer using a peristaltic pump (Watson Marlow). Firstly, 2.5mls of 1.25g/ml NaBr, then 6mls of 1.19g/ml NaBr. Distilled water was added to make up the volume to 11mls. Samples were centrifuged in a Beckman Optima XL100 using a 40Ti swing-out bucket rotor (Max acceleration, no brake) at 15°C for 20 hours at 40,000 RPM (200,000g). Upon completion the
tubes were placed in a Beckman fraction recovery system (Figure 2.2). A dense hydrophobic fluid, Maxidens (Nycomed, Norway) density 1.9g/ml was used to displace the gradient by introduction under the plasma layer. Using an infusion pump, running at 42ml/hr, the gradient was displaced through a microflow cell whilst being monitored continuously at 280nm in a spectrophotometer (Beckman DU650). The resulting HDL profile provides a graphical representation of the different HDL subclasses. Each subclass' percentage area is integrated by dropping perpendiculars to a flat baseline. These percentages can be applied to the total HDL-C measurement, to provide a relative value of HDL$_2$ and HDL$_3$ in mmol/l.

2.5 Determination of erythrocyte membrane fatty acid phospholipid composition

Lipids were extracted from the erythrocyte membranes with a mixture of chloroform: methanol (2:1; vol/vol), containing butylated hydroxytoluene (0.01 % BHT) as an antioxidant, according to the Folch method (Folch et al 1956). Fatty acid methyl esters were prepared with sodium methoxide (0.5 M solution in methanol), incubated at 60°C for 15 minutes, acidified with glacial acetic acid, and finally extracted with hexane. The resulting fatty acid methyl esters were analysed with a gas chromatography (GC) using a 3400 Gas Chromatograph (Varian). PAG Capillary Column: 30m x 0.25mm x 0.25μm film thickness (Supelco, Inc) and a flame ionization detector (250°C), with helium as the carrier gas. Temperature was programmed at 4°C/min from 150 to 220°C. The fatty acid peaks were identified against a standard fatty acids mixture (Supelco 37 Component FAME Mix, Sigma Chemical Co. UK) and run on the same column under identical conditions. Individual standards (Docosapentaenoic acid: 22:5n-3, Docosatetraenoic acid: 22:4n-6) were also used as these two fatty acids were not included in the standard mixture. The injection volume was 2 μL and the run 45 min. The maximum CVs for repeated injections were 0.4% for FA retention time and 0.3% for FA peak AUC. Results are expressed as percentages of the sum of all identified peaks. This work was carried out by a colleague Dr Eric Ah-Sing, University of Surrey.
2.6 Comparison of HDL subclasses as isolated by HDL-DGUC and electrophoretic separation.

HDL is structurally heterogeneous, such that HDL subclasses can be isolated on the basis of their lipid composition, apoprotein content, their electrophoretic mobility and density.

Gradient Gel Electrophoresis is a sensitive technique based on separating HDL by their particle size. A non-denaturing polyacrylamide gradient gel is used, and has shown at least five HDL subclasses: HDL\textsubscript{3c}, HDL\textsubscript{3b}, HDL\textsubscript{3a}, HDL\textsubscript{2a} and HDL\textsubscript{2b}. The 3 HDL\textsubscript{3} subclasses can be grouped together as can the 2 HDL\textsubscript{2} subclasses to give a ratio of HDL\textsubscript{2} to HDL\textsubscript{3}. (Blanche et al 1981, Vézina et al 1988, Williams et al 1992).

There are several ultracentrifugation methods for measuring HDL: analytical, preparative, density gradient and rate zonal centrifugation. The equipment required is expensive and preparative methods for isolating HDL can be time consuming, as they require repeated centrifugation often over a period of days. The density gradient ultracentrifugation (DGUC) method can be performed in one step over 24 hours. The method described above was used to compare the new Lipoprint HDL System using identical samples.

2.6.1 A new gel rod electrophoresis system for the determination of HDL subclasses: HDL Lipoprint system

The Lipoprint HDL System (Quantimetrixs Corporation, USA) was a new technique for separating HDL subclasses using polyacrylamide gel electrophoresis (PAGE). The gel is pre-cast and contained within small glass tubes, and all the reagents for method are contained within the system kit.
2.6.1.1 Method

The buffer salts were reconstituted by dissolving one vial (20g) in 1200mls of distilled water. Final pH 8.2 – 8.6. The required numbers of tubes were placed in the preparation rack (Figure 2.3) with the unfilled end face up. A maximum of 12 tubes can be run at one time. Storage buffer was removed from the top of the tubes by gentle tapping onto an absorbent cloth. 25μl of plasma were added to the top of the gel, 300μl of the loading gel was layered on the top. Once all samples had been loaded, the tops of the tubes were temporarily sealed and the whole rack inverted several times to ensure complete mixing of the sample and loading gel.

![Figure 2.3: HDL Lipoprint loading and preparation rack.](image)

The tubes were polymerised by the application of light. The preparation rack was placed in the centre of the preparation light with the loading gel portion of the tubes touching the light tube. Polymerisation took 30 minutes. Upon completion, tubes were removed and placed in the upper segment of the electrophoresis chamber. A maximum of 12 tubes could be run at any one time. If less than 12 samples were being run, empty slots were filled with glass rods containing only air. All tubes containing samples were checked for air bubbles, which were removed by gentle tapping. 1000mls of buffer solution was added to the bottom chamber, and the remaining 200mls in the top chamber. The apparatus was then connected to a power supply. Gels were run at 3mA per tube at 300V for approximately 80 minutes. Running times
varied depending on the number of tubes present, but as a rule, electrophoresis was stopped when the albumin fraction was approximately 1cm from the end of the tube. The tubes were removed from the chamber and placed back in the preparation rack. The albumin front, visible as a pale yellow band, was marked on the exterior of the tubes with a thin marker pen. Identification of this front allowed analysis of the HDL subclasses to be performed relative to the albumin front. The optical density of the tubes was determined using a densitometer (Pharmacia, Biotech). An image of the gels in the preparation rack was captured using the LIPCAP capture application (Pharmacia Biotech). Capture was via a CCD video camera using white background light. The captured images were analysed using the Imagine Master ID Prime software (Pharmacia Biotech). HDL fractions were identified using Rf values. The VLDL and LDL band at the top of the tube was labelled Rf 0 and the albumin front, as Rf 1. HDL\textsubscript{2} was identified as having an Rf of 0.18 and HDL\textsubscript{3} as an Rf of 0.27. A diagram of how the gel tube looks after electrophoresis is shown in Figure 2.4. A manual baseline was inserted and the area under the curves of the two HDL sub-fractions calculated by dropping perpendiculars to the baseline. Areas under the curve were expressed as optical density units and then converted in percentages (Figure 2.5).

*Figure 2.4: Diagram of electrophoresis gel tube from HDL Lipoprint kit illustrating the Rf bands for HDL\textsubscript{2} and HDL\textsubscript{3} subfractions.*
2.7 Validation of HDL subclasses using HDL-DGUC and the Lipoprint HDL system

2.7.1 Aims

To characterise electrophoretic banding patterns (Lipoprint) by direct comparison with HDL subclasses co-isolated by density gradient ultracentrifugation.

2.7.2 Study Design

A small group of volunteers were selected from within the university student population to compare the HDL DGUC method with the new HDL Lipoprint System. The expectation was that similar values for HDL$_2$ and HDL$_3$ would be found from each method.
2.7.2.1 Subjects:

7 Male and 3 Female subjects took part in the validation study each providing a sample of fasting blood for duplicate analysis using the HDL Lipoprint System, and density gradient ultracentrifugation.

2.7.3 Results:

Measurement of HDL subfractions using the PAGE method, resulted in lower % AUC values for HDL₃ than the density gradient method, and higher % AUC values for HDL₂. Both methods clearly identified the differences in the HDL₂:HDL₃ ratio observed when comparing female to male subjects. Mean values for AUC for HDL₂ and HDL₃ from both methods are shown in Table 2.1

Table 2.1: mean values for AUC for the page and density gradient methods

<table>
<thead>
<tr>
<th>No.</th>
<th>Sex</th>
<th>PAGE HDL₂</th>
<th>PAGE HDL₃</th>
<th>DGUC HDL₂</th>
<th>DGUC HDL₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=7</td>
<td>Male</td>
<td>33.6 (13.0)</td>
<td>66.4 (13.)</td>
<td>8.5 (3.0)</td>
<td>92.5 (3.0)</td>
</tr>
<tr>
<td>n=3</td>
<td>Female</td>
<td>61.0 (4.6)</td>
<td>39.0 (4.6)</td>
<td>36.3 (15.5)</td>
<td>63.7 (15.5)</td>
</tr>
</tbody>
</table>

Correlation between HDL subclass data using PAGE and HDL-DGUC is shown in Table 2.2. and Figures 2.6 and 2.7. An example of a male and female HDL subclass profile as generated using DGUC is shown in Figure 2.8.

Table 2.2: Correlation between PAGE and DGUC methods.

<table>
<thead>
<tr>
<th>Correlation</th>
<th>HDL₂</th>
<th>HDL₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAGE vs DGUC</td>
<td>r=0.77</td>
<td>r=0.77</td>
</tr>
<tr>
<td></td>
<td>p&lt;0.01</td>
<td>p&lt;0.01</td>
</tr>
</tbody>
</table>
Figure 2.6: AUC (%) values for HDL$_2$ PAGE compared to HDL$_2$ DGUC, from the same sample.

**DGUC vs PAGE in same subjects (HDL$_2$)**

![Graph showing AUC (%) values for HDL$_2$ PAGE compared to HDL$_2$ DGUC.](image)

Figure 2.7: AUC (%) values for HDL$_3$ PAGE compared to HDL$_3$ DGUC, from the same sample.

**DGUC vs PAGE in same subjects (HDL$_3$)**

![Graph showing AUC (%) values for HDL$_3$ PAGE compared to HDL$_3$ DGUC.](image)

Figure 2.8: HDL profiles generated using DGUC (Grey = Female, Red = Male)

![Graph showing HDL profiles generated using DGUC.](image)
2.7.4 Comparison of HDL-DGUC and gel rod electrophoresis in the separation of HDL subclasses in 3 subjects.

HDL subclasses were separated from 3 subjects by the two methods. Blood was taken at baseline and 12 weeks and plasma prepared as described in Chapter 2.

2.7.5 Results

Both methods were able to distinguish between HDL\(_2\) and HDL\(_3\) subfractions. Results for AUC differed depending on the method used. Changes over time were detected by both methods. Co-isolation of samples using the Lipoprint system (Table 2.3) and HDL-DGUC (Table 2.4) are shown.

Table 2.3: Results of %AUC for HDL Lipoprint at 0 and 12 weeks

<table>
<thead>
<tr>
<th>Subject</th>
<th>% AUC @ 0 Weeks</th>
<th>% AUC @ 12 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HDL(_2)</td>
<td>HDL(_3)</td>
</tr>
<tr>
<td>A</td>
<td>21</td>
<td>79</td>
</tr>
<tr>
<td>B</td>
<td>17</td>
<td>83</td>
</tr>
<tr>
<td>C</td>
<td>20</td>
<td>80</td>
</tr>
</tbody>
</table>

Table 2.4 Results of %AUC for DGUC at 0 and 12 weeks.

<table>
<thead>
<tr>
<th>Subject</th>
<th>% AUC @ 0 Weeks</th>
<th>% AUC @ 12 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HDL(_2)</td>
<td>HDL(_3)</td>
</tr>
<tr>
<td>A</td>
<td>6</td>
<td>94</td>
</tr>
<tr>
<td>B</td>
<td>4</td>
<td>96</td>
</tr>
<tr>
<td>C</td>
<td>15</td>
<td>85</td>
</tr>
</tbody>
</table>

Subjects A and B showed similar changes in HDL\(_2\) and HDL\(_3\) in both the HDL-DGUC and PAGE methods. This was not observed with subject C. Correlation between the two methods was not significant (p=0.28) \(r=0.53\). The relative percentages of HDL\(_2\) and HDL\(_3\) from each method were converted into HDL cholesterol mmol/l equivalents and are shown in Table 2.5.
Table 2.5: Calculation of mmol values of HDL\(_2\) and HDL\(_3\) using the HDL Lipoprint system and HDL-DGUC methods.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Total HDL (mmol/l)</th>
<th>Lipoprint</th>
<th>DGUC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HDL(_2) (mmol/l)</td>
<td>HDL(_3) (mmol/l)</td>
<td>ΔHDL(_2) (mmol/l)</td>
</tr>
<tr>
<td>A (wk 0)</td>
<td>1.07</td>
<td>0.23</td>
<td>0.84</td>
</tr>
<tr>
<td>A (wk 12)</td>
<td>1.00</td>
<td>0.16</td>
<td>0.84</td>
</tr>
<tr>
<td>B (wk 0)</td>
<td>0.97</td>
<td>0.17</td>
<td>0.80</td>
</tr>
<tr>
<td>B (wk 12)</td>
<td>1.11</td>
<td>0.17</td>
<td>0.94</td>
</tr>
<tr>
<td>C (wk 0)</td>
<td>0.85</td>
<td>0.17</td>
<td>0.68</td>
</tr>
<tr>
<td>C (wk 12)</td>
<td>0.89</td>
<td>0.21</td>
<td>0.68</td>
</tr>
</tbody>
</table>

These data show that plasma concentration values for HDL\(_2\) and HDL\(_3\) are different when calculated from the AUC data using total HDL values. PAGE results are greater for HDL\(_2\) and less for HDL\(_3\) when compared to the HDL-DGUC method.

2.7.6 Discussion

While both methods were able to distinguish between HDL\(_2\) and HDL\(_3\) subfractions, and also determine changes in composition over time, the AUC of each method differed greatly. While the HDL-DGUC method relies on the protein content, the PAGE method quantifies HDL on its lipid content. The ratio of lipid:protein in the two subclasses varies, leading to different graphical representations of the same sample, depending on which method is used. HDL\(_2\) is generally TAG rich and protein poor, as opposed to HDL\(_3\) which is smaller, contains less lipid and is relatively high in protein. This is reflected in the results which show higher values for HDL\(_2\) using PAGE vs HDL-DGUC, and similar higher values for HDL\(_3\) comparing HDL-DGUC to PAGE. Despite the differences, both methods were able to distinguish gender differences between subjects, as well as quantitative changes in HDL subclass populations. It is clear that the mathematical conversion of AUC for each HDL subclass may not provide an accurate representation of the cholesterol content of each HDL subclass. Even in this small group of samples there appeared to be significant variation in the %AUC between the two methods.

The PAGE method proved a reliable, quick and efficient method with the advantage over HDL-DGUC of offering high through-put of samples per day. The HDL-DGUC method is limited to 6 samples per 24 hours per
ultracentrifuge. The reliability of the data would be increased by analysis of larger numbers of samples.
Prior to the start of the principal study a pilot study was designed primarily to test the feasibility of methods for ALA delivery in flaxseed oil in a small group of normolipidaemic, healthy volunteers. The investigation served to provide insight into the logistics of delivering a high dose of flaxseed oil effectively to free living subjects. It also served to look at the palatability and general acceptance of these foods, and methods by which compliance to the dietary protocol may be improved.

An additional aim was to measure the dietary n6:n3 ratio achieved by increasing ALA and decreasing n-6 PUFA in the diet, and to examine whether a reduction in this ratio is associated with "fish-oil" like effects on CHD risk. To determine dietary compliance and any indirect conversion of ALA to EPA and DHA, erythrocyte membrane phospholipid fatty acid composition was measured. The pilot also served as a means to train study staff with the materials and methods that would be used in the main intervention study.

This pilot study was approved by the ethics committee of the University of Surrey, Guildford, and the Royal Surrey County Hospital. Informed consent was obtained from the participating subjects.

A total of nine normal, healthy male volunteers from within the University of Surrey aged 21 – 58 were recruited and undertook a 4 week, free living dietary intervention. Subjects were recruited through advertisements placed within the university, and several of the staff involved with the study also took part. Despite attempts to increase the size of the study group no more than 9 volunteers could be recruited.
3.2.2 The study diet

The intervention consisted of one diet based on 30mls of flaxseed oil per day against a background of low dietary n-6 PUFA intake. The aim was to achieve a dietary n6:n3 ratio of <1. It was decided to do this with 16g of ALA per day. This intake was considered large enough to have a potential effect on EPA formation (Matzioris et al 1994, Allman et al 1995, Li et al 1999) but still be manageable by volunteers as a dietary supplement. All subjects followed this diet for 4 weeks. Subjects were provided with cold pressed flaxseed oil (Flora, distributed by Savant) in 500ml bottles and written information in the form of recipes and tips on how to use the flax oil and flax oil containing products.

Subjects were instructed to use combine or mix the oil with everyday foods or to take the oil directly on a spoon with meals. To simplify the dose of ALA and to aid compliance, intakes of flax oil were determined using a unit system. One unit was equivalent to 3.8g of ALA or 6mls of flax oil. In addition to this, flaxseed oil enriched butter (2:1 butter:flax oil ratio) and mayonnaise (3:1 low fat mayonnaise:flax oil ratio) was made and given to subjects to use whenever possible. The unit system also applied to these products. Subjects were instructed to follow a low n-6 PUFA diet, and to facilitate this were provided with rapeseed oil (Goldenfields, Cargill Foods) for cooking and frying. There were clear instructions not to bake, cook or fry with the flax oil or flax oil products due to the oil oxidising above 70°C. One tablespoon of rapeseed oil was equivalent to 0.5 units. Subjects were also instructed to avoid oily fish and fish oil supplements and to follow as near their normal diet as possible outside of these restrictions. The aim was to consume 8 units per day. All the products supplied to the subjects, and their respective unit values are shown in Table 3.1.

<table>
<thead>
<tr>
<th>Food</th>
<th>Amount</th>
<th>Unit Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flaxseed Oil</td>
<td>6 ml</td>
<td>1.0</td>
</tr>
<tr>
<td>Enriched Mayonnaise</td>
<td>1 Tablespoon</td>
<td>1.0</td>
</tr>
<tr>
<td>Rapeseed Oil</td>
<td>1 Tablespoon</td>
<td>0.5</td>
</tr>
<tr>
<td>Enriched Butter Cube</td>
<td>1 cube</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Table 3.1: Unit System for determining ALA intake in differing food products.
3.2.3 Dietary Analysis

Two 7-day food diaries were completed, and dietary intakes before and during the study analysed using the dietary analysis software Dietplan 5 (Forestfield Software Ltd). Intakes of macronutrients and dietary n-6 and n-3 fats were calculated. Data input was carried out by Jenny Hunniset.

3.2.4 Measurement of lipids, lipoproteins and anthropometrics

Blood samples were taken at the start of the study (0 weeks), mid-point (2 weeks) and at the end (4 weeks). Blood was taken and prepared as described in Chapter 2 and analysed for TAG, TC, HDL-C and glucose as described in chapter 2. LDL-C was calculated using the Freidwald equation (Freidwald et al. 1972). Low-density lipoprotein subclasses were separated by density gradient ultracentrifugation as described in chapter 2 at baseline and 4 weeks post diet.

3.2.5 Analysis of erythrocyte membrane phospholipid fatty acids

This was determined using gas chromatography as previously described in chapter 2 and analysis performed by Caroline Emery.

3.2.6 Statistics

Statistical analysis was performed using SPSS version 10. The statistical significance of differences in baseline, mid-point and end-point plasma biochemistry and weights were tested by ANOVA with repeated measures over time. Pre versus post study differences were determined by paired student t-test.
3.3 RESULTS

No subjects reported any side effects when taking the oil. It was easily added into the diet, although more frequently by adding it to drinks or taking it with food as opposed to incorporating it into the food matrix. One subject was able to detect the oil even when blended with other foods to mask the flavour, but generally palatability was good, and subjects found the recipes included in the written information useful. Subjects found it difficult to judge quantities accurately when using the oil alone and despite the additional use of enriched products, some subjects reported difficulty in achieving the desired number of units per day. The unit system itself was also considered a poor idea. Many subjects found the butter cubes too large to use in one sitting and expressed problems using them as they needed to be kept frozen until 20 minutes before use. A similar problem occurred with the mayonnaise with subjects rapidly becoming frustrated with the monotony of this mode of delivery.

A majority of subjects expressed difficulty in adhering to the study dietary protocol when eating out, and suggested that more comprehensive advice be given before embarking on the principal intervention. Subjects also recorded other ways of incorporating the oil into the diet; the most common method was to include the oil in a milkshake drink.

3.3.1 Differences between the nutritional composition of baseline and study diets

A comparison between baseline and study diets revealed an increase in energy from fat (p=0.01) and a decrease in energy from carbohydrate (p=0.01). Energy derived from PUFA increased from 6.9% total energy to 11.5% (p=0.003). A decrease in the n-6:n-3 ratio was from 16.2 in the habitual diet, to 0.35 in the dietary intervention (p=0.02) was measured. The mean intake of ALA per day was 23.1g (±4.1g), this was equal to 6.2 units. The original aim of 8 units was not achieved for reasons stated above. Variability in the intake of ALA came from other foods containing ALA and differences in
subjects compliance to the diet. A summary of habitual and study diets is shown in Table 3.2.

Table 3.2: Habitual and study diet information from the pilot study.

<table>
<thead>
<tr>
<th>Total Energy/ % Energy</th>
<th>Habitual Diet (S.D.)</th>
<th>Study Diet (S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Energy (MJ)</td>
<td>9.5 (2.2)</td>
<td>11.1 (2.0)</td>
</tr>
<tr>
<td>Protein</td>
<td>15.4 (1.0)</td>
<td>14.0 (2.4)</td>
</tr>
<tr>
<td>Fat</td>
<td>33.9 (4.1)</td>
<td>39.1 (4.9)**</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>40.9 (6.0)</td>
<td>35.7 (6.0)**</td>
</tr>
<tr>
<td>SFA</td>
<td>10.9 (2.4)</td>
<td>11.4 (2.9)</td>
</tr>
<tr>
<td>MUFA</td>
<td>11.1 (1.0)</td>
<td>10.9 (1.8)</td>
</tr>
<tr>
<td>PUFA</td>
<td>6.9 (0.9)</td>
<td>11.5 (2.9)***</td>
</tr>
<tr>
<td>n6:n3 ratio</td>
<td>16.2 (16.8)</td>
<td>0.35 (0.1)*</td>
</tr>
<tr>
<td>Dietary Unit equivalents</td>
<td></td>
<td>6.2</td>
</tr>
</tbody>
</table>

* p = 0.02 ** p = 0.01 *** p = 0.003 (Paired t-test)

There were no significant differences measured in plasma TAG, Cholesterol, HDL-Cholesterol, LDL-Cholesterol or Glucose at any intervention time point (Table 3.3).

Table 3.3: Biochemical parameters and weights of all subjects at weeks 0, 2 and 4.

<table>
<thead>
<tr>
<th></th>
<th>Week 0 (S.D.)</th>
<th>Week 2 (S.D.)</th>
<th>Week 4 (S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAG mmol/l</td>
<td>1.42 (0.58)</td>
<td>1.24 (0.33)</td>
<td>1.37 (0.23)</td>
</tr>
<tr>
<td>Cholesterol mmol/l</td>
<td>4.91 (0.99)</td>
<td>4.73 (0.82)</td>
<td>4.77 (0.80)</td>
</tr>
<tr>
<td>HDL mmol/l</td>
<td>1.17 (0.31)</td>
<td>1.20 (0.38)</td>
<td>1.16 (0.33)</td>
</tr>
<tr>
<td>LDL mmol/l</td>
<td>3.09 (0.86)</td>
<td>2.96 (0.75)</td>
<td>2.98 (0.88)</td>
</tr>
<tr>
<td>Glucose mmol/l</td>
<td>5.52 (0.35)</td>
<td>5.52 (0.31)</td>
<td>5.49 (0.29)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>77.0 (6.1)</td>
<td>77.2 (5.8)</td>
<td>76.7 (5.8)</td>
</tr>
</tbody>
</table>

The changes in erythrocyte membrane fatty acid phospholipids were related to changes in dietary intake. The percentage of ALA in erythrocyte membrane fatty acid phospholipids increased from 0.4% total fatty acids to 1.3% (p=0.02). There was also an increase in EPA from 1.1% to 2.1% by week 4 (p=0.004). On the other hand, DHA decreased from 4.9% to 3.4% (p=0.004). The composition of erythrocyte membrane fatty acid phospholipids at week 0 and week 4 is shown in Table 3.4.
Table 3.4: Fatty acids in erythrocyte membrane phospholipids at week 0 and week 4.

<table>
<thead>
<tr>
<th>Fatty Acid Type</th>
<th>% Week 0 (S.D.)</th>
<th>% Week 4 (S.D.)</th>
<th>Δ%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic Acid 16:0</td>
<td>26.0 (1.78)</td>
<td>27.2 (6.0)</td>
<td>4.6%</td>
</tr>
<tr>
<td>Stearic Acid 18:0</td>
<td>10.1 (1.6)</td>
<td>11.8 (4.2)</td>
<td>16.8%</td>
</tr>
<tr>
<td>Oleic Acid 18:1 n-9</td>
<td>3.2 (6.4)</td>
<td>5.7 (10.2)</td>
<td>78.1%</td>
</tr>
<tr>
<td>Linoleic Acid 18:2 n-6</td>
<td>14.8 (1.9)</td>
<td>14.4 (2.3)</td>
<td>-2.7%</td>
</tr>
<tr>
<td>Alpha-Linolenic Acid 18:3 n-3</td>
<td>0.4 (0.29)</td>
<td>1.3 (0.97)*</td>
<td>225.0%</td>
</tr>
<tr>
<td>Arachadonic Acid 20:4 n-6</td>
<td>13.8 (4.7)</td>
<td>11.5 (5.0)</td>
<td>-16.7%</td>
</tr>
<tr>
<td>EPA 20:5 n-3</td>
<td>1.1 (0.64)</td>
<td>2.1 (1.14)**</td>
<td>90.9%</td>
</tr>
<tr>
<td>DHA 22:6 n-3</td>
<td>4.9 (1.4)</td>
<td>3.4 (1.94)**</td>
<td>-30.6%</td>
</tr>
</tbody>
</table>

* p = 0.02  ** p <=0.01

Comparison of percentage change in dietary intake of fats between baseline and intervention with changes in erythrocyte membrane phospholipid fatty acid composition is shown in Figure 3.1.

Figure 3.1: Percentage change in dietary intake between baseline and intervention of fats and observed changes in erythrocyte fatty acid membrane composition.

The mean percentage of each LDL subclass at baseline and 4 weeks are shown in Table 3.5.
Table 3.5: Mean distribution of LDL subclasses at week 0 and week 4.

<table>
<thead>
<tr>
<th>Distribution of LDL subclasses</th>
<th>Week 0 Mean (SD)</th>
<th>Week 4 Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% LDL 1</td>
<td>19 (9.9)</td>
<td>22 (17.4)</td>
</tr>
<tr>
<td>% LDL 2</td>
<td>56 (12.2)</td>
<td>57 (7.8)</td>
</tr>
<tr>
<td>% LDL 3</td>
<td>25 (17.4)</td>
<td>21 (7.3)</td>
</tr>
</tbody>
</table>

At baseline 8 out of the 9 subjects exhibited a pattern A phenotype. One subject (subject 6) exhibited a pattern B phenotype. Overall no significant changes were observed in LDL subclass profiles. Figure 3.2 illustrates all the LDL subclass profiles pre dietary intervention (blue) and post dietary intervention (red). Subject 6 demonstrated a shift from the “high-risk” pattern B phenotype to pattern A.
Figure 3.2: LDL subclass profiles for all 9 volunteers pre and post diet.
3.4 Discussion

The pilot study succeeded in its initial aims to test the feasibility of routes of delivery in providing ALA in combination with a diet low in n-6 PUFA diet. Verbal feedback from all the subjects who took part led to changes that may improve compliance in the principal study. The addition of ALA to foods after cooking or as part of the ingredients provided an effective route of delivering the oil. The use of written information and recipes aided compliance as verbally reported by subjects and as shown by increases in erythrocyte membrane FA ALA. Several subjects suggested that having the oil packaged in known quantities would aid accurate delivery, and also provide greater flexibility when eating outside of the home as such more written information on eating out was added into the principal study along with abandoning the unit system, which was not well liked, for pre-packaged oils in known doses.

The most successful means of delivery was using the oil directly, either adding it to food after it had been cooked, or using it as part of a cold dish such as a salad dressing. The idea of incorporating the oil into milkshakes was not initially considered in the pilot, but was added to methods of delivery for the principal study after a majority of subjects found it useful.

The twofold increase in ALA measured in erythrocyte membrane phospholipids provides evidence for effective absorption and incorporation. The increases in the abundance of ALA in erythrocyte membrane phospholipids in those subjects taking the flax oil diet could be used as an indicator of compliance to the diet. The failure to measure any increases in DHA are consistent with the findings of other studies (Table 1.9). However, the intervention period of this pilot was only 4 weeks, whereas the principal study would last 12. It is important to note that the mean life span of an erythrocyte is 120 days (4 months), and that reliance on this over a 28-day period may not provide an accurate indication of dietary compliance and ALA conversion to its longer chain products. The effects of the metabolic transport and storage pools of FA discussed in Chapter 1 should also be considered.
Increases in the erythrocyte fatty acids ALA, and EPA, but decrease in DHA may be the result of insufficient metabolic elongation, rather than insufficient ALA from the diet. The use of the 7 day food diaries supported this by showing a decrease in the dietary n-6:n-3 ratio from 16.2 in the habitual diet to 0.35 in the study diet. Changes in the composition of erythrocyte fatty acid membranes provided a valuable insight into incorporation and metabolic elongation of ALA. The absence of any changes in dietary saturates and monounsaturated fats suggest that changes observed in membrane fatty acids are as a result of substitution following the alteration of other dietary fatty acids, most notably ALA. For example, oleic acid increased by 78% following a 2% reduction in MUFA dietary intake, whereas LA remained virtually unchanged.

The significant increase in EPA in the absence of any dietary sources suggests that elongation of ALA has occurred. However, there is no evidence of continued elongation as demonstrated by the 31% decrease in DHA. These data may suggest that decreases in AA and DHA in the erythrocyte membrane phospholipids have been replaced to some extent by oleic acid.

Despite some extensive changes in dietary composition, there were no significant increases in total energy intake. The significant rise in total energy from fat (+5%) was to a certain extent compensated for by a reduction in overall energy from carbohydrate (-4.8%). There were very small non-significant changes in the total energy from SFA and MUFA, but as expected a significant rise in total energy from PUFA. There were no changes in overall weight. It is unclear how subjects maintained energy balance (demonstrated by weight maintenance) when no specific advice was given other than that stated earlier.

There were no significant changes in LDL subclasses measured over the 4 week period. This is likely to be due to 8 out of 9 subjects being normolipidaemic. They presented with Pattern A phenotypes at the start of the study. Had ALA exerted fish oil like effects on LDL subclass distribution, it
would have been difficult to measure in subjects already exhibiting the pattern A phenotype. Subject 6, who began the study as pattern B (Figure 3.7), shifted towards pattern A by week 4, this was represented by a decrease from 77% LDL-3 at baseline, to 31% LDL-3 at the end. This was accompanied by a decrease in TAG from 2.89 mmol/l at the commencement of the study, to 1.64 mmol/l at the end. It is not possible to conclude if the effects observed in the pilot study would occur in other hypertriacylglycerolaemic subjects. The principle study population however, would consist of dislipidaemic subjects.

3.5 Conclusion

Overall, the numbers investigated were too small to determine statistically the effects of the diet on any of the parameters measured. What it did achieve was valuable insights into the logistical and practical issues surrounding the delivery of dietary ALA for the principal study. It provided evidence for incorporation of ALA into erythrocytes, and how this may potentially be useful as an indicator of dietary compliance. The study also provided some evidence for metabolic elongation of ALA to EPA.
4 CHAPTER 4: THE IMPORTANCE OF ALPHA-LINOLENIC ACID AS A SOURCE OF N-3 PUFA: ASSESSMENT OF DIETARY COMPLIANCE.

4.1 Introduction

The need for good dietary compliance in a dietary intervention study is essential. It is made more difficult in free-living studies, where intakes cannot be regulated as accurately as they can in a metabolic kitchen. These studies are nevertheless important, as they are more representative of the types of behaviours that would be witnessed should such dietary advice be given to the population in general. Enhancing dietary compliance is an important consideration when devising a free-living dietary intervention study. Barnard et al (1995) identified a number of factors which enhance dietary compliance, including involving family members, encouraging food monitoring, providing instruction on cooking, nutrition and meal plans. Lewis et al (1990) also found that adherence on less palatable diets could be enhanced with written information on how to make them taste better. Providing specific foods to individuals also improves dietary compliance (Jeffery et al 1993). Gorbach and co-workers (1990) suggested that motivation to change was an important factor, this is relevant as a number of studies target populations who are aware of their risk factors or diagnoses. The most common factors which decrease dietary compliance are that people enjoy the food they are currently eating and/or they already think their diets are healthy (Contugna et al 1992). While this may be an important consideration in long-term dietary advice, it is less likely to affect those who have volunteered to take part in a study of known duration and who are considered to be motivated.

4.2 Methods

To determine habitual and study dietary intakes, validated (Bingham et al 1997) 7 day food diaries were used. Based on the finding of other studies and on the experience of other investigators who have previously worked on dietary intervention studies, a list of factors which may improve or reduce dietary compliance were considered (Table 4.1).
Table 4.1: Factors that may positively or adversely affect compliance

<table>
<thead>
<tr>
<th>Factors which aid compliance</th>
<th>Factors which may reduce compliance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Good information &amp; education</td>
<td>Time frame (3 month intervention)</td>
</tr>
<tr>
<td>Dietary supplements that are simple to use</td>
<td>Palatability</td>
</tr>
<tr>
<td>Time required each day to comply</td>
<td>Quality of life (such as eating out)</td>
</tr>
<tr>
<td>Support during intervention (written &amp; verbal)</td>
<td>Side effects of supplementation</td>
</tr>
</tbody>
</table>

From these, practical and educational strategies to enhance compliance were devised. These were categorised into education and information, and into practical and dietary strategies.

4.3 Education & Information Strategies

Volunteers were provided with information in 4 ways. Firstly, an open evening was organised where the investigators involved explained the basic concepts of the study; this included partners who for many were considered instrumental in maintaining compliance at home, primarily as they were heavily involved in food preparation. Secondly, and to enhance the latter point, written information was given to all volunteers (see Appendix 3). This not only described the rationale for the study, but also gave clear advice on maintaining low intakes of n-6 PUFA and fish oils for the duration of the study. The written advice not only included information on what not to eat, but also suitable alternatives. Ways in which to use the oils and margarines provided were also included. The third method was an individual consultation with a State Registered Dietitian. This was conducted on a one-2-one basis with the volunteer. It involved a detailed diet history to identify foods which may not have been recorded in the 7 day habitual diet diary and advice more specific to that individual on how to maintain a low intake of dietary n-6 PUFA and fish oils excluding those provided by the study. The fourth method was through verbal communication. Contact telephone numbers, including out of hours, were provided and volunteers actively encouraged to contact study personnel should they have any queries about certain foods. Volunteers were also encouraged to ask questions during regular re-supply of supplements, during
acquisition of blood samples or if participating in additional investigations running alongside the main intervention.

4.4 Dietary and Practical Strategies

Delivering the oils into the diet was difficult. Flaxseed oil is inherently unstable and cannot be heated as it oxidises (Chen et al. 1994). It is also unstable at room temperature for long periods. This presented some problems in terms of delivery. While the pilot study has issued each volunteer with a bottle of flaxseed oil and instructed them on how to measure it out, using this method for the main intervention was considered impractical for many reasons. Firstly, there may be volunteer errors in measuring the correct dose of oil, secondly inappropriate storage may affect fatty acid stability and as such restrict the "portability" of the oil during the day. Flaxseed oil stored in a bottle is sealed under nitrogen when packaged, but once opened even if stored correctly is still subject to oxidation.

To overcome these problems surrounding dose, stability and portability the oil was packaged in light proof, nitrogen filled foil lined packets in individual doses. This approach would improve compliance in a number of ways; by allowing the oil to be used outside of the home, reducing the time needed to measure out the correct dose, ensuring the delivery of a known amount (dose) of oil and potentially reducing oxidative changes in the oil which may affect palatability and efficacy.

While the sunflower oil did not need to be subjected to such rigorous storage conditions it was packaged in the same way as the flaxseed oil for reasons of portability and ease of use. As the packaging was the same, apart from a single letter code, it removed the problem of subjects deducing which dietary supplement they were on.

A majority of the volunteer's habitual dietary n-6 PUFA was derived from oil and margarine. As such, the study also provided replacements for cooking oils and margarines during the intervention period. This potentially would
improve dietary compliance by providing a specific product, rather than a list of suitable margarines and oils to use.

4.5 Assessing dietary compliance

A number of strategies were created to assess dietary compliance. A majority of these were from information provided by the volunteers themselves, either verbally or in a written format (Table 4.2).

<table>
<thead>
<tr>
<th>Strategies used to determine dietary and study protocol compliance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food diaries (7 days) x3</td>
</tr>
<tr>
<td>Debriefing during re-supply of supplements or during blood sample acquisition</td>
</tr>
<tr>
<td>Questionnaires sent out upon completion of the study</td>
</tr>
<tr>
<td>Counting returns of oil sachets and fish oil capsules</td>
</tr>
<tr>
<td>Erythrocyte membrane fatty acid analysis</td>
</tr>
</tbody>
</table>

The number of sachets of oil required by each volunteer to complete their intervention period was calculated. As the sachets required storage in a freezer until used, volunteers would regularly attend the investigation unit to acquire more supplies. A record of the number of sachets of oil or fish oil capsules dispensed was recorded and all volunteers asked to return anything left over when they completed the study.

4.6 Volunteers assessment of their compliance

To determine volunteer impressions of the study, and to ascertain how well they thought they complied anonymous questionnaires were sent out to all participants. Questions covered impressions of the study, dietary and practical compliance and the opportunity for individual comments. The questionnaires also provided insight into the effectiveness of the strategies developed to aid compliance in this particular study.

4.7 Results

4.7.1 Questionnaires

55 questionnaires were sent out to volunteers (Appendix 1) and 17 were returned completed (31%). The results are summarised in Tables 4.3 to 4.8.
Maintaining a low dietary intake of n-6 PUFA and fish oil during the intervention was essential. 30% found it hard or very hard to eliminate foods identified as being high in these during the intervention. Compliance with meeting the required doses of oils was also very important; 29% of those taking fish oil capsules said they forgot to take them frequently and 24% said they forgot to take the oil sachets occasionally. None of the volunteers reported complete compliance with respect to sachets or capsules.

It is reasonable to assume that a decrease in compliance may be associated with increasing difficulty consequently when asked how easy it was to take the sachets of oil, fish oil capsules and margarines all volunteers stated the latter two were average or easy to use, only 36% thought the sachets were an effective mode of delivery. Using the sachets of oil in drinks was favoured more than incorporating them into food.

Feedback on the use of food diaries was also mixed. 56% found them hard or very hard to complete, with 33% filling them in retrospectively the following day. Most volunteers thought they were an accurate reflection of their dietary intake.

The use of an open evening was reported as being useful by 93%, and 81% stated they would take part in another intervention study (Figure 4.1). Despite expressing some difficulties with the food diaries and modes of supplement delivery, only 12% stated the study was hard or very hard to complete. 12% stated they had made lifestyle changes prior to the commencement of the study, based simply on the fact they had met the entry criteria and had been given access to their blood screening results.
Table 4.3: Responses from volunteer questionnaire – General Impressions

<table>
<thead>
<tr>
<th>Question</th>
<th>Yes (%)</th>
<th>No (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Did you find the first 6 weeks easier than the second?</td>
<td>29</td>
<td>71</td>
</tr>
<tr>
<td>Did you weight yourself each week?</td>
<td>88</td>
<td>12</td>
</tr>
<tr>
<td>Did you make any diet or lifestyle changes between your screening blood test and the start of the study?</td>
<td>12</td>
<td>88</td>
</tr>
<tr>
<td>If you did make lifestyle changes, was it a result of your screening test results?</td>
<td>88</td>
<td>12</td>
</tr>
<tr>
<td>Has selection for this study prompted you to visit your GP for health checks?</td>
<td>24</td>
<td>76</td>
</tr>
<tr>
<td>Did you find the blood taking an inconvenience?</td>
<td>24</td>
<td>76</td>
</tr>
<tr>
<td>Has your diet returned to normal since completing the study?</td>
<td>47</td>
<td>53</td>
</tr>
<tr>
<td>Would you consider participating in another intervention study?</td>
<td>81</td>
<td>19</td>
</tr>
<tr>
<td>Did you find the introduction evening at the University useful in allaying any uncertainties regarding your participation in the study?</td>
<td>93</td>
<td>6</td>
</tr>
<tr>
<td>Was there information you felt was missing in relation to the study that you would have benefited from had you been made aware of it at the start?</td>
<td>*12 88</td>
<td></td>
</tr>
</tbody>
</table>

* Additional Comments:
More information on how much time was involved/level of inconvenience
Less technical presentation of study aims and clearer idea of what study was trying to achieve

Table 4.4: Responses from volunteer questionnaire – assessing compliance

<table>
<thead>
<tr>
<th>Question</th>
<th>Very Easy</th>
<th>Easy</th>
<th>Average</th>
<th>Hard</th>
<th>Very Hard</th>
</tr>
</thead>
<tbody>
<tr>
<td>How did you find taking the fish oil capsules every day?</td>
<td>57%</td>
<td>14%</td>
<td>29%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>How did you find the study to complete overall?</td>
<td>18%</td>
<td>24%</td>
<td>46%</td>
<td>12%</td>
<td>0%</td>
</tr>
<tr>
<td>How did you find using the sachets in food?</td>
<td>6%</td>
<td>25%</td>
<td>31%</td>
<td>25%</td>
<td>13%</td>
</tr>
<tr>
<td>How did you find using the sachets in drinks?</td>
<td>24%</td>
<td>24%</td>
<td>18%</td>
<td>18%</td>
<td>18%</td>
</tr>
<tr>
<td>How did you find suing the margarine provided?</td>
<td>64%</td>
<td>12%</td>
<td>24%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>How difficult did you find it to cut out the foods indicated by study personnel?</td>
<td>18%</td>
<td>34%</td>
<td>18%</td>
<td>24%</td>
<td>6%</td>
</tr>
<tr>
<td>How would you rate completing the food diaries?</td>
<td>6%</td>
<td>19%</td>
<td>19%</td>
<td>37%</td>
<td>19%</td>
</tr>
<tr>
<td>How would you rate the sachets as a means of delivering the oil?</td>
<td>12%</td>
<td>6%</td>
<td>18%</td>
<td>47%</td>
<td>18%</td>
</tr>
<tr>
<td>Question</td>
<td>Never</td>
<td>Rarely</td>
<td>Occasionally</td>
<td>Lots</td>
<td>All the time</td>
</tr>
<tr>
<td>-------------------------------------------------------------------------</td>
<td>-------</td>
<td>--------</td>
<td>--------------</td>
<td>------</td>
<td>--------------</td>
</tr>
<tr>
<td>How often did you forget to take your capsules?</td>
<td>0%</td>
<td>57%</td>
<td>14%</td>
<td>29%</td>
<td>0%</td>
</tr>
<tr>
<td>How often did you forget to take your sachets?</td>
<td>41%</td>
<td>29%</td>
<td>24%</td>
<td>6%</td>
<td>0%</td>
</tr>
<tr>
<td>How often would you enter foods into the diary the day after you had eaten them?</td>
<td>13%</td>
<td>20%</td>
<td>34%</td>
<td>13%</td>
<td>20%</td>
</tr>
</tbody>
</table>

Table 4.6: Responses from volunteer questionnaire – food diaries

<table>
<thead>
<tr>
<th>Question</th>
<th>&lt;25%</th>
<th>25-50%</th>
<th>51-75%</th>
<th>76-99%</th>
<th>100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall how accurate do you think your food diary was compared to your actual diet over the week it was completed?</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>53%</td>
<td>47%</td>
</tr>
</tbody>
</table>

Table 4.7: Responses from volunteer questionnaire – overall compliance with instructions

<table>
<thead>
<tr>
<th>Question</th>
<th>None</th>
<th>A little</th>
<th>Often</th>
<th>Most of the time</th>
<th>100% of the time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall how compliant would you say you were to all the instructions and advice given to you whilst on the study?</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>47%</td>
<td>53%</td>
</tr>
</tbody>
</table>

Table 4.8: Responses from volunteer questionnaire – overall study rating

<table>
<thead>
<tr>
<th>Question</th>
<th>More</th>
<th>Less</th>
<th>Same</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall was the study more difficult, less or the same to complete as you thought?</td>
<td>24%</td>
<td>65%</td>
<td>12%</td>
</tr>
</tbody>
</table>
Figure 4.1: Questionnaire responses to general impressions of the study

Did you find the open evenings useful?
- Yes: 93%
- No: 7%

Would you take part in another study?
- Yes: 81%
- No: 19%
4.8 Discussion

The very nature of a free-living study is that there will be considerable individual variability. This variability can manifest itself in a number of ways: lack of understanding, poor compliance and inaccurate recording and processing of dietary intakes.

Issues around "lack of understanding" can be reduced but not eliminated by good verbal and written communication. The socio-economic group of the volunteer may also have an impact on their ability to follow dietary advice (Reid et al 1984). The principal study provided a broad framework of support for those taking part and their partners if appropriate. It was felt that no additional practical measures could have been implemented to improve understanding in the group studied.

Poor compliance due to lack of interest or difficulty in complying with the study diet may be reduced with good written and verbal support (Masley 1998). In as far as was possible those volunteers that were having difficulties were encouraged to contact the study investigators. Daytime and out of hours contacts were available. Good, clear information on the duration and nature of the study were made available as part of an open day prior to commencing the study, it was considered that in an adult population of this nature, this information would enable them to decide whether 3 months was too long for them to comply with the diet. Poor compliance due to lack of interest or practical difficulties may have resulted in failure to take the required dose of supplements in parallel with a low n-6 PUFA diet. The method of counting returns was used, but this did not take into account losses or damage to supplements. Some volunteers failed to return supplements they had remaining at the end of the study. The use of the margarines and oil for cooking was not limited for use by volunteers and so were frequently used by the rest of the family; this made it impossible to determine intakes based on the numbers of returns and made the study more reliant on the data presented in the food diaries.
4.8.1 Over and under reporting of dietary intake

Completion of the food diaries by volunteers is another potential source of inaccuracy. The quality of the food diaries ranged between volunteers in terms of the accuracy with which they recorded intakes, 10 food diaries across all 3 diets were not completed. As discussed some were completed retrospectively, which may decrease accurate recall of that day, or previous day's, dietary intake. The quality of the diaries also varied widely with some recording more detailed information than others, making over or under reporting a possibility. Seven-day food diaries are considered the most closely associated with weighed food intake when compared to other forms of dietary assessments (Bingham et al 1994). With respect to this study, it was felt that compliance may have been reduced if volunteers were required to weigh every item they consumed for 3 months.

Other discrepancies to the data collected from the food diaries were added by operator error. Three people were involved in entering the foods recorded into the computer program for analysis. Discrepancies were minimised by using standard portion sizes for foods where no amount was specified in the diary, however the literature is limited in the number of foods listed and some entries required an educated guess from the operators. One operator was a qualified dietitian and the remaining two student dietitians. Discrepancies between the output of the dietary analysis program were exacerbated by incomplete nutritional data on some foods, particularly fatty acid composition. While the MAFF fatty acid data was included in the software, this was limited to a certain number of foods. As such, data on the intakes of dietary ALA, LA, EPA, DPA and DHA may be falsely low. The variability of the dietary data collected is likely to be high, with errors from volunteers, operators of the software, and omissions in the nutritional data of foods. As such, conclusions based on dietary intakes should be considered carefully when interpreting the results and their effects in terms of CHD risk presented in Chapter 5.
4.8.2 Outcome of post dietary compliance questionnaires.

The use of the questionnaire to obtain feedback from subjects who had completed the study provided valuable insight into compliance to the written and verbal instructions given as well as any difficulties they had following the diet. It also provided insight into possible changes that may facilitate future dietary interventions. Although the present study was conducted with free-living individuals, there were constraints on participants with regard to their dietary intake. This had obvious consequences when eating out, for example. Apart from written information, the diets were designed to be as “user friendly” as possible, packaging the oil in portioned sachets and replacing subjects usual cooking oil and margarine. Responses from the questionnaire indicate that the method of delivering the oil in sachets was not as well received as the investigators predicted with 65% stating it was a hard or very hard way of delivering the oil. Equally, taking the oil in drinks was favoured over using in or on food. Freese and Mutanen (1997) used capsules as a means of delivering flax oil, and 71% of those on the present study taking fish oil capsules rated them as easy or very easy to take every day. Likewise, the margarine as a means of delivery was well received with 76% of respondents stating it was easy or very easy to use. This suggests that capsules and margarines may be a better way of delivering flaxseed oil in terms of compliance, particularly if dietary intervention is in free living populations and over a long period of time. Despite the unpopularity of the sachets as a means of delivery, only 12% of those asked stated that the study was hard to complete overall.

The use of food diaries is a necessity in dietary intervention studies. In this case a 7-day diary was used to ascertain habitual and then dietary intervention intakes. Fifty-six percent of those asked found competing the diaries hard or very hard, with a third completing the diaries the day after they had eaten and a further third reporting they “occasionally” completed the diaries the following day. This undoubtedly would have some effect on accuracy, increasing the chances of
a subject forgetting or recalling inaccurate portion sizes. There seems to be little that can be done to change this, as the importance of not completing the diaries retrospectively was emphasised both verbally and in written materials. Those volunteers who had made lifestyle changes following the initial screening session stated it was because they had been given access to their screening results. Future studies may benefit from non-disclosure to those selected to participate in the study, until all investigations have been completed. Those not selected to take part would not require such safeguards. The large number of subjects who stated they would take part in a future investigation suggests that some of the criticisms expressed in the 3-month intervention period were not prohibitively difficult. It is noteworthy that 24% had returned to their GP for further health checks and 53% stated their diet had not returned to normal upon completion of the study. This must be considered if other dietary intervention studies are to use the same subjects in future investigations. The use of an open evening pre and post intervention was considered by 93% to be useful. The opportunity to meet staff and investigators "behind the scenes" and to have academic staff explain the origins of the research and what it hopes to demonstrate were considered very helpful. This means of communicating information to subjects and partners brought volunteers together and allowed them and their partners to ask questions in both a formal and informal manner. The role of partners in motivating volunteers was not investigated by the questionnaire, but in retrospect may be beneficial should such a questionnaire be used again.
CHAPTER 5 PRINCIPLE STUDY: THE IMPORTANCE OF ALPHA-LINOLENIC ACID AS A SOURCE OF N-3 PUFA: INFLUENCE ON RISK FACTORS OF CARDIOVASCULAR DISEASE

5.1 Introduction

It is now well established that long chain n-3 polyunsaturated fatty acids offer protection against CHD (Burr et al 1989, Connor et al 1997, GISSI 1999). Most of these fatty acids are of marine origin, and in order to achieve intakes that confer protection, oily fish or fish products need to be consumed. There is much resistance to consumption of oily fish within populations for a variety of reasons including taste, cultural, financial or dietary restrictions. The fatty acid alpha-linolenic acid (ALA) 18:3 n-3 is the shorter chain pre-cursor to the longer chain n-3 PUFA. As humans possess the ability to elongate these shorter chain n-3 fats (Gerst H 1988) it follows that if sufficient ALA were consumed, conversion to the longer chain n-3 PUFA might occur and potentially offer the same benefits as fish oils. As previously discussed, prospective and case control trials have shown some, little or no benefit associated with consumption of ALA. Other studies have shown conversion to longer chain n-3 PUFA and others have failed to measure this.

This study aim was to measure the effect of dietary supplementation of ALA on a sub-set of an all male, free-living group exhibiting an ALP within the context of the FSA’s objectives, which were to seek suitable dietary alternatives to long chain n-3 PUFA. Dietary long chain n-3 PUFA have been associated with favourable changes in CHD risk factors, primarily through decreases in TAG, reductions in the numbers of small, dense LDL particles in the circulation and small increases in HDL-C. In order to further reduce CHD risk in the UK population, ALA the shorter chain pre-cursor to EPA and DHA could potentially be very useful. Alpha-linolenic acid is one of the most abundant fatty acids in the natural world. Within the present study, if adequate conversion of ALA to EPA
and DHA could be achieved, potentially beneficial effects in terms of reversing an ALP might occur.

Three high PUFA diets were tested each with different PUFA compositions. The high short chain n-3 PUFA diet was achieved through ALA supplementation from flaxseed oil, the high n-6 PUFA diet was achieved through sunflower oil and the high fish oil diet through sunflower and fish oil capsule supplementation. The aim of the ALA enriched diet was to increase the intake of ALA and decrease the intake of LA in order to reduce the dietary n-6:n-3 ratio below 1.0. It was postulated that higher intakes of ALA against a lower intake of LA may result in greater conversion of the shorter n-3 PUFA to longer chain n-3 PUFA via the delta-6-desaturase enzyme. This enzyme is essential to both ALA and LA for elongation, its affinity for ALA being greater than LA. The aims of the high n-6 fat diet were reflect the habitual dietary n-6 intakes within the UK but also to achieve similar intakes of PUFA to that of the flax oil diet; as a result energy derived from total fat and PUFA would be similar. The two diets would differ only in the ratio's of n-6 and n-3 fatty acids. The aim of the sunflower and fish oil diet was to act as a control. It was anticipated that those subjects allocated the fish oil diet would be associated with improvements in some indicators of CHD risk such as TAG and LDL heterogeneity. Lack of significant changes using longer chain n-3 PUFA may indicate that the use of shorter chain n-3 PUFA from flax oil to promote "fish oil" like effects may not be feasible in the population investigated in the present study.

5.2 Methods

5.2.1 Recruitment and Screening

This pilot study was approved by the ethics committee of the University of Surrey, Guildford, and the Royal Surrey County Hospital. Informed consent was obtained from the participating subjects. Volunteers were recruited through local GP practices, via the media, or the University's intranet and internet sites.
Researchers from the study, contacted local GP practices via letter (Appendix 2) and e-mail. Presentations were also offered to those who wished to learn more about the study's aims and objectives. Potential volunteers were then identified by the GPs using computerised biochemistry records, or anonymously by study personnel following data from the local hospital's biochemistry department. These individuals were then contacted by the GP informing them of the study and if they chose to take part all they had to do was return a slip in a pre-paid envelope to the researchers. Until this time, the identities of those initially selected were only known to the GP, thus maintaining GP-Patient confidentiality. The initial selection criteria for identification of potential volunteers as described to the GPs are detailed in Table 5.1.

<table>
<thead>
<tr>
<th>Table 5.1: Initial selection criteria for potential volunteers.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volunteers must not be on any lipid lowering medication or have history of heart disease</td>
</tr>
<tr>
<td>Volunteers must be non-smokers</td>
</tr>
<tr>
<td>Volunteers must not be suffering from diabetes</td>
</tr>
<tr>
<td>Recent fasting triglyceride levels below 1.5mmol/l</td>
</tr>
<tr>
<td>Recent total serum cholesterol level ≤6.5mmol/l</td>
</tr>
<tr>
<td>If available, recent HDL cholesterol levels &lt;1.1mmol/l</td>
</tr>
</tbody>
</table>

Once volunteers had agreed to take part, they were invited to provide a blood sample. Blood samples were taken by the study’s nurse either at their local GP practices, or at the university’s investigation unit. Fasting lipids and glucose were then checked, and if within the initial selection criteria, were further analysed to determine LDL subclass phenotype. If the volunteer's lipids and LDL phenotype met the entry criteria (Table 5.2), they were then formally invited to take part in the study by letter. Any volunteers who fell below the criteria were given a record of their blood results and an explanation of why they were unsuitable for the study by letter. GPs were asked to contact volunteers whose blood results appeared abnormal, after the results were made available. These volunteers were not invited to take part in the study.
Table 5.2: Entry criteria for the study after screening blood samples.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting triglyceride</td>
<td>&gt;1.5mmol/l</td>
</tr>
<tr>
<td>Total plasma cholesterol</td>
<td>£6.5mmol/l</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>&lt;1.1mmol/l</td>
</tr>
<tr>
<td>Fasting Glucose</td>
<td>&lt;6.5mmol/l</td>
</tr>
<tr>
<td>LDL-3 subclass</td>
<td>&gt;40%</td>
</tr>
<tr>
<td>BMI</td>
<td>&lt; 30 kgm^-2</td>
</tr>
</tbody>
</table>

Potential volunteers were also invited to take part in the study following press releases. Articles appeared in the local newspaper and on local BBC radio (Southern Counties Radio). This method resulted in a large number of queries by phone, letter and e-mail. Those that met the initial inclusion criteria (excluding blood results) were then invited to attend the investigation unit at the university to have screening blood samples taken. The GPs of those volunteers who had abnormal results were contacted, and the individuals urged to make an appointment to see them to discuss their results. Those who met the inclusion criteria for lipids had their LDL subclass phenotype determined, only subjects with LDL-3 >40% were eligible to take part in the study. Three dietary cohorts took part (Table 5.3) the first cohort was recruited entirely from local GP’s, the second from the media, and the third from both sources and also from within the university. All those who were selected were invited to attend an open evening along with their partners. Study investigators presented a summary of the current literature, and explained why the study was being conducted.

5.2.2 Study Design

Table 5.3 summarises the 3 dietary intervention periods and number of subjects run within each cohort.

Table 5.3: Numbers of volunteers completing each diet.

<table>
<thead>
<tr>
<th>Diet / Cohort</th>
<th>Numbers within each cohort</th>
<th>Total Volunteers who completed each diet (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>X (Flax-Oil)</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>Y (Sunflower Oil)</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Z (Fish oil + Sunflower Oil)</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Total Each Cohort</td>
<td>16 (28%)</td>
<td>22 (37%)</td>
</tr>
</tbody>
</table>
5.2.3 Diet design

The study was a randomised single, blind study with three parallel diets. It was assumed that the recruitment population was reasonably homogenous in terms of their habitual dietary intake prior to commencing the study, as such there was no run-in diet prior to starting the intervention. Volunteers were randomly assigned to their treatment diets and everyone instructed to follow a low n-6 PUFA diet during the intervention. To enable them to achieve this written information and practical advice was provided, as well as individual direction from a State Registered Dietitian more specific to their lifestyle. The investigators substituted the volunteer's usual cooking oil with vegetable oil (Sainsbury's 100% rapeseed oil) to cook and fry with. Volunteers on the sunflower oil and the fish oil diets received unmarked margarine high in monounsaturated fats and low in n-6 fat (Mono, St-Ivel). Volunteers on the flax oil diet received the same margarine, with a higher content of alpha-linolenic acid included by the manufacturers (additional 1.7g per 100g of spread). Seventeen mls of flaxseed oil or sunflower oil were packaged in nitrogen filled, lightproof aluminium lined sachets (Gravely Packaging Cambs, UK). Each sachet was labelled with a single letter code depending on the oil it contained. No other markings were present. Sachets labelled Y and Z contained sunflower oil. Sachets marked X, contained flax oil. All sachets were kept frozen at -18°C until used by the volunteer. Each volunteer was instructed to consume 2 sachets per day, and use only the margarine and cooking oil provided. Volunteers supplemented with fish oil consumed the same as those on the sunflower oil diet with the addition of 6 x 1g Pikasol (Pronova, Biocare) capsules per day. These provided 1.7g and 1.4g of EPA and DHA per day respectively. Recipes and instructions provided to volunteers can be found in Appendix 3. The nutritional content of the margarines, oils and fish oil capsules used are shown in Table 5.4.
Table 5.4: Nutritional content of the margarines, oils and fish oil capsules supplied to volunteers.

<table>
<thead>
<tr>
<th>Nutrients per 100g</th>
<th>Flaxseed Oil</th>
<th>Rapeseed Oil</th>
<th>Sunflower Oil</th>
<th>Mono Oil</th>
<th>Altered Mono</th>
<th>Pikasol Oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (MJ)</td>
<td>3.78</td>
<td>3.78</td>
<td>3.78</td>
<td>2.84</td>
<td>3.02</td>
<td>3.78</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>99.9</td>
<td>100</td>
<td>99.9</td>
<td>75</td>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>SFA (g)</td>
<td>6.6</td>
<td>6.7</td>
<td>12</td>
<td>11.5</td>
<td>11.5</td>
<td>-</td>
</tr>
<tr>
<td>MUFA (g)</td>
<td>31.5</td>
<td>60</td>
<td>20.5</td>
<td>35</td>
<td>35</td>
<td>-</td>
</tr>
<tr>
<td>PUFA (g)</td>
<td>57.5</td>
<td>30</td>
<td>63.3</td>
<td>14.7</td>
<td>19.7</td>
<td>60.3</td>
</tr>
<tr>
<td>Total n-6 (g)</td>
<td>14.8</td>
<td>9.6</td>
<td>63.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total n-3 (g)</td>
<td>57.5</td>
<td>19.8</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
<td>60.3</td>
</tr>
<tr>
<td>C18:2 (g)</td>
<td>14.8</td>
<td>19.6</td>
<td>63.2</td>
<td>1.0</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>C18:3 (g)</td>
<td>57.5</td>
<td>9.6</td>
<td>0.1</td>
<td>3.4</td>
<td>5.1</td>
<td>-</td>
</tr>
<tr>
<td>C20:5 (g)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>27.9</td>
</tr>
<tr>
<td>C22:4 (g)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3.7</td>
</tr>
<tr>
<td>C22:6 (g)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>22.3</td>
</tr>
</tbody>
</table>

5.2.4 Determining dietary intakes

To determine habitual intake, volunteers completed a 7-day food diary prior to starting the study. They were asked to complete a further 7-day food diary at week 6 and at week 10 for dietary analysis. Intakes were determined using the dietary analysis program Dietplan 5 (Forestfield Software Ltd).

5.2.5 Blood samples and anthropometric measurements

Baseline (0 weeks), midpoint (6 weeks), and 12 week blood samples were taken and prepared as described in Chapter 2.

Blood pressure was taken supine, prior to venepuncture by the study nurse. Body composition was determined by Bio-Impedance Analysis (BIA) using the Bodystat 1500 (Bodystat Ltd, UK). Subjects were instructed to empty their bladder and remove any metallic objects from their person before analysis began. The Bodystat machine was calibrated using a known resistance standard every morning before measurements began. Subjects were provided with breakfast after venepuncture. Investigators used this time to provide volunteers with supplies to take home and also to consult with them on any problems they may be experiencing in adhering to the diet.
All subjects were provided with e-mail and telephone numbers (including out of hours) should they run into any problems during the course of the intervention. At the start of the study they were given recipe booklets on how to use the oil, information on eating away from home and a weight diary. Subjects were instructed to weigh themselves once a week and to notify study personnel if they had gained or lost more than 1.5kg.

5.3 Determination of haemostatic variables

Batches of blood samples, stored at -80°C until the end of each cohort, were transported to the MRC Medical Epidemiology & Medical Care Unit in London, for analysis of Factor VII, fibrinogen and PAI-1 by methods previously described by (Meade et al 1993, Miller et al 1996).

5.4 Endothelial dysfunction measurements by flow mediated dilation

Ultrasound measurements were conducted by a trained radiologist at the Royal Surrey County Hospital and by Dr M Griffin at the University of Surrey. Blood flow and arterial diameter were measured non-invasively using high-resolution ultrasound (Celemajer et al 1992). Arterial diameter was measured from 2-demensional images with a 7.0Mhz liner array transducer and a standard 128XPi 10 System. Subjects had fasted for at least 2 hours and were rested supine for 15 minutes before measurements were taken. Scans were measured at rest and during cuff-induced, reactive hyperaemia (endothelium ‘NO-dependent’ dilatation, then again at rest and after sublingual GTN (300μg) (endothelium independent dilation). A blood pressure cuff was inflated to 200mmHg for 5 mins before being released. Readings were taken at rest and then 30, 90 and 180 seconds after cuff release and 3.5 and 5 mins after administration of GTN. Recovery time between measurements was 5 minutes. Arterial blood flow was measured by pulsed Doppler signal at a 70° angle to the artery. Vessel diameter was measured at 6 different sites in the same segment of vessel by ultrasonographer
that was blinded to the study groups. Comparisons were made between baseline and the mean post cuff or post GTN measurements and expressed as percentage change.

5.5 Statistical Tests
Analysis was performed using SPSS 11.0 for Macintosh. The group was studied for covariates to check the homogeneity of baseline values. Differences within each dietary group were analysed using paired student t-tests. Differences between the 3 dietary groups were analysed using ANOVA, plus post hoc tests for "paired" differences. Univariate associations between continuous variables were determined by correlation and simple linear regression. P-values were considered significant at less than the 5% level.

5.6 Results
5.6.1 Dietary Analysis of Habitual and Study Diets
There were no significant differences in the habitual dietary intakes of macronutrients and energy in all three groups at baseline. The fish oil group had a marginally higher intake of total energy per day compared to the control group but this was not significant. Nutritional data for all three diets are shown in Table 5.5. All three study diets differed in relation to percentage energy derived from n-3 PUFA (Figure 5.1). An increase in the percentage energy from MUFA in the flax oil group (p<0.001) compared to the sunflower and the fish oil groups was observed. Intakes of n-6 PUFA were lower in the flax group (p<0.001) compared to both the fish oil and sunflower oil groups (Figure 5.1). All three dietary groups differed significantly in their n6:n3 ratios (p<0.001) (Figure 5.1), whilst intakes of SFA were similar.
5.6.2 Habitual versus study diet composition

In the flax oil group there was an increase in energy derived from MUFA (p<0.001) occurred compared to baseline. There was also an increase in energy from and intake of n-3 PUFA (p<0.001). The n6:n3 ratio decreased from 8.7±5.3 to 0.5±0.2 on the study diet (p<0.001). Intakes of n-6 PUFA did not change significantly.

In the sunflower oil group there was an increase in the percentage energy derived from fat (p<0.01) occurred compared to the habitual diet. Saturated fat intake decreased from 14.5±3.4% energy to 11.3±2.4% on the study diet (p<0.001), whilst intakes of n-3 PUFA (p<0.05) and n-6 PUFA (p<0.001) increased. The n6:n3 ratio increased from 10.0±4.2 to 26.4±19.8 (p<0.05).

In the fish oil plus sunflower oil group the percentage energy derived from protein fell slightly but was still significant (p<0.01). Energy from SFA decreased (p<0.01) and intakes of n-6 PUFA (p<0.001) and n-3 PUFA (p<0.001) increased. There was a decrease in the n6:n3 ratio (p<0.05). Energy derived from alcohol decreased from 8.6±5.7% to 6.2±4.5% (p<0.01).
Table 5.5: Intakes of Macronutrients on Habitual and Study diets

<table>
<thead>
<tr>
<th></th>
<th>Flaxseed Oil Diet (DIET X)</th>
<th>Sunflower Oil Diet (DIET Y)</th>
<th>Fish Oil + Sunflower Oil Diet (DIET Z)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Habitual Diet</td>
<td>Study Diet</td>
<td>Habitual Diet</td>
</tr>
<tr>
<td>Energy MJ per day</td>
<td>9.9 (1.9)</td>
<td>10.8 (2.7)</td>
<td>10.1 (1.2)</td>
</tr>
<tr>
<td>% Energy from Fat</td>
<td>37.7 (6.9)</td>
<td>42.8 (6.4)</td>
<td>36.8 (5.6)</td>
</tr>
<tr>
<td>% Energy from Carbohydrate</td>
<td>41.9 (7.3)</td>
<td>37.5 (6.6)</td>
<td>42.8 (7.3)</td>
</tr>
<tr>
<td>% Energy from Protein</td>
<td>16.5 (2.9)</td>
<td>15.2 (3.0)</td>
<td>14.6 (1.8)</td>
</tr>
<tr>
<td>% Energy from SFA</td>
<td>13.4 (3.4)</td>
<td>11.8 (1.9)</td>
<td>14.5 (3.4)</td>
</tr>
<tr>
<td>% Energy from MUFA</td>
<td>13.0 (2.1)</td>
<td>17.8 (2.7)***</td>
<td>12.3 (2.2)</td>
</tr>
<tr>
<td>n-3 g/d</td>
<td>1.2 (2.3)</td>
<td>19.0 (1.4)***</td>
<td>0.7 (0.4)</td>
</tr>
<tr>
<td>n-6 g/d</td>
<td>6.0 (5.4)</td>
<td>8.6 (3.4)</td>
<td>7.2 (6.3)</td>
</tr>
<tr>
<td>% Energy from n-3 PUFA</td>
<td>0.7 (1.3)</td>
<td>6.9 (1.4)***</td>
<td>0.3 (0.2)</td>
</tr>
<tr>
<td>n6:n3 PUFA ratio</td>
<td>8.7 (5.3)</td>
<td>0.5 (0.2)***</td>
<td>10.0 (4.2)</td>
</tr>
<tr>
<td>% Energy from Alcohol</td>
<td>5.1 (4.2)</td>
<td>5.4 (4.7)</td>
<td>6.8 (6.7)</td>
</tr>
</tbody>
</table>

(SD) Standard Deviation *p<0.05 **p<0.01 ***p<0.001 (compared to habitual diet)
Figure 5.1: Intakes of MUFA, n-6 and n-3 PUFA and the n-6:n-3 ratio of all 3 dietary groups on their respective study diets.

*p<0.01 (compared to flax diet) **p<0.001 (compared to flax diet) †p<0.001 (compared to control diet)
5.6.3 Erythrocyte membrane phospholipid fatty acid composition

Erythrocyte membrane fatty acid composition for all three diets, at all three time points are shown in Figure 5.2. There were no significant differences between the 3 diet groups in the abundance of any of the fatty acids measured at baseline. A decrease (pre vs post diet) in palmitic acid on the flax oil diet (-13%, p=0.04) and the fish oil diet (-15%, p=0.02) was measured. Oleic acid decreased at week 12 on the flax oil diet (-14%, p=0.02) and the fish oil diet (-23%, p<0.001) compared to baseline respectively. The abundance of LA decreased on the flax oil diet when compared to the control diet (p<0.01). The abundance of ALA (pre vs post diet) increased in the flax oil group (p<0.0001), this increase was significant at week 6 (p<0.001) and 12 (p<0.001) when compared to the fish oil and sunflower oil diets. Increases in EPA (pre vs post diet) on the flax oil diet (+153%, p=0.01) and the fish oil diet (+124%, p<0.01) were also measured as were increases in DPA (pre vs post diet) on the flax oil diet (+26%, p<0.05) and the fish oil diet (+64%, p<0.01). At week 6 the abundance of EPA on the flax and fish oil diets was greater than the sunflower oil diet (p=0.01). By week 12, this was between the fish oil and sunflower oil diets only (p<0.01). The abundance DHA increased on the fish oil diet (pre vs post) (+60%, p<0.01); this remained significantly higher than those measured on the flax and control diets at week 6 (p<0.001) and 12 (p<0.001). A positive (p=0.01, r=0.64) correlation was observed between the change in n-3 PUFA intake and the change in the abundance of 18:3 n-3 in erythrocyte membrane phospholipids (habitual versus study diets) (Figure 5.3).
Figure 5.2: Erythrocyte membrane phospholipid fatty acids: relative abundance (SD) at baseline (0 weeks), 6 and 12 weeks in all three dietary treatment groups.

*Diet X: Flax Oil Group*

*Diet Y: Control (Sunflower Oil) Group*

*Diet Z: Fish Oil + Sunflower Oil Group*
5.6.4 Lipids, glucose, lipoproteins, haemostatic, LDL and HDL subclasses.

5.6.4.1 Plasma glucose and plasma apolipoprotein B

There were no significant changes in fasting plasma glucose levels either within or between any of the groups at any time point. Similarly, there were no significant differences at any time point between all three dietary groups in the concentration of plasma apolipoprotein B. (Table 5.6).

5.6.4.2 Plasma Lipoproteins

There were no significant differences between dietary groups in lipids, glucose, lipoproteins, LDL and HDL subclasses or haemostatic values at baseline. The fish oil group had marginally higher but non-significant levels of TAG (+16%) and total serum cholesterol (+9%) compared to the control group at baseline (Figure 5.4).

Changes in TC, LDL-C, TAG and HDL at baseline, 6 and 12 weeks are shown in Figure 5.4. There were no significant differences in LDL-C between any diet
group at 6 and 12 weeks. There was a non-significant decrease in LDL cholesterol within all groups compared to their respective baselines (Figure 4.3) flax (-9%), control (-8%) and fish (-5%). This fall was consistent over the intervention period. There were no significant differences in HDL-C between groups at baseline and at 6 weeks. There was a consistent but non-significant decrease in HDL-C at 12 weeks relative to baseline on the flax (-15%) and sunflower oil (-6%) diets, and a small increase in HDL-C on the fish oil diet. There were no significant differences in the TC:HDL-C ratio between the three dietary groups at 6 or 12 weeks. Changes within each group at 6 and 12 weeks were also not significantly different from baseline. There was a significant decrease (p<0.001) in the TC:HDL-C ratio in the fish group (Table 5.6) at 12 weeks compared to baseline.

There were no significant differences in total plasma cholesterol between any dietary group at 6 and 12 weeks. There was however, a consistent, but non-significant fall in total serum cholesterol within all groups (Figure 3) compared to their respective baseline values (pre vs post diet) flax (-12%), control (-11%) and fish (-8%).
Table 5.6: Anthropometric data, glucose, ApoB and TC:HDL ratio at baseline, 6 weeks and 12 weeks.

<table>
<thead>
<tr>
<th></th>
<th>Baseline (0 weeks)</th>
<th>Midpoint (6 weeks)</th>
<th>Endpoint (12 weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fish Diet (n=21)</td>
<td>Control Diet (n=17)</td>
<td>Fish Oil Diet (n=19)</td>
</tr>
<tr>
<td>TC:HDL-C ratio</td>
<td>5.9 (1.4)</td>
<td>5.7 (1.1)</td>
<td>6.0 (1.2)</td>
</tr>
<tr>
<td></td>
<td>5.8 (1.3)</td>
<td>5.4 (1.3)</td>
<td>5.6 (1.1)</td>
</tr>
<tr>
<td></td>
<td>5.7 (1.2)</td>
<td>5.6 (1.2)</td>
<td>5.4* (1.1)</td>
</tr>
<tr>
<td>ApoB g/l</td>
<td>1.57 (0.37)</td>
<td>1.40 (0.33)</td>
<td>1.53 (0.30)</td>
</tr>
<tr>
<td></td>
<td>1.00 (0.63)</td>
<td>0.99 (0.50)</td>
<td>1.17 (0.53)</td>
</tr>
<tr>
<td></td>
<td>0.96 (0.55)</td>
<td>1.02 (0.57)</td>
<td>1.14 (0.56)</td>
</tr>
<tr>
<td>Glucose mmol/l</td>
<td>6.09 (0.98)</td>
<td>6.88 (1.84)</td>
<td>6.02 (0.65)</td>
</tr>
<tr>
<td></td>
<td>6.00 (0.95)</td>
<td>6.50 (1.22)</td>
<td>6.22 (0.68)</td>
</tr>
<tr>
<td></td>
<td>5.89 (1.07)</td>
<td>6.01 (0.80)</td>
<td>8.21 (0.60)</td>
</tr>
<tr>
<td>Anthropometric Data</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight kg</td>
<td>87.2 (17.0)</td>
<td>89.9 (-17.4)</td>
<td>90.3 (12.6)</td>
</tr>
<tr>
<td></td>
<td>87.6 (17.0)</td>
<td>89.9 (16.8)</td>
<td>91.2 (12.2)</td>
</tr>
<tr>
<td></td>
<td>87.9 (18.1)</td>
<td>90.9 (16.9)</td>
<td>91.7 (12.8)</td>
</tr>
<tr>
<td>BP Diastolic mmHg</td>
<td>99.2 (28.6)</td>
<td>100.3 (23.9)</td>
<td>101.9 (30.9)</td>
</tr>
<tr>
<td></td>
<td>98.5 (26.8)</td>
<td>96.9 (24.6)</td>
<td>100.0 (31.6)</td>
</tr>
<tr>
<td></td>
<td>94.5 (23.9)</td>
<td>96.1 (29.1)</td>
<td>98.7 (28.9)</td>
</tr>
<tr>
<td>BP Systolic mmHg</td>
<td>110.4 (23.9)</td>
<td>119.5 (32.6)</td>
<td>118.6 (26.4)</td>
</tr>
<tr>
<td></td>
<td>112.2 (25.3)</td>
<td>119.3 (25.3)</td>
<td>120.2 (29.1)</td>
</tr>
<tr>
<td></td>
<td>111.5 (27.1)</td>
<td>120.1 (24.5)</td>
<td>120.3 (30.1)</td>
</tr>
<tr>
<td>Body Fat %</td>
<td>23.8 (4.4)</td>
<td>26.1 (5.6)</td>
<td>23.7 (4.7)</td>
</tr>
<tr>
<td></td>
<td>24.5 (4.3)</td>
<td>26.0 (5.9)</td>
<td>24.6 (4.5)</td>
</tr>
<tr>
<td></td>
<td>24.6 (4.3)</td>
<td>26.3 (5.8)</td>
<td>24.5 (4.9)</td>
</tr>
</tbody>
</table>

(SD) Standard Deviation  *p<0.001 (compared to baseline value)
Figure 5.4: Plasma triacylglycerol, total cholesterol, HDL-C and LDL-C on all 3 diets at baseline, 6 and 12 weeks (SEM).
5.6.4.3 Plasma Triacylglycerol

Total plasma TAG in the control group increased by 15% at week 6 and had failed to return to baseline by week 12. There was a consistent decrease in TAG in the flax group compared to baseline (-11% by week 6 and -18% at week 12). However, these changes were not significant when compared to the fish and control diets. There was a pronounced and statistically significant reduction in TAG on fish oil (p<0.001), which was apparent by week 6 and was still evident at week 12 (Figure 5.4). Changes in TAG compared to baseline are shown in percentages in Figure 5.5.

Figure 5.5: Changes in TAG expressed as percentages compared to baseline on all three diets at weeks 6 and 12

5.6.4.4 Lipoprotein Subclasses

Plasma TAG and small dense LDL-3 showed a positive relationship (p<0.015, r=0.32) in all subjects at baseline (Figure 5.6). A negative (p=0.02, r=-0.33) correlation was measured between plasma TAG and % LDL-1 in all subjects at baseline.
No significant changes in the distribution of LDL subclasses between groups at any time point (Figure 5.7) were measured. Small, dense LDL 3 decreased within each dietary group with the fish oil group and sunflower oil group decreasing by 16% at 12 weeks. The flax group had decreased by 12% at 12 weeks. An increase in % LDL 1 was measured in the fish oil group (p<0.05), it was apparent at 6 and 12 weeks. A reduction in the preponderance of LDL 3 in the fish oil group compared to baseline levels at week 6 (p<0.01) and week 12 (p<0.01) was measured. A positive correlation at baseline between TAG and LDL-3 (r=+0.6 p<0.05) and TAG and LDL-1 + LDL-2 (r=-0.6 p<0.05) was measured in the flax group, however this relationship was not observed at any other time point. A positive correlation between change in TAG and LDL-3 (pre vs post diet) (r=+0.51 p<0.05) and a negative correlation between LDL-1 + LDL-2 (r=-0.52 p<0.05) was measured on the fish oil diet.
Figure 5.7: Percentage change in LDL subclasses from baseline at 6 & 12 weeks
*p<0.05  **p<0.01 (compared to baseline)
No significant changes were observed in HDL$_2$ or HDL$_3$ particles in the flax group when expressed as percentages (pre vs post diet). Both the control and the fish oil groups showed an increase in HDL$_2$ and decrease in HDL$_3$ particles ($p<0.05$) compared to baseline at week 12 (Figure 5.8). When subclasses were expressed in terms of cholesterol content (HDL$_2$-C and HDL$_3$-C), the changes were not significant. In the fish and control diets HDL$_2$-C tended to increase and HDL$_3$-C decrease, whereas the flax diet an opposite change was observed.

![Figure 5.8: HDL subclasses at baseline and 12 weeks](image)

* $p<0.05$ (compared to respective baseline value)

5.6.4.5 Anthropometric findings

Anthropometric measurements are shown in Table 5.6. There were no significant differences between any dietary group at baseline or any significant changes in weight, blood pressure or percentage body fat between groups at weeks 6 and 12.
5.6.4.6  Haemostatic variables

The concentration of plasma/serum Factor VII, fibrinogen and PAI-1 were not significantly different between intervention groups at baseline, nor were any significant changes measured between or within diets at 6 and 12 weeks (Figure 5.9).

5.6.5  Effects of erythrocyte DHA on plasma TAG and TC:HDL ratio

A positive correlation (p<0.001, r=0.69) was measured in the fish oil group between the change in plasma TAG (pre vs post diet) and the abundance of DHA in erythrocyte membrane phospholipids (Figure 5.10).

5.6.6  Effects of dietary intakes on vascular function (Endothelial Dysfunction) by flow mediated dilatory (FMD) ultrasound

Flow-mediated dilation was measured before and after dietary intervention in a small subset of subjects (Figure 5.11). Cuff-induced reactive hyperaemia (NO-dependent) and GTN (endothelium independent) produced mean increases of 2% and 5% in arterial diameter respectively in the fish group and decreases of 4% and 3% in the flax group. There were no significant changes on any of the three diets (pre versus post).
Figure 5.9: Haemostatic results at baseline, week 6 and week 12 for the flax, sunflower and fish oil diets

**Factor %VIIc (SD)**

**PAI-1 µg/l (SD)**

**Fibrinogen mg/l (SD)**
Figure 5.10: Absolute change in plasma TAG in the fish oil group (pre-post diet) versus abundance of DHA in erythrocyte membrane phospholipids $r = 0.69$ ($r^2 = 0.48$) $p<0.001$
Figure 5.11: Vascular endothelial function measured by flow-mediated dilation at baseline and 12 weeks.

Endothelium Dependent Vasodilatation (-/+ cuff inflation)

Endothelium Independent Vasodilatation (-/+ GTN)

101
When examining all the baseline data, irrespective of diet allocation, there was a significant correlation ($p<0.001$, $r=0.53$) between the TC:HDL-C ratio and the abundance of DHA in erythrocyte membrane phospholipids. The percentage abundance of DHA explained 28% of the variation in the TC:HDL-C ratio. (Figure 5.12).

Figure 5.12: Relationship in all subjects at baseline between the abundance of DHA in erythrocyte membrane phospholipids and the TC:HDL-C ratio $r=0.53$ ($r^2=0.28$) $p<0.001$
5.7 Discussion

The aim of this study was to examine whether increasing the dietary intake of ALA and reducing the n6:n3 ratio would promote fish oil like changes in cardiovascular risk factors and modify features of the ALP.

While potentially favourable changes were observed on all three test diets with respect to CHD risk, these were most notable on the fish oil diet. The study provided valuable practical information into means of increasing and delivering ALA in the diet, against a background of low n-6 PUFA intake.

5.7.1 Habitual and study dietary intakes.

The habitual intakes of macronutrients in all three dietary groups was average for the UK population. The dietary ratio of n6 PUFA to n3 PUFA was lower at 9:1 compared to the UK average of 10:1 (Gregory 1990). Intakes of energy, protein, carbohydrate, alcohol and saturated fats were not significantly different on the study diets. Increases in energy from fat, and decreases in energy from carbohydrates and saturates occurred within all three diets; therefore it seems reasonable to speculate that the effects on endpoint measures, if any, would be similar for each group. Consequently, any changes in the variables measured may be more closely associated with MUFA, n-3 and n-6 PUFA intakes.

Energy derived from fat increased on all three diets, but only significantly within the sunflower oil diet (pre vs post). Some of the favourable effects on lipids and lipoproteins, usually attributed to reductions in total fat and/or SFA may therefore be attributed to changes in the type rather than the absolute amount of fat. The intake of carbohydrate, protein and saturates were not significantly different between the study diets. This is important, as increasing energy from carbohydrate may be associated with the phenomenon of carbohydrate-induced
hypertriglyceridaemia (Harris 1984, Abbasi et al 2000). The cholesterol lowering properties of PUFA or MUFA (Mutanen 1997, Hodson et al 2001) when replacing SFA should also be considered.

SFA intakes on the intervention diets were not significantly different between groups at baseline, the decrease within groups was significant in both fish and sunflower diets when compared to baseline. These reductions may have contributed to the decrease in TC observed on these diets (Gylling & Miettinen 2001, Hodson et al 2001). This is difficult to substantiate as all 3 diets resulted in decreases in cholesterol, with fish oil diet being the least affected.

The flax oil diet had a significantly higher intake of MUFA (p<0.001) than the fish and sunflower diets, due primarily to the higher MUFA content of flax oil (31%), compared to sunflower oil (20%). A higher MUFA content supplied to the flax oil group diet from the margarine (additional 5g per 100g) would have made an additional contribution to this. The increase in MUFA would have been difficult to compensate for without adjusting the fatty acid content of the fish and sunflower oil diets and thus potentially increasing further the energy from fat in these groups. Historically, it was thought that MUFA was neutral in terms of its effects on lipids (Keys et al 1957). However, more recently similar effects on serum cholesterol to those achieved with PUFA have been reported (Ginsberg 1990). Berry et al (1991) reported a 10% decrease in TC after 12 weeks on a high MUFA diet; a significant reduction in fasting TAG was also reported. Wardlaw & Snook (1990) noted a similar hypotriacylglycerolaemic effect when SFA was replaced with MUFA or n-6 PUFA.

Intakes of n-6 PUFA on the sunflower and fish oil diets changed significantly with two and three-fold increases respectively. This makes it difficult to establish whether the decrease in TC and LDL-C were due to changes in SFA intake or n-6 PUFA intake. Increases in energy from n-6 PUFA were similar in the sunflower group when compared to the fish group, and saturates fell similarly. The greatest
decrease in TC was in the flax group, which did not alter its intake of n-6 PUFA relative to baseline, though the latter was significantly lower than the fish and control groups during the dietary phases (p<0.001). This might suggest that the hypocholesterolaemic effect of ALA is independent of n-6 PUFA. This supports other evidence that dietary ALA can reduce serum lipids by the same extent as LA (Chan et al 1991, Harris 1997).

The overall intake of n-3 PUFA in the flax group, predominantly from ALA, was significantly higher than both the fish and sunflower diets. Other studies have reported no change in total serum cholesterol on enriched ALA diets (Kelly et al 1993, Layne et al 1996), mild reductions (Abbey et al 1990, Freese and Mutanen 1997, Chan et al 1991, Cunnane et al 1995) or significant decreases (Bierenbaum et al 1993, Valsta et al 1995). This variety of responses may be explained in part by studies using different sources of ALA such as rapeseed oil, flaxseed oil and ground flaxseeds. The quantities consumed and length of intervention should also be considered. The use of whole flaxseeds as a means of delivering ALA was investigated on 15 volunteers with mild hyperlipidaemia by Bierenbaum et al (1993). Over 3 months, intakes of 4.6g of ALA per day were achieved through 3 slices of flaxseed containing bread and 15g of ground flaxseed. Significant reductions in TC and LDL-C were seen, with a non-significant reduction in TAG. The study demonstrates the potential hypocholesterolaemic effects of flaxseed, but is confounded by the contribution of 15.5g of fibre per day from the seed component. The use of flaxseed oil, as opposed to flax seeds avoids the potentially confounding hypocholesterolaemic effect of fibre (Knopp et al 1999). This was the main reason why flaxseed oil was used in the present study over the use of seeds.

In a number of earlier studies investigating the effects of ALA on lipids, baseline values of TC, LDL-C and TAG were within healthy ranges. This raises the possibility that rendering further reductions by dietary manipulation in such individuals might be difficult. The use of flaxseed within the current investigation
was aimed specifically at reducing risk factors in those individuals expressing an ALP, a condition associated with moderately raised TAG and TC. In the present study TC was greater at baseline than those reported by Layne et al (1996), Nydahl et al (1994), Valsta et al (1996) and Mantzioris et al (1994) and values for TAG at baseline greater than those of Abbey et al (1990) and Bierenbaum et al (1993). In an almost identical study design to Layne et al (1996) Goh et al (1997) provided ALA (35mg/kg per day) against a high (0.65) P/S ratio and a low (0.44) P/S ratio, but in non-insulin dependant diabetics. While in the present study, subjects were not diabetic, fasting TAG and TC levels in the Goh et al (1997) study were similar to our own at ≈2.2mmol/l and ≈5.8mmol/l respectively. The study failed to show any significant effects on TAG, TC or LDL-C. An increase in EPA and a significant increase in ALA in lipoprotein cholesterol ester fractions was seen. A fish oil supplemented diet was run in parallel with the high ALA diet (35mg/kg EPA+DHA per day). Significant reductions in fasting TAG and increases in EPA and DHA in lipoprotein cholesterol ester fractions were seen, though total cholesterol and LDL-C were not affected.

5.7.2 Influence of dietary n-3 PUFA on biomarkers of CHD.

While intakes of n-3 PUFA were not separated into ALA, EPA and DHA, sources of dietary EPA and DHA were negligible due to a total restriction on fish and fish oil products on all diets, the only source of EPA and DHA was from the fish oil capsules. In the flax diet, the average intake was 19g of n-3 PUFA, of which flaxseed oil contributed 17.2g of ALA. The remaining ALA was from rapeseed oil and the margarine. The control diet was used to determine the contribution of ALA from the margarine and rapeseed oil. As the margarine was identical to that used by the fish oil group, and a known dose of 3g EPA + DHA was supplied, the contribution of ALA within the fish oil group was not significantly different to that of the sunflower group (1.7g and 1.4g per day respectively). The increases in n-3 PUFA in the flaxseed oil diet and the fish oil diet, when compared to the control diet were from different n-3 fatty acids. Since intakes of n-6 PUFA did not differ
significantly from baseline in the flax group, this might suggest that the effect of diet may have been attributable to changes in MUFA or ALA intake.

Intakes of n-6 PUFA on the sunflower and fish oil diets during the intervention were similar; but significantly greater when compared to their respective habitual intakes. Intakes of ALA were similar between the fish and sunflower oil diets. However, n-3 PUFA intake on the fish oil diet was higher due to additional EPA and DHA. The hypotriacylglycerolaemic effects of the fish oil diet, when compared to control, are therefore likely to be attributed to the increase in EPA and DHA rather than the total n-3 PUFA intake.

The results of the present study suggested that ALA was slightly more effective at lowering TC and LDL-C than LA. It also suggests that when SFA are substituted with LA additional EPA and DHA from fish oil may have opposing actions on LDL-C blunting the hypocholesterolaemic effect of LA.

5.7.3 Effects of dietary intake on erythrocyte membrane fatty acid composition

The effective incorporation of ALA into erythrocytes on the flax diet was rapid and statistically significant at 6 weeks, doubling the relative abundance of ALA within the cell membrane phospholipids. This provided good evidence for compliance and absorption of ALA from the diet. Equally the fish oil diet was associated with increases of 124% and 60% in EPA and DHA respectively. There were no changes in the relative abundance of LA within any of the diets, nor were there any statistical changes in relative abundance of any fatty acids on the control (high sunflower) diet. The increases in EPA and DHA in erythrocytes observed on the fish oil diet, were largely at the expense of palmitic (16:0) and oleic (18:1n-9) acids. A similar decrease in palmitic and oleic acid was observed on the flax oil diet, being replaced by ALA as well as EPA and DPA. It is interesting that despite a significant increase in MUFA intake on the flax diet, erythrocyte oleic acid content fell significantly. Williams et al (1999) found a significant increase in MUFA in plasma phospholipid fatty acids following supplementation. This may
suggest that compliance with MUFA enriched diets cannot necessarily be determined by erythrocyte fatty acid content alone, not at least in the presence of increased n-3 fats, which seem to be preferentially incorporated into erythrocyte membrane fatty acids.

There was convincing evidence for the conversion of ALA to EPA and DPA on the flax diet. Intake of EPA on the fish oil diet would have been 1.7g per day compared to zero on the flaxseed oil diet. Despite this difference in intake, the increase in the erythrocyte membrane fatty acid EPA on the flax diet was greater than that of the fish oil diet: 153% versus 124% at week 12 respectively. Significant increases in DPA were also seen in both the fish and flax oil diets, despite no DPA being present in the flax diet and only 22mg per day in the fish oil. While this would suggest further elongation of EPA in the flax group from 20:5n-3 to 22:5n-3, it is difficult to determine the direction in which DPA was formed in the fish oil diet. A 60% increase in DHA was observed on the fish oil diet only. Other studies have shown increases in EPA when only preformed DHA is fed (Nelson et al 1997b, Mori et al 2000, Grimsgaard et al 1997), this may be caused by EPA “backing up” or accumulating conversion to DHA is not necessary. When EPA was fed to volunteers (4g/d) significant increases in EPA were measured in plasma phospholipids but no increase in DHA was detected (Mori et al 1999). Although conversion of EPA to DHA occurs, it would seem that conversion towards shorter chain rather than longer chain fatty acids can also take place.

5.7.4 Differential effects of dietary EPA and DHA on plasma lipids and lipoproteins

Studies examining the separate effects of DHA and EPA are becoming more common. In the present study, the fish oil group consumed both EPA and DHA. Mori et al (2000) found that both EPA and DHA reduced TAG, but that DHA was more effective though not significantly. Grimsgaard et al (1997) reported similar findings. In the present study, TAG fell in both the fish and flax oil diets, but the
effect (pre-post diet) was greater, and only significant in the fish oil diet. Interestingly, the positive association between the abundance of DHA in erythrocytes and the TC:HDL-C ratio would suggest that greater levels of DHA in erythrocyte membrane phospholipids may be associated with increased risk of CHD. One explanation for this finding may be that DHA is the fatty acid responsible for increases in LDL-C that have been reported during fish oil feeding. Some of the hypercholesterolaemic effects of long chain n-3 PUFA may be masked by the high n-6 PUFA background diet. However, if such a relationship existed between DHA and TC (LDL-C) it may still be detectable. In accord with Mori et al (2000) both diets were associated with enrichment in long chain n-3 fats, but only DHA was increased in the fish oil diet. In the present study no correlation between EPA and TAG was found on the flax oil diet. However, in the fish oil group a significant correlation was found between the change in TAG (pre-post diet) and the abundance of DHA at 12 weeks (Figure 5.12). No significant correlations were found on the flax oil diet between the erythrocyte fatty acid content of ALA or EPA and change in TAG. This may suggest that the TAG lowering effect of the fish oil diet was a result of DHA rather than EPA. The inefficiency of the conversion of ALA to DHA and the enrichment of red blood cell membranes with EPA but not DHA on the flaxseed-oil diet is consistent with the idea that the level of DHA in cell membranes and lipoproteins is a more important determinant of fish-oil-induced changes in blood lipids and lipoproteins than EPA.

5.7.5 Dietary effects on lipids, lipoproteins and lipoprotein heterogeneity.

As discussed in Chapter 1, the TAG lowering effects of long chain n-3 PUFA are well documented. In the present study, the decrease in the concentration of plasma TAG on the fish oil diet (-34%) was in the order of magnitude observed in other studies supplementing with long chain n-3 PUFA (Contacos 1993, Mori et al 2000, Leigh-Firbank et al 2001). A mean absolute reduction in plasma TAG of
0.6mmol/l was seen in the present study after 6g per day of EPA and DHA supplementation. Hokanson and Austin (1996) found that a 1mmol/l reduction in fasting TAG was associated with a 32% reduction in CHD risk in men. Consequently, reductions in TAG measured in the fish oil group in the present study should be considered clinically significant. It is unlikely, however, that the magnitude of plasma TAG reduction achieved with fish oil capsules could be achieved through dietary means only, the large quantities of oily fish that would have to be consumed would make it impractical. While significant reductions in plasma TAG were measured in the fish oil group, the diet did not reduce plasma TAG below 1.5mmol/l. The relevance of the 1.5mmol threshold in modifying the distribution of LDL subclasses was discussed in Chapter 1, but may offer some insight into why the fish oil diet, even with its potent TAG lowering effects, was unable to modify the ALP of this group (Griffin et al. 1994).

The effects on TC and LDL-C following fish oil supplementation are still equivocal, variable responses to fish oil feeding have been measured in different lipid phenotypes. In the current study, fish oil supplementation was associated with a reduction (NS) in TC and LDL-C. The subjects in this group were not hypercholesterolaemic and although Nestel (1990) suggested that in normolipidaemic subjects changes in TC tended to be small and often of no clinical importance, it is questionable whether in the present study, the 7% reduction in TC following fish oil supplementation should be labelled “clinically unimportant”; as a 10% reduction in TC is associated with a 15% reduction in CHD mortality (Gould et al. 1998). Phillipson et al. (1985) showed that the effects of fish oils on lowering TC were more pronounced in subjects with raised TAG primarily though a reduction in VLDL-C and IDL-C. None of the dietary groups in the present study were hypertriacylglycerolaemic. While reductions in TC in response to fish oils have been reported, increases in LDL-C, possibly through increased conversion of VLDL (Huff & Telford 1989) or competition between VLDL and LDL for the LDL receptor (Gianturco & Bradley 1991) have also occurred. The present study not only observed reductions in TC but also LDL-C
after fish oil supplementation. Shifts in the distribution of LDL subclasses from LDL-3 towards the more receptor active LDL-2 particle in the present study may explain some of the reductions observed following fish oil supplementation.

In the present study, consuming 34mls of flaxseed oil per day resulted in a 21% decrease in plasma TAG by week 12, an effect not observed consuming an identical amount of sunflower oil. The effects of linoleic acid on fasting TAG have been reported as being inconsistent (Grundy & Denke 1990), high LA intakes may reduce hepatic synthesis of VLDL TAG, or be a better substrate for LPL. In the present study the absolute change in plasma TAG on the ALA diet was – 0.4mmol/l, this was not statistically significant but may be clinically relevant with respect to CHD risk (Hokanson and Austin 1996). In comparison, both flax and sunflower oil diets were associated with reductions in TC and LDL-C with the ALA diet being more effective. The effect on TC of increasing ALA in the diet was of a similar magnitude to adding 25g of soy (Setchell & Cassidy 1999) or 2-3g of plant stanols to the diet per day (Gylling & Miettinen 1999). The ALA diet, like the fish oil diet, failed to modify the ALP but did produce changes in the concentration of plasma lipids. Overall the study was underpowered to be able to demonstrate significant changes. The power value for the sample size used was 0.38, and 0.41 for TAG and TC respectively.

Enrichment of erythrocyte membrane fatty acids with DHA correlated with changes in plasma TAG in the fish oil group, no such correlation could be determined with any fatty acid in the flax group. It is difficult to determine the reason behind the reduction in fasting TAG in the flax group. Valsta and co-workers (1995) fed 5.9g of ALA per day as rapeseed oil and observed a 19% decrease in plasma TAG. Freese and co-workers (1997) fed the same amount but observed no changes in plasma TAG. Length of intervention did not differ markedly at 6 weeks and 4 weeks respectively. Our findings are similar to those of other studies that have shown no significant effect on fasting plasma TAG.

Prospective trials (Stamfer et al 1996) have shown that small, dense LDL is associated with an increased risk of MI, while case control studies (Austin et al 1988) have shown small dense LDL to be predictive of future coronary events. A reduction in circulating levels of small, dense LDL may be achieved through a concomitant reduction in TAG (Griffin et al 1992, Gaw et al 1994). Of particular relevance is the 1.5mmol/l threshold for TAG, beyond which the preponderance of small dense LDL (LDL-3) increases (Griffin et al 1994).

The TAG lowering efficacy of n-3 fatty acids may not be as effective as drugs, but is associated with a reduction in cardiovascular risk through a variety of mechanisms (Nestel 1990, Harris 1989). The fish oil diet on the present study was associated with significant reductions in TAG (pre versus post) and a reduction in the number of LDL-3 particles. Reductions in LDL-3 are a result of shifts towards larger, less dense LDL particles (Griffin 2001). This is of importance in the present study as all volunteers expressed an ALP, and thus had >40% LDL-3 at baseline. While decreases in LDL-3 were measured on all three diets, only the decrease on the fish oil diet was significant. None of the diets was able to reduce TAG below 1.5mmol/l which may account for the preponderance of LDL-3 remaining above 40% (pattern B phenotype) (Griffin et al 1994). The greatest reduction in LDL-3 occurred in the fish oil group, which also exhibited the greatest reduction in TAG, this relationship was not present in the flax and sunflower oil diets. In the flax group an 18% reduction in TAG was associated with a 12% reduction in LDL-3, in the sunflower oil diet, a 4% increase in TAG was associated with a 16% reduction in LDL-3.

Three desirable effects were observed in the fish oil group: a decrease in TAG, a reduction in TC and LDL-C and a significant reduction in LDL-3. The cholesterol response to fish oil feeding suggests an overriding effect of dietary LA in the
background diet, essentially superimposing a high long chain n-3 PUFA diet onto a high n-6 PUFA diet. Linoleic acid is still commonly recommended in the dietary management of hypercholesterolemia (Ascherio et al. 1996) and in the current study was one of the main replacements of SFA on the test diets.

5.8 Effects of diet on haemostatic factors.

There were no significant effects observed in PAI-1, Factor Vllc% or fibrinogen in the present study. While evidence exists that suggests both positive and negative effects of diet on haemostatic variables, it should be noted that all three dietary groups presented with values within the normal reference ranges for each of the variables measured. The anti thrombotic effects of fish oils are mediated in part through decreased platelet aggregation and reduction in plasma fibrinogen (Hostmark et al. 1998, Mutanen et al. 2001). Fibrinogen levels generally increase with age, smoking and LDL-C, but are decreased by physical activity, alcohol intake and HDL-cholesterol levels (Scarabin P et al. 1998). The current study showed no changes in fibrinogen as a result of diet. Allman-Farinelli et al (1999) investigated differing ratios of ALA:LA on coagulation and fibrinolysis. A ratio of 1:1.1 produced a three-fold increase of EPA in platelets, but no effects on haemostasis. In the present study a 2.5 fold increase in EPA in erythrocyte membrane phospholipids was seen. The mean ALA intake was 3.5g per day (Allman-Farinelli et al. 1999) as opposed to 18g per day on the present study. Fatty acid composition of platelets was not measured on the present study so a direct comparison cannot be made.

Olle et al (1998) reported significant increases in PAI-1 after fish oil feeding, an effect considered undesirable, but in agreement with Mahrabian et al (1990) and Boberg et al (1992) who postulate that impaired fibrinolysis is mediated through an increase in PAI-1 levels. While there is evidence that fish oils may increase PAI-1 levels, Mehta et al (1998) reported a significant correlation between decreases in TAG and decreases in PAI-1. Higher circulating levels of PAI-1
have also been shown to be associated with body mass index (Mehta et al 1987), but unlike Factor VIIc, are also elevated by alcohol intake and smoking (Scarabin et al 1998). While all volunteers on the present study were non-smokers, it is worth noting that the energy derived from alcohol in the fish oil group fell significantly during the dietary intervention (p<0.01) it therefore seems reasonable to speculate that any increases in PAI-1 levels in response to fish oil feeding in present study may have been counter-acted upon by the hypotriacylglycerolaemic effects of the diet and decreases in alcohol consumption.

Leigh-Firbank et al (2002) showed a significant correlation between reduction in fasting TAG and the increase of EPA in platelets after feeding similar amounts of EPA and DHA as the present study. While there were similarities in intakes of fish oils, the present study diet was run against a background of increased n-6 PUFA and their study was not. Leigh-Firbank et al (2002) also determined enrichment in platelets, whereas the present study used erythrocytes. This aside, the present study supports much of the literature with regard to responses of TAG to DHA but not EPA. Nelson et al (1997b) supplemented volunteer’s diets with DHA only, (6g/d) and found no significant changes in haemostatic variables. Interestingly a 7-fold increase in platelet EPA was observed, despite EPA being absent from the diet.

Physical activity is associated with a reduction in PAI-1 levels (Mehta et al 1988, Scarabin et al 1998). In the case of the current intervention, no record of physical activity was kept. The effects of dietary fats on haemostasis remain equivocal, it should be considered that some of the beneficial effects observed may be a result of these fats on other variables such as TAG and lipoproteins. Other factors such as physical activity and alcohol intake, which are difficult to control in free-living studies, may also be affecting the results.
The Food Standards Agency stated in 2000 that: "to contribute to the improvement of the health of the UK population it is important to encourage and facilitate the adoption by the population of a healthy balanced diet". Poor diet is a risk factor for a number of diseases, and in particular cardiovascular disease and some cancers. The incidence of these diseases is highest among socially and economically deprived groups. Because diet is only one of a number of risk factors for these diseases it is not possible to establish outcome measures in terms of reduced incidence of morbidity or mortality. However, if all other risk factors remain unchanged we would expect improvements in diets to be reflected over the long term in reduced rates of cardiovascular disease and cancer. In the Government's white paper Saving Lives: Our Healthier Nation, clear targets have been set to reduce deaths from CHD by 40% by the year 2010 (DOH, 1999).

The main aims of the dietary interventions conducted in the pilot, dietary compliance and principal studies (Chapters 3 4 and 5) were to test the hypothesis that short chain n-3 PUFA could promote improvements in cardiovascular risk factors similar to that produced by longer chain n-3 PUFA in fish oils (Connor et al 1997, GISSI 1999). The fatty acid alpha-linolenic acid (ALA) can be converted into longer chain n-3 PUFA in vivo (Gerst, 1998), and thereby, potentially exert the same effects as fish oils on CHD risk. The experimental diets in both the pilot and principal studies achieved a dietary n-6:n-3 ratios <1.0, the purpose of the former was to look more at the practical feasibility of supplementing ALA in a free living population, rather than the effects of the diet on CHD risk. Most notably the principal study differed from the pilot in subject numbers, duration and volunteers who were expressing a dyslipidaemia or ALP. This group was chosen as the most appropriate target population for testing the effects of dietary ALA for the following reasons: it is at increased risk of CHD (Austin et al 1990), has a relatively high prevalence in the general
population and expresses coronary risk factors that are known to be responsive
to fish-oil feeding (Minihane et al 2000).

In the principal study, 12 weeks of a diet enriched with ALA did not reproduce the
characteristic effects of fish-oils on blood lipids, haemostatic risk factors or tests
of vascular function reported in previous studies (Phillipson et al 1985, Schmidt
et al 1990, Mori et al 1999). Moreover, even the effects produced by the fish-oil
diet, the purpose of which was to serve as 'positive control' were inconsistent
with that previously shown in this group (Minihane et al, 2000). In explanation for
these findings, it is conceivable that the usually pronounced effects of fish-oils on
an ALP and haemostatic variables were in some way obscured by combining the
fish-oil supplement with a high intake of n-6 PUFA. Alternatively, any lack of
dietary response could be ascribed to the relative normality of the study
volunteers with respect to their baseline characteristics as compared to a more
clinically defined group i.e. that expressing values outside the physiological
reference range. It remains difficult to ascertain whether the reductions in total
cholesterol observed on the fish oil diet were in response to increases in n-3 and
n-6 PUFA and/or through the more 'permissive' effects produced by the
replacement of SFA in the habitual diet. The fact that plasma TAG was not
reduced below the value of 1.5mmol in any dietary group may explain, in part,
why there was the lack of response in the ALP, or more specifically, a decrease
in the abundance of small, dense LDL-3 below 40%, since this level of plasma
TAG represents a critical threshold for changes in these variables (Griffin et al
1994, Griffin 2001). It is now well known that genotypic variation influences the
response to dietary components. This is particularly relevant with respect to the
variable effects of dietary n-3 PUFA on lipid metabolism. Several common
polymorphisms have been implicated as modifiers of the response to dietary long
chain n-3 PUFA, the most well known of which is the apo E polymorphism
(Minihane et al, 2000, Khan et al, 2002). However, whilst data on the impact of
this polymorphism on the response to the shorter chain ALA is limited,
unfortunately the address of nutrient-gene interactions lay beyond the scope of
the present study.

While all study volunteers selected for the principal intervention were positive on
screening for the expression of an ALP, examination of their habitual diet
revealed a ratio for dietary n-6:n-3 PUFA that was marginally lower than the
national average. This posed the question as to how much more “modifiable” the
CHD risk factors in these subjects would be through further dietary manipulation?
All study volunteers were recruited from a population in and around the Guildford
area. Surrey is well known to be one of the more affluent areas in the south of
England, and as such is associated with a lower incidence of CHD (Coronary
Heart Disease National Service Framework, 2000). Based on the FSA National
Diet and Nutrition Survey (2001) the habitual diet of all the subjects was slightly
higher than the UK average in energy derived from total fat, saturated fat and
monounsaturated fat and lower in n-3 and n-6 PUFA. This might suggest that
there was a degree of selection bias in the recruitment with respect to diet, that
is, that the trial selected individuals whose diets were higher in fat and lower in n-3
PUFA than the UK mean pre-disposing this group to increased CHD risk. Mean
body mass index (BMI) for all subjects was 28.5 kg/m² (data not shown); values
above 25 kg/m², are considered to be overweight. Total and LDL-cholesterol are
positively associated with increases in BMI, whereas HDL-Cholesterol is
inversely associated (Schroder et al 2003).

The superimposition of a high intake of n-6 PUFA on the fish oil supplemented
diet may well have confounded the inclusion of fish-oil diet as a positive control
i.e. a diet that would produce marked and predictable changes in the outcome
measures of risk. In contrast, the ALA enriched diet was not enriched with n-6
PUFA in order to achieve a n-6:n-3 ratio <1.0. In retrospect, a fourth diet,
supplemented with fish oil but with low in n-6 PUFA may have provided a more
satisfactory comparison of short versus long chain n-3 PUFA without the
additional influence of n-6 PUFA, the effects of which were clearly in evidence
from its cholesterol-lowering action. Alternatively, the high n-6 PUFA could have been removed from the fish oil diet altogether to leave just three diets as before.

The single greatest confounding factor in all human dietary intervention studies is dietary compliance. This is extremely difficult to assess in any reliable way in a free-living study group. Although the dietary questionnaires gave some insight into this area, the number returned was low. Food diaries provided information on both habitual and study dietary intakes, but the quality of information recorded varied greatly between subjects. There was also an interval of 10 weeks when no information was documented, and it is conceivable that other foods containing n-6 PUFA or EPA and DHA could have been consumed during this period. In addition to this, the nutritional data currently available on the fatty acid contents of foods in the UK is still limited. This would tend to undermine the accuracy of the dietary analysis for intakes of ALA, LA, EPA and DHA and also affect the reliability of the calculated ratios of dietary n-6 to n-3 PUFA.

The manner in which most subjects took the oil should also be examined, as effectively it became two boluses of 17mls of oil twice a day. While subjects were encouraged to use the oil by adding it to food after cooking, a majority took it in a milkshake. This could be considered more of a supplement or even pharmacological in nature and it does not represent the manner in which ALA would be consumed if introduced into foods. In this case, intakes would range over the day depending on the type of products available. Future studies may wish to add flax to foods that can be consumed cold such as pasta salads or desserts, not only to spreads and oils. In this way potentially beneficial intakes of ALA could be achieved over the course of the day and integrated as part of the food matrix rather than as one or two doses.

The overall implications of these studies in terms of the modification of cardiovascular risk in the general population are difficult to interpret. Decreases in plasma cholesterol and TAG were seen in the flax oil group, and though
insufficient to reverse the expression of an ALP, may still be linked, by extrapolation with CHD mortality data, to decreased CHD risk (Hokanson & Austin 1996, Gould et al 1998). While some studies have suggested that ALA (n-3 PUFA) is as effective as LA (n-6 PUFA) in reducing CHD risk (Chan et al 1991), the LA test diet was associated with no changes in TAG. The potentially favourable but non-significant changes in blood lipids in the flax group might indicate that a greater number of subjects would have produced more statistically significant results (Type II error). As changes in TAG are relatively large when using fish oils at the doses used in the present study, it is reasonable to assume the study was sufficiently powered with regard to the fish oil supplemented group. At the time of the study design there was limited information on the effect of ALA on TAG. Harris (1997) stated that as little as 3g of fish oils may reduce serum TAG by up to 30%. It was postulated that should dietary ALA be as potent at reducing TAG as fish oils, a similar reduction in TAG may be observed. A reduction in TAG was seen on the ALA supplemented group, and the retrospective power calculation suggests that this reduction may have been significant but that a greater number of subjects would have be required to determine if this was the case (figure 6.1). In order achieve a power of 0.8 and detect a 25% decrease in TAG or TC between dietary groups using ANOVA, a sample size of 50 per group (n=150) would have been required (Figure 6.1)

Figure 6.1: Power values to detect a medium size difference in TAG, between the three treatment groups.
Diet forms the cornerstone in all primary preventative strategies for reducing CHD mortality and morbidity, particularly in those who are at high and intermediate risk (~20% absolute risk). Many of these people will be asymptomatic for CHD but harbour sub-clinical atherosclerosis that is undetectable at a population level (>20% of the UK population). The principal intervention study aimed to identify and intervene in a group of free-living volunteers that could be classified within this vulnerable group, that is, men expressing silent, sub-clinical risk factors for CHD that are amenable to dietary modification. The findings of the present study indicate that ALA is not as effective as EPA and DHA in reducing lipid-mediated risk in this particular experimental model. Nevertheless, it was equally efficacious in lowering total and LDL cholesterol as the high LA / n-6 PUFA diet. While the study was inconclusive with respect to the effects of dietary ALA on markers of haemostasis and vascular function, it was significantly underpowered to examine the latter.

Supplementation with fish oils, especially DHA, may help prevent arrhythmias and sudden cardiac death in healthy men (Hagstrup et al 1999). The evidence that ALA promotes enrichment of cell membranes with EPA may suggest greater attention to dietary ALA and markers of inflammation and cardiac arrhythmia. (Segal-Isaacson & Wylie-Rosett 1999). As with the hypotriacylglycerolaemic effects of fish oils, more research is needed to determine the combined and independent effects of EPA and DHA on cardiac arrhythmias.

Increased knowledge of the effects of dietary fatty acids on metabolic risk factors for CHD in vulnerable groups underlies the formulation of dietary guidelines for the reduction of CHD risk in the population at large. However, the practical implementation of dietary change based on this knowledge remains the greatest challenge of all. The use of functional foods is becoming more widespread and may offer a means of producing subtle changes in the food chain required to deliver potentially beneficial nutrients to the population as a whole. Unfortunately
food policy and what is actually achieved in practice is driven largely by business and not science. Hence, the successful implementation of any scientific outcome will not only depend on communication and co-operation with government but also with profiteering food manufactures and retailers.
REFERENCES

Km Reaven GM. (2000) High carbohydrate diets, triglyceride-rich lipoproteins,
and coronary heart disease risk. American Journal of Cardiology 85(1) 45-48

Abbey M, Belling GB, Noakes M, Hirata F and Nestel PJ (1993) Oxidation of low
density lipoproteins: intra-individual variability and the effect of dietary linoleate
supplementation. American Journal of Clinical Nutrition 57 391-398

lipoproteins, lecithin:cholesterol acyltransferase, and lipid transfer protein activity

Adam O, Wolfram G, Zollner. (1986) Effect of a-linolenic acid in the human diet in

sunflowerseed oil in healthy young men consuming a low fat diet: effects on
178.

Comparison of the effects of two low fat diets with different alpha-linolenic:linoleic
acid ratios on coagulation and fibrinolysis. Atherosclerosis 142, 159-168

American Heart Association dietary guidelines. Revision 2000: a statement for
healthcare professionals from the Nutrition Committee of the American Heart


Ball KP, Hannington E, McAllen PM (1965) Low fat diet in myocardial infarction: a controlled trial *Lancet* **2:**501-504


Food Standards Agency's Nutrition Remit: PAPER FSA 00/03/06 (JULY 2000)

128


Gerst H (1998) Can adults adequately convert alpha-linolenic acid (18:3n-3) to Eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3)? International Journal of Vitamin and Nutritional Research 68, 159-73.


Goldstein JL, Ho YK, Basu SK, Brown MS. (1979) Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. Proc Natl Acad Sci USA 76:333-7


Griffin BA (2001) The effect of n-3 fatty acids on low density lipoprotein subfractions *Lipids* 36 S91-S97


Harris WS (1989) Fish oils and plasma lipid and lipoprotein metabolism in humans, a critical review *Journal of Lipid Research* 30, 785-807


Institute of Biology (2003) The Environmental Effects of Marine Fisheries: A Response to the Royal Commission on Environmental Pollution from the Institute of Biology in association with the Scottish Association for Marine Science and the Nutrition Society, on behalf of the Biosciences Federation.


Khan S, Minihane AM, Talmud PJ, Wright JW, Murphy MC, Williams CM & Griffin BA (2002). Dietary long chain n-3 PUFAs increase LPL gene expression in adipose tissue of subjects with an atherogenic lipoprotein phenotype. Journal of Lipid Research 43, 979-985

Kris-Etherton PM, Harris WS, Appel LJ (2002) Fish Consumption, Fish Oil, Omega-3 Fatty Acids, and Cardiovascular Disease *Circulation* 106: 2747-2757


Layne KS, Goh YK, Jumpsen JA (1996) Normal subjects consuming physiological levels of 18:3(n-3) and 20:5(n-3) from flaxseed or fish oils have characteristic differences in plasma lipids and lipoprotein fatty acid levels. *Journal of Nutrition* 126:2130-2140.


139


Parthasarathy S, Barnett J and Fong LG (1990) High density lipoprotein inhibits the oxidative modification of low density lipoprotein *Biochem Biophy Acta* 1044 275-283


Sanders TAB, Oakley FR, Miller GJ, Mitropoulos KA, Crook D, Oliver MF. (1997) Influence of n-6 versus n-3 polyunsaturated fatty acids in diets low in saturated fatty acids on plasma lipoproteins and hemostatic factors. *Arteriosclerosis, Thrombosis and Vascular Biology* 17 3449-3460


Tackling Obesity in England, National Audit Office, 2001


The Heights and Weights of Adults in Great Britain, ONS, 1980


Young SG (1990) Recent progress in understanding Apolipoprotein B Circulation, 82 1484-1490

Appendix 1

Principal Investigation
Volunteer Questionnaire
ALP OIL QUESTIONNAIRE

This questionnaire is designed to help us determine how you found participating in the study. It will help us improve compliance issues and provide us with information on ways in which to improve future dietary intervention investigations. The questionnaire is anonymous and you will not be contacted regarding your answers, so please be as honest as possible when completing them.

Questions asking you to rate something between 1 and 5 are designed so that 1 is the lowest rating and 5 is the highest.

The only information we require from you before you start the questionnaire is whether you were taking the capsules as well as the sachets of oil.

Thank you in advance for your co-operation.

1. Were you on the fish oil diet? Yes [ ] No [ ]

1a. (if yes) how easy did you find it to take the capsules every day? (1 very easy, 2 easy, 3 average, 4, hard 5, very hard) Score [ ]

2. How easy was the study, overall to complete? (1 very easy, 2 easy, 3 average, 4, hard 5, very hard) Score [ ]

3. Did you find the 1st 6 weeks easier than the 2nd? Yes [ ] No [ ]

4. How easy did you find it to use the sachets in foods? (1 very easy, 2 easy, 3 average, 4, hard 5, very hard) Score [ ]

5. How easy did you find it to use the sachets in drinks? (1 very easy, 2 easy, 3 average, 4, hard 5, very hard) Score [ ]

6. How often did you forget to take your capsules? (1 never, 2 rarely, 3 occasionally, 4, lots 5, all the time) Score [ ]

7. How often did you forget to take your sachets? (1 never, 2 rarely, 3 occasionally, 4, lots 5, all the time) Score [ ]

8. How difficult did you find it to cut out the foods indicated by the study personnel? (1 very easy, 2 easy, 3 average, 4, hard 5, very hard) Score [ ]
9. How would you rate completing the food diaries?  
(1 very easy, 2 easy, 3 average, 4, hard 5, very hard) Score []

10. How often would you enter foods into the diary the day after you had actually eaten them?  
(1 never, 2 rarely, 3 occasionally, 4, lots 5, all the time) Score []

11. How useful was continuous support from study personnel in helping you complete the study?  
(1 not useful, 2 of some use, 3 useful, 4, very useful 5, indispensable) Score []

12. Did you weigh yourself every week? Yes [ ] No [ ]

13. How easy did you find it to use the margarine provided?  
(1 very easy, 2 easy, 3 average, 4, hard 5, very hard) Score []

14. How palatable was the margarine?  
(1 not at all, 2 just, 3 palatable, 4, fairly palatable 5, delicious) Score []

15. How would you rate the sachets as a means of packaging and delivering the oil (1 to 5)? Score []

16. How often did you eat something you know you should not have eaten whilst on the study?  
(1 never, 2 less than once a month, 3 less than once a week, 4, once a week, 5 more than once a week) Score []

17. Did you make any diet or lifestyle changes between your screening blood test and the start of the study? Yes [ ] No [ ]

18. If the answer to Q.17 was yes, was that as a direct consequence of these results? Yes [ ] No [ ]

19. How instrumental was a partner or spouse in your decision to participate in this study?  
(1 N/A, 2 none, 3 some, 4, a lot 5, their idea) Score []

20. If you attended, did you find the introduction evening at the University useful in allaying any uncertainties regarding your participation in the study? Yes [ ] No [ ]
ALP OIL QUESTIONNAIRE

21. Has selection for this study prompted you to visit your G.P. for any health checks?  
   Yes [ ]  No [ ]

22. Did you find the blood taking an inconvenience?  
   Yes [ ]  No [ ]

23. Has your diet returned to normal since completing the study?  
   Yes [ ]  No [ ]

24. Did you ever experience any unpleasant side effects from the oil or sachets other than those mentioned?  
   Yes [ ]  No [ ]

25. How accurate do you think your food diary was compared to your actual diet over the week it was completed?  
   (1 <25%, 2 26-50%, 3 51-75%, 4 75-99% 5 100%)  Score [ ]

26. Overall how compliant would you say you were to all the instructions and advice given to you whilst on the study?  
   (1 not at all, 2 a little, 3 often, 4, most of the time 5, 100%)  Score [ ]

27. Overall was the study more or less difficult to complete than you thought?  
   More [ ]  Less [ ]  Same [ ]

28. Would you consider participating in another intervention study in the near future?  
   Yes [ ]  No [ ]

29. Is there any information you felt was missing in relation to the study that you would have benefited from had you been made aware at the start?  
   Yes [ ]  No [ ]

30. If you answered yes, please describe what you felt was missing below.

__________________________________________________________________________________________

__________________________________________________________________________________________

Thank you for taking time to complete this questionnaire. Please return it in the prepaid envelope supplied.
Appendix 2

GP Study Information & Sample Letters
The Importance of α-Linolenic Acid (α-LA) as a Source of Long-Chain n-3 Polyunsaturated Fatty Acids (PUFA) and its Influence on Risk Factors of Cardiovascular Disease.

The Centre for Nutrition & Food Safety at the University of Surrey & Royal Surrey County Hospital is currently seeking volunteers for a dietary intervention study. The study is designed to examine the effects of dietary n-3 polyunsaturated fatty acids (essentially fish-oils and α-linolenic acid from flaxseed oil) on cardiovascular risk factors in 60 healthy, male volunteers with moderately raised serum triglycerides (>1.5mmol/l). Please find enclosed further details of the study in the form of a Volunteer Information Sheet and Study Summary. We hope to recruit these patients through local general practices and write to enquire of your willingness to participate.

The study would involve you selecting suitable candidates from your database. You would either then supply us with their names and addresses and a letter stating your involvement in the study. Alternatively we would provide you with letters about the study to send to potential volunteers along with a covering letter from yourself. That way we have no information at all about potential volunteers until they express an interest in the study. At no time would we have access to their medical records. A small blood sample for screening purposes would then be taken from those interested. We would supply you with all biochemical data obtained from these patients, including a LDL profile on those selected. We would also keep you informed of the results of the trial, its outcome for your patients and publications arising from it. If you are interested, we would be very grateful if you could return your name and contact address on the enclosed slip in the FREEPOST envelope or alternatively contact Dr. Amelia Feredy at the University of Surrey (Tel / Fax 01483-879735 or e-mail a.feredy@surrey.ac.uk).

Many thanks in advance for your help.

Yours sincerely,

I would be interested in hearing more about the dietary intervention trial.

Doctors name:

Contact address:

Telephone number:

Best time to phone:

e-mail:

Please return in the S.A.E.
The Importance of α-Linolenic Acid (α-LA) as a Source of Long-Chain n-3 Polyunsaturated Fatty Acids (PUFA) and its Influence on Risk Factors of Cardiovascular Disease.

Summary

Dietary long chain n-3 PUFA from fish-oils have beneficial effects on cardiovascular risk factors. Unfortunately, efforts to take advantage of these effects have been hindered by resistance to the increased consumption of oily fish. The supply of the precursor of these oils, α-linolenic acid in enriched foods offers an alternative and realistic way of achieving the same benefits. The efficacy of this approach in humans remains unproven because high levels of dietary n-6 PUFA in our diet from vegetable oils prevents conversion of the α-LA to its longer chain and biologically active products. There is evidence to suggest that diets enriched with α-LA produce mild ‘fish-oil’ like effects on blood clotting by decreasing platelet aggregation, but no convincing data, as yet, of effects on serum lipids or lipoproteins. This is expected since the form of these dietary interventions and nature of the experimental subjects have been inappropriate to demonstrate such an effect.

We postulate that decreasing the ratio of dietary n-6:n-3 PUFA and increasing the source of dietary α-LA will increase the conversion of α-LA in the body and thus promote ‘fish-oil’ like changes in cardiovascular risk factors. Subjects expressing an atherogenic lipoprotein phenotype (ALP) are uniquely responsive to fish-oils and are thus the most appropriate target group for this kind of intervention. Volunteers expressing an ALP will be recruited through local GP clinics and invited to take part in a 12 week dietary intervention trial. Subjects will be randomly allocated to either a control diet, based on monounsaturated fat, a test diet with increased α-LA or a fish-oil supplemented diet (control diet + fish-oil capsules). We will replace 45 grams of fat with a fat supplement supplied in the form cooking oils, spreads and sachets of oil. Our endpoints of cardiovascular risk are based on the high risk features of an ALP, and will be assessed by examining changes in serum lipoprotein subclasses, a range of blood clotting factors and blood flow and arterial diameter by ultrasound, a non-invasive measure of endothelial dysfunction. Metabolic tracer studies using stable isotopes will be carried out in a small number of subjects to measure the conversion rate of α-LA within the body.

Bruce A. Griffin
Lecturer in Nutritional Metabolism
Dear Mr

We have been approached by Dr Bruce Griffin (Reader in Nutritional Metabolism) and Paul Wilkinson (Research Dietitian) from the School of Biological Sciences at the University of Surrey in Guildford. They are in the final year of an intervention study examining the effects of diet on cardiovascular risk factors. The project, funded by the Ministry of Agriculture, Fisheries and Foods, requires volunteers to participate in a 3-month dietary intervention planned to commence in _______

We have contacted you as you meet some of the criteria needed for selection on the project, which is aimed primarily at non-smoking, middle-aged men with moderately raised serum cholesterol.

If you think you might be interested in taking part, and would like to find out more about the project, without any obligation, please return the reply slip attached with this letter directly to Paul Wilkinson in the freepost envelope provided.

If they do not hear from you within 2 weeks of you receiving this letter, you will not be contacted again and they will assume you do not wish to participate in the study.

Yours sincerely
Appendix 3

Volunteer Information & Dietary Instructions
Volunteer Information Sheet

Study Title and Summary

The Importance of α-Linolenic Acid (α-LA) (linseed oil from flax) as a Source of Long-Chain n-3 Polyunsaturated Fatty Acids (fish oils) and its Influence on Risk Factors of Cardiovascular Disease.

You have been asked to volunteer for a dietary intervention trial run jointly through the University of Surrey and the Royal Surrey County Hospital in Guildford. The study will look at the effects of dietary fats on risk factors for coronary heart disease in 60 male volunteers. It will involve changing your diet in your own home for 12 weeks by eating a range of specially manufactured foods and oils, in place of some of the foods which you normally eat. You may also be asked to take fish-oil supplements in the form of capsules. We will supply you with all of the foods and fish-oil supplements and provide regular dietary advice and guidance throughout the trial. It is important that you do not lose or gain weight on this diet and your calorie intake will be carefully controlled to ensure this does not happen. The supplied foods & oils, which have been manufactured by well known food companies, have been made with different types of fat which when eaten will modify the fat in your blood in a way that should reduce your risk of heart disease. We will monitor the effects of these dietary fats on risk factors for heart disease by taking small blood samples at regular intervals (a total of 150 ml ~1/4 of a pint over 12 weeks), by making measurements of blood flow by ultrasound (a procedure similar to having your blood pressure taken), and by giving selected volunteers some fat in the form of an oil capsule labelled with a harmless tracer so that we can follow the way the fat is handled within the body. You will be paid for the inconvenience of taking part in the trial and for your travel expenses.

Background information on the study

Heart disease remains the greatest cause of premature death in middle aged men in the U.K. The risk of dying from a heart disease is largely determined by genetic factors (family history) but also by lifestyle factors such as diet. We know that raised levels of cholesterol in the blood increases the risk of heart disease but this is not the whole story. Cholesterol is transported in the blood circulation in different fractions some of which deposit in the walls of coronary arteries. This eventually blocks the flow of blood to the heart which causes an often fatal heart attack. In contrast, other cholesterol fractions actively remove cholesterol from artery walls and prevent heart attacks. Fish oils are very effective in modifying the balance of these different cholesterol fractions in the blood in favour of the 'good' cholesterol. They also prevent blood from clotting in 'clogged' arteries and protect the walls of coronary arteries from damage caused by fat and physical stress (high blood pressure). This is how fish-oils are thought to provide protection against heart disease, and why we are currently being encouraged to eat more oily fish such as herring, mackerel and sardines. However, there is a problem in that oily fish are not eaten in a quantity sufficient to provide the necessary protection against disease. An alternative way of acquiring this protection is by getting our own bodies to make the oils found naturally in fish. We can do this by supplying the building blocks for the biologically active constituents of fish oils in the form of an oil from linseed known as α-LA. Unlike fish-oil, α-LA can be easily incorporated into a wide range of food products and can be eaten in quantities which, we predict, will reduce the risk of developing heart disease. It should be emphasised that fish oils and α-LA are perfectly safe to eat and have been used in numerous human dietary intervention studies in the past.

What do we hope to achieve in this study?

We aim to show that dietary α-LA, a product of linseed oil, can be converted in the body into the active constituents found in fish oil, and when incorporated into foods and consumed can produce 'fish-oil' like changes in the blood that will reduce the risk of heart disease.

Why have you been selected?

You have been chosen primarily because you are male and middle aged (30-60 years) and represent the group most likely to benefit from the experimental diets. To be suitable for our study you must also be a healthy non-smoker who is not taking any drug medication or dietary supplements which may adversely affect our results. We have been notified by your GP of your interest and potential suitability for this trial but must stress that we do not have access to your medical records which remain confidential.
What will happen once you have agreed to take part in the trial?
If the level of fat in your blood is suitable for you to participate in the study you will be asked to provide written consent of your willingness to participate. This consent does not in any way infringe your right to discontinue the trial at any stage. Before you enter the trial we need to obtain information on your normal background diet. To do this you will be given full instructions and guidance on how to complete a 7-day food diary. This diary will provide information on the fat content of your normal diet and daily calorie intake and help us to design the experimental diets. It is vitally important that you do not change your diet or any other lifestyle habits from this point until the start of the dietary intervention period.

When and where will the dietary intervention trial take place and what support will be provided?
The dietary intervention period is planned to start in April/May 2001. The period of time prior to this will allow us to study your background diet and recruit more volunteers for the dietary study. You will be notified well in advance of the start date. Immediately before the trial commences you will be asked to attend the University of Surrey to provide a blood sample (35ml ~3.5 dessert spoon full). A small number of volunteers will be asked to undergo a ‘non-invasive’ assessment of blood flow. This involves placing a blood pressure cuff around the upper arm and measuring blood flow by means of an ultrasound probe placed on the skin. This measurement will be made before and after a tablet of glyceryl trinitrate (GTN), a harmless substance used to dilate blood vessels. After your hospital visit, which should take about 60 minutes, you will visit the University to collect your food supplies / fish-oil supplements and to receive dietary advice and information before starting your diet. The 12-week intervention period will take place in your own home. A dietitian will be on call at the University to provide dietary advice and counseling throughout the 12-week period. The principal scientific investigators will also be available at the University to deal with your queries on any aspect of the trial. Telephone (01483) 879716 or 879724.

What types of food will you be expected to eat?
We are aiming to replace approximately 50% of your dietary fat by supplying you with cooking oils and spreads. All of our food products will have been made by well known food companies and must be acceptable and palatable to our volunteers if we are to maintain their interest and dietary compliance throughout the trial. If there is evidence of non-compliance to the diet at any stage, the volunteer’s participation in the trial may be terminated.

Interim blood sample and dietary check
You will be asked to provide an interim blood sample after 6 weeks on the diet. This will enable us to check on your dietary compliance and to measure your blood fat level. It will also provide an opportunity for further food collection, though foods will be delivered directly to our volunteers throughout the trial on request.

What will happen at the end of the 12 week intervention?
At the end of the 12 weeks you will be asked to attend the University to provide a final blood sample and repeat the blood flow measurements. In the same visit, a small number of volunteers will be asked to take part in an additional study which involves swallowing a small capsule of oil (α-LA) that has been labelled with a harmless tracer substance. This allows us to study the rate at which α-LA from linseed is converted inside the body into the types of oils found in fish. After this you can go home and the next day (24 hours later) a final blood sample is taken. This completes your involvement in the study.

Participation in this trial is voluntary and subjects may withdraw at any time. All subjects who successfully complete the study will receive £150. In the case of subjects who withdraw prior to the end of the study, the level of remuneration may be reduced. Extra remuneration will be given to those who participate in the assessment of blood flow studies.

The Local Research Ethics Committee has approved the above statement.
VOLUNTEER CONSENT FORM

Please read this form carefully before signing. Your signature on this consent form is taken as evidence that you have read and understand the statements on the Volunteer Information Sheet and are willing to take part in the research study.

The Importance of α-Linolenic Acid (α-LA) (linseed oil from flax) as a Source of Long-Chain n-3 Polyunsaturated Fatty Acids (fish oils) and its Influence on Risk Factors of Cardiovascular Disease.

I have read and understand the Volunteer Information Sheet and acknowledge the following points:

1. The purpose of the research study.
2. The expected duration of my participation in the study.
3. The nature of the experimental procedures involved.
4. The nature of any foreseeable risks or discomforts.
5. The nature of any benefits to medical science or the volunteer which may arise from this research.
6. The circumstances under which my participation in the study may be terminated.
7. The fact that my participation in this study is voluntary and refusal to participate will not involve any penalty or loss of benefits to which I am otherwise entitled.
8. That the investigators of this study do not have access to my medical records which will remain confidential.

I, the undersigned, hereby agree to participate as a volunteer in the above research study.

Volunteer

________________________  _______________________
Signature                Name (In BLOCK LETTERS)

Independent Witness to Consent

________________________  _______________________
Signature                Name (In BLOCK LETTERS)

Thank You For Volunteering to Take Part.
ALP-OIL STUDY
Subject Information Booklet

THANK YOU for agreeing to participate in the study. This booklet aims to give you all the information you need for the study. However, if you ever have any queries and can't find the answer in the booklet then please ask. The most important thing to remember is IF IN DOUBT ASK!

If you have any queries the people to contact are:

Amelia Fereday or Paul Wilkinson

= 01483 879735

There is an answerphone at the office if we are not there when you ring. However if you have a problem and urgently need to speak to someone out of work hours, you can contact:

= Amelia 01483 425118 or
= Paul (0956) 470695
Use this sheet to write down anything which you think would be useful for us to know about: good ways to use the oils, any effects from the oil such as decreased indigestion.

The Dietary Intervention

The aim of the dietary intervention is to replace as much of the fat in your diet as possible with fat that we know the composition of. To do this we will provide you with alternative cooking oils and spreads and sachets of oil.

The people participating in the study are divided into 3 diet groups, each with a different letter. One of these diets will contain the flax seed oil, the other two will not. One of those that doesn't will also contain fish oil capsules.

Many manufactured foods are made with sunflower or corn oil, the oils we want you to avoid. We will give you advice on which food to avoid and alternatives.

Following analysis of your normal diet an individual diet plan has been devised for you. The amount of the test foods we want you to consume has been calculated based on your normal intake. We also have a number of suggestions of foods to avoid. But don't worry, we'll do our best to keep your diet as interesting as possible!!
Foods and Oils Supplied

- **Sachets of oil.** These are the main change to your diet for the study. Please use 2 sachets a day.
The sachets of the test oil are provided frozen. Place your sachets in the fridge overnight to defrost. This oil cannot be used for cooking but may be added to cooked foods before eating. See later for other suggestions on how to incorporate it into your diet.

- **Cooking Oil.** This can be used for all types of cooking and frying in place of your normal cooking oil.

- **Margarine.** Please aim to use one tub every 10 days. It may be used for spreading or baking. Store it in the fridge or frozen.

- **Nesquik milkshake powder.** A great way to use up the oil sachets is in a milkshake taken with a meal. Great for those times when you've not managed to use them any other way.

- **Capsules.** Some of you will also be asked to take 6 capsules a day. Take them at mealtimes, for instance 2 each at breakfast, lunch and tea. Try to avoid all at one meal. We suggest you take the capsules with a cold drink. A hot drink will cause the capsule to melt and may result in a slightly unpleasant taste.

Recipes With Oil.

*Milk Shake*
Mix a sachet of oil with skimmed milk and your favourite Nesquik flavour.
Or alternatively mix oil, skimmed milk, fruit and dairy ice cream in a blender for a lovely thick shake.

*Salad Dressing*
1 sachet of oil, 1 teaspoon lemon juice and 1 teaspoon of vinegar
Beat together with a fork.
Add herbs such as dill or herbal seasoning, honey, soy sauce or mustard.

*Quick Tomato Sauce*
Cook onions, courgettes and peppers in rapeseed oil. Add pasata and heat through. Cool to serving temperature and mix in sachet of oil. Serve with pasta topped with cheese.

*Angel Delight*
Beat together the angel delight powder and skimmed or semi skimmed milk. When smooth mix in a sachet of oil. Refrigerate for 15 minutes to allow to set.

*Fruit Fool*
Mix 1 sachet of oil with 1 cup of low fat natural yoghurt. Add chopped fruit of your choice, chopped almonds, a few drops of vanilla essence and a sprinkle of cinnamon. Sweeten with honey or maple syrup.
Ways To Use The Sachets.

- Add to your main meal. Mix in just before eating. Great with curry, pasta sauce, chilli, casserele, baked beans or mashed potato.
- Salad dressing. Make your own or mix some oil with a fat free dressing. Make sure you mop it all up with some bread after.
- Yoghurts can act as a great carrier. Virtually fat free or lower fat yoghurts are ideal for this.
- Milk shake. Try to have this with food rather than on its own.
- Try mixing with orange juice, it improves the flavour.
- Angel Delight. Add to the milk when making up some Angel Delight.
- Add to your breakfast cereal with the milk
- Alternatively, you can just take it on a spoon if you really want to! Try to do this at mealtimes though.

Dietary Changes: General Guidelines

<table>
<thead>
<tr>
<th>foods to avoid</th>
<th>foods to use instead</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>✓</strong> sunflower and corn oils</td>
<td>the cooking oil supplied</td>
</tr>
<tr>
<td><strong>✗</strong> margarine, spread or butter alternatives</td>
<td>the spread provided</td>
</tr>
<tr>
<td><strong>✗</strong> ready made meals containing or cooked in the above oils</td>
<td>those cooked with olive oil or the lower fat alternatives</td>
</tr>
<tr>
<td><strong>✗</strong> crisps or peanuts</td>
<td>reduced fat or baked crisps e.g. Go Ahead, Doritos or French Fries</td>
</tr>
<tr>
<td><strong>✗</strong> retail cakes or biscuits</td>
<td>make your own using the spread provided or choose lower fat alternatives</td>
</tr>
<tr>
<td><strong>✗</strong> french dressings, salad creams or mayonnaise</td>
<td>make dressings with the sachets provided or use fat free alternatives (ideally mixed with a sachet)</td>
</tr>
<tr>
<td><strong>✗</strong> oily fish such as mackerel, trout, salmon</td>
<td>use white fish such as cod, haddock or plaice</td>
</tr>
</tbody>
</table>
Eating Away From Home.

Sandwiches and Other Snacks
- Choose lower fat alternatives. These tend to use a low fat spread and often don't have mayonnaise.
- Avoid pasties, pies and sausage rolls because of the pastry.
- Choose lower fat salads or those without any dressing.

Fast Food / Take Away
- Fish and Chips. Avoid if at all possible. Nearly all chip shops use sunflower oil.
- Chinese. Avoid those foods that are deep fried such as pancake / spring rolls, sweet and sour balls, prawn crackers.
- Curry. Most curries are high in fat, go for the dry fried variety and have rice. Avoid poppadums, samosas and bhajis.
- Pizza. Watch out for the garlic bread unless they use butter.
- MacDonaldis / Burger King. Avoid the fries & dressing.

Eating Out
- Try to avoid fried foods, choose something baked instead.
- Choose potato rather than chips.
- Choose butter rather than margarine for your bread roll.
- Take your sachets with you if you can
If found, please return this diary to
Dr. A. Fereday
School of Biological Sciences
University of Surrey
Guildford
Surrey
GU2 5XH
Telephone: 01483 879735

NAME: _______________________

ALP-OIL STUDY

WEIGHT DIARY
General Points

- Measure your weight at the same time of day. This is best done when you first get up, after you have been to the toilet.
- Always use the scales on the same surface.
- Record your weight immediately, before you forget.
- Weigh yourself once a week, preferably on the same day of the week.
- If your weigh increases or decreases by more than 2Kg or 4I then let us know IMMEDIATELY. If in doubt, contact us.
- Bring this diary with you when you come to see us.
<table>
<thead>
<tr>
<th>WEEK</th>
<th>DATE</th>
<th>WEIGHT</th>
</tr>
</thead>
<tbody>
<tr>
<td>example</td>
<td>Monday 13 September</td>
<td>14 stone 7lb</td>
</tr>
</tbody>
</table>