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DIETARY AND GENETIC FACTORS AFFECTING THE CHOLESTERYL ESTER TRANSFER PROTEIN AND LIPOPROTEIN METABOLISM

**A Thesis submitted for the Degree of
Doctor of Philosophy (Ph.D)**

by

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at

University of Dublin, Trinity College

October 2000

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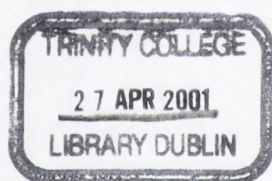
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Declaration

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Summary

The cholesteryl ester transfer protein (CETP) plays an inherent role in the redistribution of neutral lipids between high density lipoproteins (HDL) and very low density lipoproteins (VLDL) and low density lipoproteins (LDL). This is an important step in the process of reverse cholesterol transport. The activities of CETP may possibly be atherogenic as CETP activity transfers CE from HDL to VLDL and LDL with the reciprocal transfer of triglyceride (TAG). The consequence of CETP activity is increased LDL and VLDL cholesterol concentrations, and increased HDL TAG and reduced HDL cholesterol concentrations. This is a lipoprotein profile which is associated with an increased risk for the development of CAD.

Taq 1B is a known polymorphism of the CETP gene. An investigation as to whether the presence or absence of this polymorphism exhibited any impact on CETP mass, activity and related plasma lipoproteins in normolipidaemics was carried out. Those individuals who exhibited the absence of the Taq 1 B polymorphism had a significantly lower CETP mass and activity and increased HDL cholesterol concentrations than did those who were homozygous or heterozygous for the presence of Taq 1B. These results indicate that genetic variants of CETP determine CETP mass and activity and HDL cholesterol concentrations in a normolipidaemic population.

The effect of postprandial lipaemia and cholesterol concentrations on CETP mass and activity in a large normolipidaemic population were examined. Chapter three demonstrates that there is an increase in CETP mass and activity during the postprandial states. Plasma cholesterol concentrations were strongly associated with CETP mass and activity. Chapter four demonstrates that individuals with moderately elevated plasma cholesterol concentrations produce an increased postprandial response in CETP mass and activity when compared to normal plasma cholesterol subjects. Increased redistribution of cholesterol into apo B lipoproteins in moderately elevated cholesterol subjects contributes to the more atherogenic lipoprotein profile of these individuals.

Intake of fish oil has been widely reported to reduce plasma TAG concentrations and the incidence of CAD. In chapter 5 an investigation into the effects of low dose fish oil intake over 16 weeks in normolipidaemics was undertaken. Fasting and 6 hr postprandial CETP mass, activity and plasma lipid and lipoprotein concentrations were measured. Low fish oil intake (0.9g/day) at the upper end of the low-dose range (0.3-0.9 g/d) resulted in significant reductions in plasma TAG concentrations in the fasted state. There was an increase in HDL₂ cholesterol concentrations in those individuals taking fish oil dietary supplements.

Finally the effects of novel fatty acid known as conjugated linoleic acid on CETP mass and activity and lipid metabolism was investigated. Recent evidence from animal studies revealed that this novel fatty acid has potential anti-atherogenic properties. The study in chapter 6 is the first human trial which investigates the effects of dietary supplementation of isomeric blends of CLA over 8 weeks on lipid metabolism and CAD risk factors in humans. Plasma TAG concentrations were reduced as a result dietary supplementation using a 50/50 isomeric blend of cis-9, trans-11 and trans-10, cis-12 CLA. VLDL cholesterol concentrations were significantly reduced as a result of dietary supplementation with an 80/20 blend of CLA. These results indicate beneficial effects of dietary supplementation using this novel fatty acid on CAD risk factors in humans.

Abbreviations

α	alpha
ANOVA	Analysis of Variance
Apo A1	Apoprotein A1
Apo B	Apoprotein B
Apo B48	apo protein B48
Apo C1	Apoprotein C1
Apo E	Apoprotein E
ATP	Adenosine Triphosphate
B	Beta
BMI	Body Mass Index
BSA	Bovin Serum Albumin
CAD	Coronary Artery Disease
cDNA	cloned Deoxyribosenucleicacid
CE	Cholesterol Ester
CETP	Cholesteryl Ester Transfer Protein
CHD	Coronary Heart Disease
CLA	Conjugated Linoleic Acid
CPM	Counts per Minute
D	Day
Δ	Delta
DHA	Dicosapentaenoic Acid
ELISA	Enzyme Linked Immunosorbent Assay
EPA	Eicosapentaenoic Acid
ER	Endoplasmic Reticulum
FAME	Fatty acid Methyl Ester
FFA	Free Fatty Acid
δ	gamma
G	Gram
GC	Gas Chromatography
H	Hours
H3	Tritiated Hydrogen
HDL	High Density Lipoprotein
HL	Hepatic Lipase
HPLC	High Performance Liquid Chromatography
IDL	Intermediated Density Lipoprotein
IgG	Immunoglobulin
IL	Interleukin
Kda	Kilodaltons
Kg	Kilogram
L	Litre
LCAT	Lecithin Co A transferase
LDL	Low Density Lipoprotein

LPL	Lipoprotein Lipase
μl	Microlitre
μmol	micromol
M	Mol
Mab	Monoclonal Antibody
Mg	Miligram
MI	Myocardial Infarction
ml	Milliliter
Mmol	millimol
mRNA	Messenger Ribo Nucleic Acid
MUFA	Monounsaturated Fatty Acids
NBD-CE	Nitrobenzoxadiol-fluorophore-cholesteryl ester
NEFA	Nonesterified Fatty Acids
NIDDM	Non Insulin Dependent Diabetes Mellitus
Nm	Nanometers
nmol	nanomol
NSD	No Significant Difference
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
%	Percentage
PLTP	Phospholipid Transfer Protein
pmol	Picomol
PPAR	Peroxisome proliferator activated receptor
PUFA	Polyunsaturated Fatty Acids
RCT	Reverse Cholesterol Transport
TAG	Triglyceride
TBS	Tris Buffered Saline
TLC	Thin Layer Chromatography
TPL	Triglyceride Poor Lipoproteins
TRL	Triglyceride Rich Lipoproteins
TWS	Top Working Standard
VLDL	Very Low Density Lipoprotein

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Chapter 1

General Introduction

1.1: Background of Reverse Cholesterol Transport

Reverse cholesterol transport is a process where cholesterol from peripheral cells is carried to the liver in lipoproteins. The cholesterol ester transfer protein (CETP) is a pivotal enzyme in this process. CETP distributes cholesterol esters and triacylglycerol between lipoproteins. A brief synopsis of the role of CETP in RCT will be explained subsequently. The structure, function and the relationship between CETP and plasma lipoproteins, coronary artery disease risk factors, and the effects of plasma lipid and diet on CETP mass and activity will be explained in more detail in subsequent sections in this literature review.

A schematic representation of CETP in relation to reverse cholesterol transport is shown in Figure 1.1. Cholesterol and phospholipid in peripheral cells are taken up by pre Beta high density lipoproteins (HDL) as they dissociate from the plasma membrane. Through the actions of lecithin Co-acyl transferase (LCAT), free cholesterol is esterified and transferred to the core of HDL. CETP, as its name suggests, transfers cholesterol esters between HDL and apo B lipoproteins, with a reciprocal exchange of TAG from apo B lipoproteins into HDL, resulting in the formation of TAG rich HDL₂ and cholesterol rich apo B lipoproteins (see figure 1.2). Cholesterol is then returned in apo B lipoproteins (chylomicron remnants, VLDL and LDL) to the liver where they are taken up via the LDL receptor. In the liver cholesterol may be excreted in the form of bile or maybe repackaged as cholesterol into lipoproteins and returned to plasma. HDL₂ are catabolised into HDL₃ or HDL remnants in the liver and are returned to the circulation.

1.2: Coronary Artery Disease – The risk factors

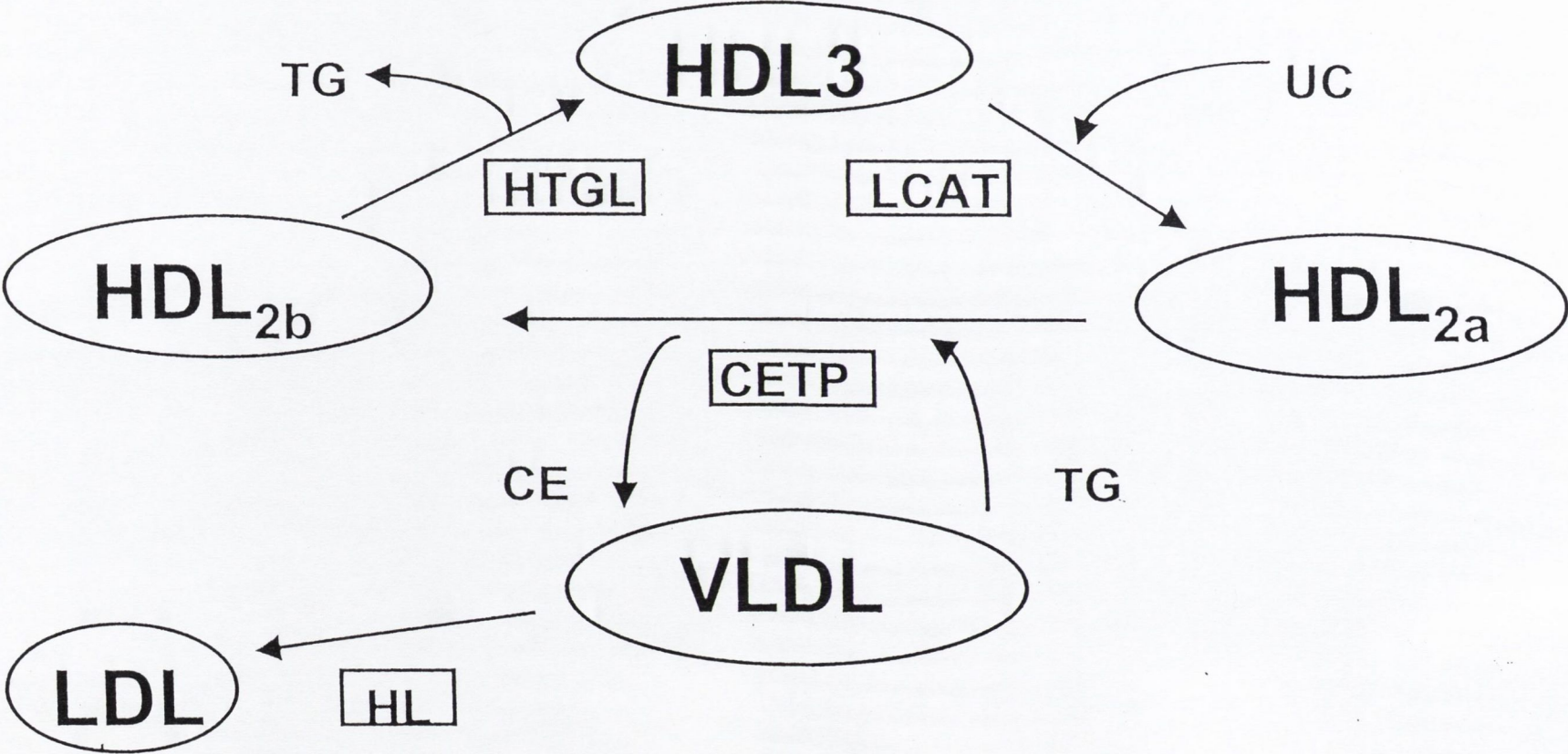
Coronary heart disease (CHD) is one of the leading causes of death in the western world accounting for 50 % of all deaths. It is a multifactorial disease, the risk factors for which include a positive family history, age, gender, smoking, hypertension, hypercholesterolaemia and obesity. The dietary dimension to the risk of CAD covers many nutrients e.g. sodium, fiber, folic acid, antioxidant vitamins, alcohol and fat intake. For the purpose of this thesis the focus will be on the dietary fat component of CAD. Western diets tend to be high in saturated and *trans*-fatty acids, fat which can promote the formation of an atherogenic lipoprotein profile. There is a large body of evidence that total plasma cholesterol is a predictor of myocardial infarction (Stampfer *et al.*

1996). Using data from the expert panel on detection, evaluation and treatment of high blood cholesterol in adults (National Cholesterol Education Program Expert Panel 1988), it is suggested that 36% of all adults in the United States between the ages of 20 and 74 years are in need of medical advice and intervention for high levels of blood cholesterol. Most of the major studies on CHD tend to focus on men for a variety of reasons. Men develop CHD earlier than women. Hormonal variations in women influence plasma lipids and the rate of progression of CHD. After the menopause estrogen levels drop and this is associated with an increase in plasma cholesterol and a decrease in HDL a lipoprotein profile very like that of men.

In a study using the Framingham data (Gordon *et al.* 1989), it was found that lowering plasma cholesterol from 7.37 mmol/l to 5.18 mmol/l in the age category 35-44 years for men and women reduced the risk of CHD death by 77%, while in the 75 - 84 age group the risk of CHD related death was reduced by 23%. It seems that with increasing age, plasma cholesterol becomes a less important predictor of CHD (Rose *et al.* 1986). Elevated HDL has a protective effect against CHD, since HDL concentrations are greater in premenopausal women. This is thought to explain the lower incidence of CHD in women. HDL exerts its protective effect against CHD through the action of reverse cholesterol transport (RCT), a process by which excessive cellular cholesterol is transported to the liver for excretion and its anti-oxidative effects on other plasma lipoproteins. More recently elevated plasma TAG concentrations have been identified as an independent risk factor for MI (Stampfer *et al.* 1996). A meta analysis of the population based prospective studies showed that elevated plasma TAG concentrations were associated with an increased risk of CHD in men but especially in women (Hoknsen and Austin 1996)

Figure 1.2

Neutral Lipid exchange between HDL and VLDL facilitated by CETP



1.3 Cholesteryl Ester Transfer Protein

1.3.1: Function

The CETP promotes the exchange of cholesterol ester (CE) from HDL to very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL), with the reciprocal transfer of TAG. It also catalyses the transfer of phospholipid between lipoproteins (Hestler *et al.* 1987; Ohnishi *et al.* 1990). Recently it has been shown that CETP can catalyse the transfer of unesterified cholesterol (Connolly *et al.* 1996b) between lipoproteins.

1.3.2: Structure

CETP is a plasma protein, which is primarily bound to plasma lipoproteins. It is a hydrophobic glyco-protein ranging in molecular mass between 66,000- 74,000 kd (Albers *et al.* 1984; Hesler *et al.* 1987; Jarnagin *et al.* 1987). A shorter variant of CETP is produced in which exon 9 is removed. This is a poorly secreted and inactive form of the protein (Yamashita *et al.* 1997). The CETP gene is located on chromosome 16 and contains 16 exons (Agellon *et al.* 1990; Lusis *et al.* 1987). The CETP cDNA encodes a polypeptide of MW. 53,000 kd. There is considerable post-translational modification of the polypeptide to produce the mature protein. This involves N- glycosylation at four sites on the protein giving rise to the mature protein (Drayna *et al.* 1987; Stevenson *et al.* 1993). The extensive post-translational modification of the protein is thought to be important for secretion of the protein. The secondary structure of CETP, as estimated by circular dichroism, contains 32% alpha helix, 35% beta sheet, 17% turn and 16% random coil (Connolly *et al.* 1996a). The polypeptide with a unique N-terminal contains a high content (44%) of hydrophobic amino acids which are thought to be an important factor in lipid binding (Drayna *et al.* 1987). The C-terminal of the CETP molecule forms the epitope where neutral lipid transfer activity occurs. Evidence for this comes from studies by Wang *et al.* (1992) in which the monoclonal antibody TP2 which had an epitope spanning from Phe-463 to Leu-475 on the alpha helix of the C terminal of the CETP molecule neutralised all CETP activity. The major production site for CETP is the liver (Quinet *et al.* 1991) but smaller amounts are produced in intestinal, adipose and skeletal tissues.

1.3.3: Distribution of CETP in plasma

In humans, CETP is found in plasma associated with all lipoproteins, however it binds primarily to HDL particles (Tall, 1993) because of the greater molar concentration of these lipoproteins in plasma (Marcel *et al.* 1990). CETP binds to HDL by electrostatic attraction between positively charged amino acids of the CETP molecule and the negatively charged phospholipid phosphate groups of the HDL. (Pattnaik *et al.* 1979). Apo A1 is a structural protein of HDL. Apo A1 causes a conformational change in the CETP molecule which results in enhanced binding of CETP to HDL, as well as increasing its activity (Mac Phearson *et al.* 1996). There is conflicting evidence as to which HDL fraction CETP is primarily associated with. Marcel *et al.* (1990) found evidence that the association of the majority of CETP was with the HDL₃ sub-fraction. Speijer *et al.* (1991) showed that when HDL was run on a gel filtration column, CETP activity was found only on the trailing edge of the HDL peak. Therefore it was concluded that the majority of CETP was in fact associated with small HDL and in particular pre-beta HDL.

1.3.4: Mechanisms for CETP transfer

The mechanism by which CETP transfers neutral lipids is unclear. However, two principal theories have been proposed to explain how this mechanism may operate. The structure of the protein (See section 1.3.2) is optimally designed to allow the enzyme to carry out its function under physiological conditions. One proposed mechanism for CETP lipid transfer is that the protein must bind to both HDL and VLDL in order for transfer to occur. This allows the CETP molecule to transfer lipid between each lipoprotein. The C-terminal of the protein allows lipid binding and the alpha helix formation operates like a flexible arm transferring lipid between the lipoproteins (Tall, 1993).

Another proposed mechanism is that CETP acts like a free carrier. It binds to a donor particle and accepts lipid (CE). It then dissociates from the donor lipoprotein and collides with an acceptor particle where it releases its bound lipid, CE and accepts TAG. It then dissociates from the lipoprotein and eventually it collides with the appropriate lipoprotein capable of accepting its bound lipid (Connelly *et al.* 1996b). This theory is quite feasible since CETP is capable of binding to all lipoproteins. Plasma CETP is not evenly distributed between the different lipoproteins. It is mainly

associated with the HDL sub fractions (Marcel, 1990 and Speijer *et al.* 1991). If this mechanism proposed by Connelly *et al.* (1996b) were probable under physiological conditions, a more even distribution of CETP would be expected between the lipoproteins.

1.3.5: Genetic variations in CETP

Brown *et al.* (1989) carried out a study on individuals with a point mutation at intron 14 (G-A) and an exon 15 missense mutation on their CETP gene which resulted in no CETP being detectable in plasma. These mutations inhibit the formation of normal CETP mRNA. This particular defect is present in about 2-5 % of the Japanese population and it is more common in those with HDL hypercholesterolaemia (Inazu *et al.* 1994). The heterozygous condition of this genetic mutation of the CETP gene results in a 35 % decrease in CETP activity (Zhong *et al.* 1996) and a moderate increase in HDL concentration. In the homozygous condition there is no detectable CETP activity and therefore elevated levels of HDL (Tall 1993). This mutation accounts for 10 % of the variation in HDL levels in heterozygous state. Population groups with these mutations show elevated HDL cholesterol levels (3 and 6 fold). Most of the increase in HDL cholesterol concentrations is accounted for in the HDL₂ fraction. The HDL₂ which is produced as a consequence of CETP deficiency is not an efficient effector of cholesterol efflux from cells (Yamashita *et al.* 1997) and therefore is not cardioprotective. The HDL produced in CETP deficient states have a higher affinity for LDL receptor than do LDL themselves (Yamashita *et al.* 1990). There is also a moderate increase in the concentrations of apo A1 and AII, CIII and apo E. This is a consequence of delayed catabolism of these proteins (Yamashita *et al.* 1997). Analysis of apo B containing particles in individuals with these CETP mutations revealed greater levels of cholesterol oleate and linoleate compared to the controls, with the concentration of apo B containing lipoproteins at reduced levels.

Lecithin cholesteryl acyl transferase (LCAT) is an HDL associated enzyme which catalyses the esterification of cholesterol, so that it can be transferred between lipoproteins. In CETP deficient states, LCAT activity is thought to switch from HDL to LDL. The reason for this is that there is no efflux of CE from HDL in the CETP deficient states and therefore LCAT activity only occurs in the LDL fraction. There is large variation in the chemical composition and distribution of LDL as a consequence of

LCAT activity (Tall, 1993; Yamashita *et al.* 1988). Evidence for this comes from the difference in the size distribution of the LDL sub-fractions in individuals with CETP deficiency. Their LDL were much more polydisperse, consisting of large and small LDL compared to normal control patients who had predominantly medium sized LDL. The apo B lipoproteins which are produced in CETP deficiency states have a lower affinity for the apo B receptor than do apo B lipoproteins produced by normal individuals (Sakai *et al.* 1995). These experiments on individuals with CETP deficiency clearly demonstrate that CETP is an important factor which regulates lipoprotein concentration and distribution in plasma.

1.3.6: Effect of the Taq 1B polymorphism on CETP and HDL cholesterol concentrations

Polymorphisms of the CETP gene are known to have an effect on CETP and HDL cholesterol concentrations. For the purpose of this thesis only the effect of the Taq 1B polymorphism on CETP mass and activity will be discussed. The Taq 1B restriction enzyme effects nucleotide 277 on the first intron in the CETP gene. It is hypothesized that Taq 1B is not functional, but is a marker for variants within the gene (Fumeron *et al.* 1995). The presence or absence of Taq 1B is associated with CETP and HDL cholesterol concentrations (Bernard *et al.* 1988; Kuivenhoven *et al.* 1998; Fumeron *et al.* 1995; Hill *et al.* 1998). B1 and B2 denote the presence or absence of the Taq 1B restriction site respectively. The frequency for the allelic variants in Caucasians is 35 % B1 B1, 49 % B1 B2 and 16 % B2 B2 (Kuivenhoven *et al.* 1997). It is considered that the presence or absence of this polymorphism is associated with CAD risk, which is mediated through the actions of CETP. The B1 B1 genotype is associated with increased CETP and reduced HDL cholesterol concentrations while the B2 B2 genotype is associated with decreased CETP and increased HDL cholesterol concentrations. The B1 B2 genotype had CETP and HDL concentrations in the middle range of the other two genotypes (Kuivenhoven *et al.* 1997).

Tall (2000) carried out an analysis of the frequency and effect of the Taq 1B polymorphism in 2416 individuals from the Framingham offspring study, found that those who were homozygous for the absence of the cutting site had increased HDL cholesterol concentrations with reduced CETP concentrations. The B2 allele had larger HDL and LDL particle sizes. The odds ratio for the B2 B2 allele for the development of

CAD was 0.692 ($P = 0.03$) for men and with the increased lipoprotein particle size and increased HDL cholesterol concentrations translated into a lower risk of B2 B2 men developing CAD

Environmental factors contribute to the effect that the Taq 1 B polymorphism has on CETP and HDL cholesterol concentrations. An investigation into the effect of Taq 1 B in obese subjects revealed that the frequency of the allelic variants of Taq 1B was similar to other populations (Vohl *et al.* 1999). The association of the B2 B2 allele with reduced CETP concentrations and increased HDL cholesterol concentrations was attenuated in obese subjects ($BMI > 27\text{kg/m}^2$) when compared to lean subjects. It is postulated that due to the increased CETP concentration in adipose tissue of obese individuals, the effect of reduced CETP concentrations and increased HDL concentrations associated with the B2 B2 allele was not apparent, due to the elevated concentration of CETP in the obese when compared to lean subjects.

The distribution of CETP/Taq 1B alleles is similar in NIDDM (Moulin *et al.* 1998) to other populations (Fumeron *et al.* 1995; Kuivenhoven *et al.* 1997). There is a relationship between Taq 1B alleles of CETP gene and CETP and HDL cholesterol concentrations. Alcohol intake appears to exacerbate the effect of B2 B2 allele on HDL cholesterol concentrations (Fumeron *et al.* 1995). Fumeron *et al.* (1995) found a similar trend in HDL cholesterol and CETP concentrations relative to the presence or absence of Taq 1B genotype. Those who drank $> 75\text{g/d}$ of alcohol with a B2B2 or B1B2 genotype had a 30 % and 13 % increased HDL cholesterol concentrations over those who had the B1B1 alleles. No relationship was found between allelic variants and HDL cholesterol concentrations in those who consumed $< 25\text{g/d}$ alcohol suggesting that the homozygous presence of the B2 allele and alcohol consumption may be beneficial towards HDL cholesterol concentrations.

Taq 1B seems to predict the outcome of drug therapy using statins to retard the rate of progression of CAD in a group of patients with angiographically documented CAD cases (Kuivenhoven *et al.* 1998). It was found that those who had B2 B2 allele, there was no reduction in the rate of progression of CAD as a result of pravastatin therapy, while the B1B1 variants were the most successful responders to pravastatin treatment. Therefore the Taq 1B genotype appears to be useful in predicting the outcome of drug therapy used for the treatment of CAD.

1.3.7: CETP and atherogenesis

Contradictory evidence exists as to whether CETP is pro-atherogenic or anti-atherogenic as it strongly influences lipoprotein lipid concentrations, content and size. A number of therapeutic agents have targeted CETP assuming that it is pro-atherogenic because it has the potential to reduce HDL cholesterol concentrations. The results from the Honolulu Heart Study found that the incidence of CHD was 21 % in those who were CETP deficient and 16 % in those who had normal CETP activity (Zhong *et al.* 1996). Moriyama *et al.* (1998) investigated a Japanese cohort with high HDL cholesterol > 80 mg/dl who were deficient in CETP and found that the prevalence of CAD was similar regardless of CETP concentrations. Similarly a study carried out by Zhong *et al.* (1996) demonstrated that only those individuals who were deficient of CETP had reduced incidence of CHD when their HDL cholesterol levels were > 60 mg/dl, when compared to individuals with normal CETP activity. Those individuals deficient in CETP with HDL cholesterol < 60 mg/dl showed an increase in CHD. Similar results were found by Inazu *et al.* (1990). Zhong *et al.* (1996) proposed that the high levels of HDL may exert an antioxidant effects, which counteracts the defect in reverse cholesterol transport. Data from these studies is not convincing that CETP deficiency is beneficial. The rate of CAD incidence in CETP normal and deficient groups appears to be similar regardless of the increased HDL cholesterol concentrations. While CETP deficiency is thought to be potentially anti-atherogenic due to the elevation of HDL, it maybe pro-atherogenic because of its inhibitory role on reverse cholesterol transport (Zhong *et al.* 1996). Furthermore, CETP may be pro-atherogenic because of its role in conjunction with LCAT, in the production of LDL particles which are a poor ligands for the apo B receptor (Sakai *et al.* 1995) and its interactions with chylomicrons, VLDL and HDL.

The formation of TAG-rich HDL₂ which are produced in CETP deficient individuals are the preferential substrates for hepatic lipase. This promotes the formation of pre-beta HDL, which are excellent effectors of cellular cholesterol efflux, thereby suggesting an anti-atherogenic effect of CETP deficiency (Fielding *et al.* 1995). In the population group from the Honolulu Heart Study group, the CETP mutants showed a higher incidence of CHD. They were however all elderly subjects and potentially the D442G mutation (a mutation of the CETP gene) could have delayed the onset of CHD and the increased in relevance in the older survivors.

1.3.8: Determinants of CETP mass and activity

A number of studies have found that CETP activity is dependent on CETP mass (Clark *et al.* 1995). The next determinant of CETP activity is the availability of substrates for the enzyme. The supply of CE is determined by the rate of cellular efflux of cholesterol and its esterification by LCAT. Rajaram *et al.* (1994) demonstrated that the unesterified cholesterol content of HDL particles determines CETP activity. The other substrate of the CETP enzyme is TAG, which is present in VLDL and LDL. Mann *et al.* (1991) demonstrated the importance of TAG as a determinant of CETP activity. These studies showed that the supplementation of normolipidaemic plasma with additional CETP caused no change in CETP activity. However when additional VLDL was added to the plasma samples, CETP activity increased until the enzyme itself became the rate-limiting factor for lipid transfer. A significant positive correlation was observed between CETP activity and VLDL TAG concentration (Mann *et al.* 1991). Tall *et al.* (1986) observed that during postprandial lipaemia, the increasing concentrations of TRL increased CE exchange. The importance of TAG as a determinant of CETP activity is further demonstrated by the fact that in hypertriglyceridaemics, the rate of CE transfer correlates with mass but does not do so in normolipidaemics, suggesting that CETP operates at maximum rate in hypertriglyceridaemics due to greater availability of substrate.

1.3.9: Variations in plasma CETP mass and activity

Using Radio Immuno Assay (RIA), and Enzyme Linked Immuno Sorbent Assay (ELISA), it has been shown that there is a difference in CETP mass and activity between genders. Normolipidaemic males have a plasma CETP concentration of 2.05 µg/ml, and normolipidaemic females have plasma CETP concentration of 2.16 µg/ml (Clark *et al.* 1995). Exercise was also found to reduce CETP plasma concentrations as did weight loss in both men and women (Seip *et al.* 1993). Plasma lipid concentrations also affect CETP, in hypercholesterolaemics CETP mass was 25 % greater than in normolipidaemics. (Tall, 1993). Hypertriglyceridaemics have CETP activity levels 17 % greater than normolipidaemics (Tato *et al.* 1995b). In nephrotic syndrome, in which VLDL and LDL cholesterol levels and apo B levels are elevated, CETP mass and activity are 58 % greater than healthy individuals (Moulin *et al.* 1992).

1.4.0: CETP and related proteins

1.4.1: Phospholipid transfer protein

Human plasma phospholipid transfer protein (PLTP) facilitates the transfer of phospholipid from liposomes VLDL and LDL to HDL. Speijer *et al.* (1991) have shown that two different proteins, CETP and PLTP, mediate lipid transfer activities. PLTP has a Mr 78,000 (Pattnaik *et al.* 1979; Tall *et al.* 1986) and is thought to be located in the very high-density region (1.20-1.26 g/l) of the HDL subfraction (Tall *et al.* 1983). Its main function is in relation to HDL size conversion and net phospholipid transfer (Pattnaik *et al.* 1979; Tall *et al.* 1986). An increase in HDL phospholipids occurs during lipolysis resulting in the formation of larger less dense HDL. In humans, there is an increase in the phospholipid content of HDL following a fatty meal (Ihm *et al.* 1982). Studies investigating the activity and effect of PLTP, using monoclonal antibodies, which block PLTP activities, showed that when PLTP was inhibited, the transfer of PL from VLDL, LDL to HDL was reduced by 50-70%. In this experiment CETP activity was not affected by the inhibition of PLTP (Cheung *et al.* 1996). PLTP is an important determinant of HDL size conversion. The importance of PLTP activity on HDL enlargement was demonstrated using anti PLTP IgG where inhibition of PLTP activity also inhibited the enlargement of HDL (Tu *et al.* 1993). As the HDL particle increases in size, PLTP activity is reduced (Screeny and Jonas, 1985). As a result of PLTP activity, HDL₃ apo A1 is released (Tu *et al.* 1993). Recently it has been observed that PLTP activity is regulated by the molar ratio of apo A1/AII in the HDL particle (Pussinen *et al.* 1997).

1.4.2: Lipoprotein lipase

LPL is a hydrolytic enzyme, and is the rate limiting step for the removal of circulating TRL from plasma. The active form of the enzyme is 55 kDa with a number of glycosylation sites. Post-translational modification of the enzyme i.e. glycosylation is thought to be important in bringing about the conformation of the active enzyme. After post-translational modification, the enzyme associates into dimers and becomes catalytically active (Vannier and Ailhaud, 1989). The enzyme is bound to the endothelial surfaces via glycoaminoglycans on the luminal surface of capillary endothelium of skeletal muscle and cardiac muscle. It is present in the adipocyte and parenchymal cells in largest quantities. When LPL goes into the circulation it binds to

cells which have the appropriate binding sites, including those which don't produce LPL. On the endothelial surface, it hydrolyses core TAG of triglyceride rich lipoproteins, releasing free fatty acids which are either stored in the adipocyte or are oxidised by cells following release into plasma. LPL activity is dependent upon fed and fasted states. Whilst in the fed state there is high LPL activity which allows the rapid production of FFA, which may be stored in the adipocyte, in the starved state, its activity is reduced. It is dependent upon apo CII for activation. Apo CIII is an inhibitor of LPL activity which is produced in the liver and intestine. Plasma TAG concentrations are proportional to the concentration of apo CIII in transgenic mice (Ito *et al.* 1990; de Silva *et al.* 1994). The concentration of the enzyme is kept low in the circulation by the hepatic uptake of the enzyme (Olivercrona, 1989). Studies by Clay *et al.* (1989) have shown that LPL exhibits substrate specificity towards hydrolysis of TAG in VLDL and chylomicrons but shows little or no activity towards the TAG component of HDL.

1.4.3: Hepatic lipase

HL and LPL probably have a common ancestor, and they both complement each other in lipoprotein metabolism (Olivercrona and Bengtsson-Olivercrona, 1990). Hepatic lipase is produced only in the liver (Semenkovich *et al.* 1989). It is an endothelial bound enzyme and the injection of heparin results in the release of the enzyme into the plasma, as is the case with LPL. HL facilitates hepatic chylomicron remnant removal. Studies using antiserum to HL in rats resulted in an increase in circulating apo B48 which indicates that CM remnant removal was inhibited (Daggy and Bensadoun, 1986). HL also controls the composition of HDL and HL inhibition leads to an increase in the phospholipid concentration of HDL (Groot *et al.* 1983). Studies in rabbits show that HL reduces the size, and apo A1 content of HDL (Clay *et al.* 1990). One of the favourite substrates of HL is TAG rich HDL₂. In human subjects with familial deficiency of HL there is excessive enrichment of LDL and HDL₂ with TAG (Breckenridge, 1987). HL plays a pivotal role in the catabolism of HDL and the release of its apo protein content, which results in the formation of pre-beta HDL. Therefore it appears that HL is an essential determinant of HDL concentration in plasma.

1.4.4: Lecithin cholesteryl acyl ester transferase.

LCAT catalyses the transfer of the acyl group from lecithin to unesterified cholesterol resulting in the formation of lysolecithin and CE (Fielding, 1985). It is the first enzyme in RCT, it promotes cellular efflux of cholesterol and allows the incorporation of cholesterol from other sources into HDL thereby promoting RCT. It is an HDL associated enzyme and increases the CE content of HDL, especially in nascent HDL particles. Pre-beta HDL accepts cholesterol from cell membranes and forms larger discoidal pre-beta HDL. These are then transported into the plasma compartment where they bind to LCAT (Castro and Fielding, 1988). LCAT esterifies the cholesterol on the surface of HDL particles and this hydrophobic CE is incorporated into the core of these particles resulting in the formation of spherical HDL particle. Apo A1 is an HDL associated protein which acts as a co-factor for LCAT. The majority of LCAT is associated with HDL A1 (Duverger *et al.* 1994). LCAT promotes the flow of cholesterol from cellular sources in to the plasma compartment (Francone *et al.* 1989). The passage of cholesterol from HDL back into cells is prevented because LCAT esterifies cholesterol within the HDL preventing it passing back into the cells (Czarneckah and Yokoyama, 1995).

LCAT activity promotes the formation of CE, which is a substrate for CETP activity. Other sources of substrates for LCAT are lipoproteins and hydrolysed TGL surface remnants. As CE accumulates in HDL particles, the rate of activity of LCAT decreases. Evidence for this comes from a study by Eisenberg *et al.* (1984) where it was found that upon removal of CE from CE rich HDL, the activity of LCAT was increased. A conditions which result from LCAT deficiency is fish eye disease. Most of their cholesterol exits in the unesterified form. HDL is very small and is devoid of CE. There is an enrichment of their cell membranes with cholesterol.

1.5.0: Post-prandial lipaemia

After ingestion of a saturated-fat containing meal lipids are digested by lipases. These include gastric lipase and pancreatic lipase, whose catalytic actions on TAG result in the formation of glycerol and free fatty acids (FFA). Cholesterol esterase activity results in the formation of cholesterol esters (CE), while phospholipase activity

results in the formation of lysophospholipids. Lipids are then assimilated into lipoprotein particles chylomicrons and very low density lipoproteins (VLDL) and then they enter the plasma where they supply tissues with an energy source.

The activities of enzymes responsible for the clearance of post-prandial lipoproteins, i.e. LPL, is correlated with the degree of postprandial lipaemia (Weintraub, 1987), as is HL activity (Patsch, 1987). Several studies have shown that the fatty acid composition of the background diet is very influential at influencing the lipaemic response to a fat load. Diets rich in polyunsaturated fat i.e. fish oil reduce the level of postprandial chylomicrons by 67%, while remnants are reduced by 53% when compared with a saturated fat diet (Weintraub, 1987). Men have a 30% greater lipaemic response than do women (Cohn *et al.* 1988). This reflects the higher activity of LPL in postprandial plasma in women when compared with men (Taskinen *et al.* 1986). Increasing age results in an increase in the duration and magnitude of postprandial lipaemia, probably due to a decrease in LPL activity and hepatic receptor function.

1.5.1: Effects of postprandial lipaemia on lipoprotein metabolism

Alimentary lipaemia gives rise to an increase in phospholipids and proteins in HDL. HDL₂ is the most responsive of the HDL sub-fractions to postprandial lipaemia. Tall *et al.* (1982) was the first to demonstrate that there is a redistribution of the HDL fractions from dense HDL₃ to less dense HDL₂ during postprandial lipaemia. Groot *et al.* (1991) has shown that the main cause of this redistribution of the HDL fractions during postprandial lipaemia is due to TAG-rich chylomicron remnants fusing with HDL₃ resulting in the formation of HDL₂.

CETP is responsible for the redistribution of CE among lipoproteins during postprandial lipaemia. Tall (1986) showed that there was 2-3 fold increase in the rate of CE transfer during postprandial lipaemia. CETP is responsible for the transfer of CE from HDL to VLDL and LDL with the reciprocal transfer of TAG. Core changes in HDL correlate positively with postprandial lipaemia (Patsch *et al.* 1984). An increase in LCAT activity was also found during postprandial lipaemia (Castro and Fielding 1985). This reflects the increase in the level of available substrate and the increase in the phospholipid composition, which increases the affinity of CETP and LCAT for HDL, all of which contribute to increased enzyme activity.

1.6.0: Lipoproteins and their relationship to CETP

1.6.1: Chylomicron synthesis

Chylomicrons are lipoproteins with a TAG rich (making up 86-92 % of particle mass) core, cholesteryl esters and a surface layer containing phospholipid, cholesterol and apo proteins. They are formed in the small intestine during post-prandial lipaemia from the products of the actions of lipolytic enzymes. Chylomicrons are produced in the enterocyte. TAG is synthesised by the actions of CoA ligase, Monoacylglycerol transferase and diacylglycerol transferase (Shiau *et al.* 1980). The cholesterol component is derived from cholesterol obtained from food intake, while the phospholipid component is made from phosphatidylcholine. Finally apo B48, combine with intestinal lipid namely TAG, cholesterol and phospholipid to form chylomicrons (Goldberg and Schonfield, 1985). These are then secreted into the thoracic duct from where they enter into the circulation causing an increase in plasma TAG concentrations. Chylomicrons are spherical particles with a density < 0.95 g/ml. The quantity and fat content of a meal ingested determines the size and lipid content of the chylomicrons. Small fat loads produce small chylomicrons while a high fat load produces large low-density chylomicrons. The fatty acid composition of a meal is also important in determining the size of chylomicrons with PUFA meals producing larger chylomicrons than meals which are comprised of saturated fatty acids (Levy *et al.* 1991). When chylomicrons are passed into the circulation they acquire apoprotein from HDL. Apo CII is an activator of LPL while apo CIII inactivates LPL and prevents hepatic uptake.

1.6.2: Catabolism of chylomicrons

Chylomicrons rapidly lose their TAG component through the action of LPL which is present on the endothelial surface of extrahepatic tissues (skeletal muscle, cardiac and adipose tissues) hydrolyses the TAG core of the particle reducing the particle to about 5% of its original size (Green and Glickman, 1981). Apo CII which is acquired by the chylomicron when it enters the circulation. Apo CII is a cofactor for the activation of LPL (Connelly *et al.* 1996c) with apo CI and CIII having an inhibitory activity on LPL activity (Havel *et al.* 1973). The chylomicron particle depleted of TAG is known as a chylomicron remnant, which consists of CE, phospholipid and protein (Green and Glickman, 1981). These remnants acquire apo E and are then removed via apo E mediated hepatic uptake (Havel, 1986; Jackle *et al.* 1991). Delivery of chylomicrons to

the liver brings about the hepatic synthesis of VLDL. Chylomicrons are removed from the circulation by about 6-8 hours after the ingestion of a meal.

1.6.3: Chylomicrons and Coronary Artery Disease

Persistent chylomicronaemia contributes to increased development of risk factors. Evidence for this exists in the Stanford five city project (Gardner *et al.* 1996) where cases of CAD had significantly ($P = 0.001$) higher TAG concentrations compared with controls. A postprandial serum TAG concentration of < 1.5 - 1.6 mmol/l produces small VLDL (30-35 nm; d 1.006-1.010 g/ml) while TAG concentration > 1.5 - 1.6 mmol/l produces much larger VLDL (>35 nm; $d < 1.006$). Large VLDL competes less efficiently for LPL, extending the residence time in plasma (Griffin *et al.* 1997). This increases TAG exchange to LDL, which ultimately become small and dense due to the activities of HL (Griffin *et al.* 1997). LDL particles diameter were significantly smaller in cases of CAD when compared with controls. Plasma TAG concentrations in the form of delayed clearance of TRLs during lipaemic state in particular contributes to CAD by promoting the formation of dense atherogenic LDL particles (Griffin *et al.* 1994). During delayed clearance of chylomicrons from plasma during lipaemia, CETP catalyses the heteroexchange of CE from LDL for TAG from TRLs. TAG in LDL is hydrolysed by HL resulting in the formation of dense LDL. Prolonged lipaemia reduces HDL cholesterol concentrations yet again mediated through the actions of CETP where CE accumulates in TRLs with the accumulation of TAG in HDL₂ reducing the antiatherogenic capabilities of the HDL₂ fraction (Patsch *et al.* 1987). TAG rich HDL are then catabolised by HL reducing the cardioprotective HDL₂ fraction resulting in the formation of HDL₃. The importance of reducing TAG concentrations particularly during lipaemia is clearly evident by the negative impact TRLs have on lipoprotein metabolism. Karpe *et al.* (1994) has shown that the production of postprandial concentrations of small chylomicron remnants after oral fat tolerance tests correlates with the 5-year progression rate of CAD as assessed by coronary angiographies in post infarction patients.

There has been debate as to whether evidence exist for the deposition of apo B 48 in aortic tissues as detected by immunosorbption techniques in atherosclerotic tissues. Chylomicron remnants have been shown to be linked to the progression of coronary lesions (Simpson *et al.* 1990; Meyer *et al.* 1996) particularly in cases of CAD and are

incorporated into the endothelium in the same way as LDL (Karpe *et al.* 1997). More recent evidence confirms these earlier findings indicating that apo B48 and B100 lipoproteins are incorporated equally into aortic tissues during postprandial lipaemia (Karpe *et al.* 1999).

1.7.0: Very Low Density Lipoproteins (VLDL)

VLDL are synthesised in the liver (Schneeman *et al.* 1993) and in the small intestine. VLDL consist of a hydrophobic core, which contains TAG and a small quantity of CE. The lipid coat of the particle is made up of phospholipid, free cholesterol and apo B100, the major structural protein (Gibbon 1990), which is thought to be important in the secretion and reabsorption of catabolised VLDL. They provide a very important source of TAG of fuel for skeletal tissue during the starved state (Gibbons, 1990)

1.7.1: VLDL subclasses

VLDL reside in the density range of $d > 1.006$ g/ml and may be divided into large and small particles based on their flotation rates using density ultracentrifugation. Large VLDL (VLDL₁) have a Svedbreg flotation of (Sf 60 - 400) and small VLDL (VLDL₂) have a Svedberg flotation of (Sf 20 - 60). The higher the TAG content of the VLDL the larger the particle produced. VLDL₂ are formed from VLDL₁ through the action of LPL (Griffin and Packard, 1994).

1.7.2: Formation of VLDL

The sources for TAG for the formation of VLDL are, lipolysis, plasma NEFA, NEFA synthesis *de novo*, TAG recycled from lipoproteins taken up by the liver and hepatic stores of TAG (Beylot *et al.* 1994). TAG, which is the major component of VLDL, is manufactured via the glycerol-3-phosphate pathway in the rough endoplasmic reticulum (ER) of the liver (Field and Mathur, 1995). In the lumen of the ER apo B100 is added, the particle is then passed on to the Golgi apparatus, where glycosylation of the protein occurs, then the protein is secreted. Nascent VLDL particles receive apo CII which is important in LPL activation. The importance of apo CII is demonstrated in those individuals who lack this protein and display hypertriglycerdaemia (Breckenridge *et al.* 1978). Apo CIII and CI play an inhibitory role on LPL activity and apo E which is

important in hepatic uptake of the particle. VLDL is manufactured from chylomicrons through LDL receptor mediated uptake of chylomicrons in the liver (Willnow *et al.* 1994). Within the liver this chylomicron-derived lipid is processed and assembled into apo B100 VLDL. An excess of TAG in the plasma leads to the formation of VLDL (Karpe *et al.* 1994). Following the ingestion of a fat meal, 80 % of the rise in plasma TAG is accounted for in particles of hepatic origin (Schneeman *et al.* 1993; Karpe *et al.* 1993). VLDL particles accumulate during postprandial lipaemia, as chylomicrons are the preferred substrate of LPL (Berr *et al.* 1992). Potts *et al.* (1991) demonstrated that there is reduced clearance of VLDL TAG in response to a fat rich test meal. Prolonged residence time of VLDL during postprandial lipaemia leads to the increased transfer of CE and TAG between HDL and VLDL in normolipidaemics as a consequence of CETP activity (Mann *et al.* 1991).

1.7.3: Catabolism of VLDL

The apo protein (apo CII, CIHI), lipid composition and size of the VLDL particle will determine the catabolism of VLDL. In normolipidemics small VLDL₂ are produced, which are an excellent substrate for LPL. As the VLDL particle shrinks it loses its apo proteins to HDL (Harvel *et al.* 1994), the VLDL is further catabolised in the liver by HL resulting in the formation of LDL. The VLDL remnants are also a very good substrate for CETP, which transfers CE from HDL to these particles in exchange for TAG. Some of the CE is hydrolysed to cholesterol for bile production, from where it is excreted. Large VLDL₁ are not a good substrate for LPL and are therefore removed primarily in the liver by apo E receptor mediated uptake (Eisenberg *et al.* 1987).

1.7.4: CETP activity and VLDL

VLDL is one of the primary substrates for CETP activity as they provide a source of TAG, one of the lipids which is redistributed between lipoproteins as a consequence of CETP activity (See section 1.2.2). They are thought to be vital in the modulation and direction of lipid transfer between lipoproteins (Griffin, 1996). During postprandial lipaemia there is an increase in the production of VLDL due to the activity of LPL and hepatic uptake of chylomicron remnants as outlined in section 1.6.3. Lassel *et al.* (1998) investigated which of the TRLs was the preferred acceptor of CE transfer from HDL during postprandial lipaemia. These models showed that chylomicrons did

not accept any CE from HDL during postprandial lipaemia. VLDL₁ was found to be the major acceptor of CE among the TRLs and this was because of the greater numbers of them present in plasma during post-prandial lipaemia. Quantitatively VLDL₂ was the primary acceptor of CE.

1.7.5: VLDL and Reverse Cholesterol Transport

VLDL provides a source of transfer of CE from HDL to VLDL through the catalytic action of CETP. The majority of cellular cholesterol undergoes RCT through this indirect RCT transport route. VLDL provides a major source of TAG for CETP transfer. It is also one of the primary acceptors of CE from HDL as a result of CETP activity (See section 1.1.4). The VLDL is then catabolised by lipase's eventually becoming LDL, which has the appropriate apoprotein, apo B100 which allows efficient hepatic uptake of these cholesterol rich particles. The transfer of CE into VLDL allows CE to be transferred into apo B100 lipoproteins which have high affinity for receptor mediated uptake. VLDL plays a pivotal role in mediating the passage of CE from HDL to LDL allowing a more efficient hepatic uptake of CE.

1.7.6: VLDL and CHD

In patients with exaggerated postprandial lipoprotein response, VLDL and chylomicrons all compete for the same lipolytic pathway during postprandial lipaemia (Bergeron and Havel, 1997). As VLDL gain apo CI and CIII both of which impair LPL activity there is a reduction in the efficiency of VLDL clearance which is the precursor step to the production of dense LDL (Krauss *et al.* 1994) due to the activities of CETP. The extent of postprandial lipaemia is important in determining the CE incorporation into VLDL, with delayed clearance of VLDL increasing interaction time with CETP thereby increasing the CE content of VLDL and the TAG content of HDL, resulting in the production of atherogenic dense LDL and dense HDL₃. This is known as an atherogenic lipoprotein phenotype and clearly implicates exaggerated VLDL production and residence in the plasma in the progression of CAD (Mann *et al.* 1991).

Dubois *et al.* (1994) demonstrated that the addition of cholesterol in a dose response study was not incorporated into chylomicrons or VLDL. Foam cells occur in individuals with persistent chylomicronaemia i.e. hyperlipoproteinemias or in those with exaggerated postprandial response or an abundance of chylomicron remnants. The

in vitro incubation of macrophage with TRL, macrophage incorporated TRL resulting in a large amount of TG and CE within them, which was capable of being stored in the arterial wall. Cholesterol is only found within these atherogenic lesions. A possible explanation for this is that the TAG may be catabolised either by the cells or by LPL leaving cholesterol within the foam cell which cannot be metabolised. This hypothesis was originally put forward by Zilversmit, (1979), where it was proposed that TRL may be atherogenic in the postprandial state and therefore capable of contributing lipid to the artery wall.

1.8: High Density Lipoprotein

Plasma HDL are small dense spherical lipid-protein complexes. They originate from the liver and the small intestine. They consist of 50 % protein and 50 % lipid. The major lipid components of HDL are phospholipid, free cholesterol, cholesterol ester and TAG. The major apoproteins of HDL are apo AI (Mr 28,000) and apo AII (Mr 17,000). There is a range of other apo proteins associated with HDL, which include apo E and apo C (C1, CII, CIII). HDL are classified according to their size ranging from the large less dense HDL₂ (9.8-12.2 nm) fraction to the smaller more dense HDL₃ (7.2-8.2 nm) fraction. HDL are inter-converted between fractions by a number of enzymes including CETP, PLTP, LCAT and LPL. The main function of HDL is to act as a carrier of cell derived phospholipid and cholesterol to the liver. This process known as reverse cholesterol transport (Glomset *et al.* 1968). HDL also acts as a reservoir for apoproteins which switch between lipoproteins during postprandial lipaemia.

1.8.1: HDL subclasses

The classification of HDL constantly changes between HDL₂ and HDL₃ subfractions by the action of enzymes such as LCAT, PLTP, CETP and LPL. HDL may be separated into sub-classes based on their size and apoprotein composition. Using gradient gel electrophoresis HDL may be separated by size into 5 distinct subclasses, HDL_{2a}, HDL_{2b}, HDL_{3a}, HDL_{3b} and HDL_{3c} (Nichols, 1986).

HDL may also be classified according to apoprotein composition of apo AI and apo AI/AII (Chung and Albers, 1985). These apoproteins modulate the action of the HDL associated enzymes e.g. LCAT, CETP and HL. Apo A IV containing HDL are another group of HDL whose function in reverse cholesterol transport is not fully

understood (Duverger *et al.* 1994). Apo E containing HDL are thought to be important for the hepatic uptake of HDL because apo E rich HDL are the preferred substrate of hepatic lipase (Thuren *et al.* 1992).

Using agrose gel electrophoresis, HDL may be separated into pre-beta and gamma mobility (van Eckardstein *et al.* 1994). Both of these HDL fractions contain apo AI and their mobility is altered by their lipid and protein composition (Davidson *et al.* 1994). Another group of HDL is the β -migrating fraction, which are rich in apo E. All of these fractions are thought to be important in initiating the first stage of reverse cholesterol transport, cellular cholesterol efflux (Castro and Fielding, 1988).

1.8.2: Formation of HDL

It is still unclear how HDL is produced. There are a number of potential pathways, which lead to the production of HDL. The main sources of apo A1 for HDL include the intestine and liver as a result of HL activity (Fruchart *et al.* 1993). This apo A1 acquires cellular free cholesterol (FC) and lipid from chylomicron remnants which are produced as a consequence of LPL activity resulting in the generation of pre-beta HDL (Hara and Yokama, 1992). They then continue to acquire more FC from cells and other lipoproteins until they become mature HDL (Skinner *et al.* 1994). A number of hypothesis have being proposed as to the mechanism which initiates the efflux of cholesterol from cells into HDL. The first is that cholesterol efflux is derived from protein kinase, which is initiated by the high-affinity binding of HDL to the cell surface HDL receptor (Mendez *et al.* 1991). Apo A1 is thought to be important in binding the HDL particle to cells, and thereby initiating cellular cholesterol efflux. Evidence for this comes from studies in bovine aortic endothelial cells which demonstrated that apo A1 rich HDL were primarily responsible for the initiation of the efflux of cellular cholesterol (Fructart *et al.* 1992). It is proposed that the apo A1 of the pre-beta 1 HDL fraction induces a conformational change which promotes the desorption of cholesterol from the cell membrane surface into the extracellular space (Fielding & Fielding 1995b). Alternatively, it is hypothesised that the cholesterol leaves the cell in an unesterified form, whereby it desorbs itself from the plasma membrane into the extracellular fluid by diffusion (Francone *et al.* 1990). Albumin binds to this extracellular cholesterol making it an attractive initial acceptor of cellular cholesterol. HDL then comes into contact with cholesterol/albumin complex and absorbs the cholesterol in to its lipid core. In

experiments by Fielding (1982), it was shown that albumin was responsible for 50 % of cholesterol efflux from cells upon removal of Apo A1.

1.8.3: Maturation of HDL

During postprandial lipaemia the activities of many enzymes including CETP, LCAT and HL have a direct effect on HDL composition. HDL maturation occurs as phospholipids, cholesterol and apoprotein produced as a result of LPL activity become associated with pre-existing or nascent HDL, or with nascent discoidal particles. LCAT catalyses the maturation of HDL by promoting the esterification of cholesterol, which then fills the core CE levels of discoidal HDL (Skinner, 1994) transforming them into mature HDL. Apo A1 is an important co-factor in LCAT activity (Jonas *et al.* 1987). Hennessy *et al.* (1993) demonstrated that the free apo A1 produced as a result of HL activity was central in the formation of new pre-beta HDL. As the particle matures LCAT then loses affinity for the HDL (Fielding and Fielding 1991). As a mature particle HDL will change between HDL₃ and HDL₂ sub-fractions through the cyclical acquisition and loss of lipids. HDL matures by the acquisition of phospholipid and cholesterol from chylomicron remnants produced as a consequence of LPL during postprandial lipaemia. CETP brings about formation of TAG rich HDL₂ by exchange of CE and TAG between lipoproteins as described in section 1.3.4.

1.8.4: Catabolism of HDL

HDL cholesterol is delivered to the liver and are themselves catabolised or undergo selective uptake of their cholesterol. As the HDL become enriched with CE they acquire apo E at the expense of apo A1. Apo E facilitates hepatic uptake of HDL by the LDL receptor via endocytosis (Skinner *et al.* 1994) or another apo E hepatic receptor. It has been suggested that apo E mediated uptake of cholesterol accounts for about 10% of total cholesterol returned to the liver (Franceschini, 1991). HDL may also undergo selective uptake of its CE in the liver via the scavenger receptor SC-B1 without the breakdown of the lipoprotein (Acton *et al.* 1996). The cholesterol content of HDL is important in determining the rate of hepatic uptake of HDL (Reichl *et al.* 1989). Hepatic lipase activity results in the catabolism of HDL in particular HDL₂. Apo A1/A11 containing HDL₂ is the preferred substrate of HL over HDL₂ containing only apo A1 (Mowri *et al.* 1992). HDL which is present in the plasma are catabolised by HL

resulting in the loss of CE and the dissociation of apo A1 (Hennessy *et al.* 1993) and its other lipoproteins. The apoproteins of HDL are recycled forming free protein, which is important in the formation of HDL as described in section 1.8.2-1.8.3. The CE component of HDL is delivered to the lysosomes (Johnson *et al.* 1991). This CE is hydrolysed to free cholesterol, which then becomes part of the intracellular pool. The cholesterol has two fates, it can be assimilated into new lipoproteins namely VLDL and LDL or it may be excreted as bile (Fielding and Fielding, 1995a). Bile is manufactured in the parenchymal cells from endogenous cholesterol production and from cholesterol received to the liver via RCT. The liver is the only organ in the body from which cholesterol may be excreted.

1.8.5: HDL and CETP in RCT

RCT is a dynamic process in which many factors affect the rate of each step: efflux, esterification, transfer and clearance of cholesterol (Fielding and Fielding, 1995a). HDL plays a pivotal role in the process of RCT (Miller, 1985). The major function of HDL is as a carrier of cellular derived cholesterol to the liver for recycling or excretion (See Section 1.8.4). When this cholesterol is returned to the liver it is either assimilated into apo B100 lipoproteins known as VLDL or else it is excreted as bile. CETP is a HDL associated enzyme, and as a result of its activities it brings about the transfer of cholesterol ester into apo B containing lipoproteins (VLDL and LDL) from where they may be returned to the liver. CETP is also important in HDL catabolism also as it enriches HDL with TAG which make them a better substrate for HL (Patsch *et al.* 1984), resulting in the regeneration of HDL₃. Alternatively, the HDL is catabolised resulting in the formation of new pre-beta HDL as described in section 1.8.2. This is potentially very important in generating cellular cholesterol efflux. In a study by Chiba *et al.* (1997) it was noted that a deficiency of CETP had major implications in HDL lipid compositions as HDL formed was particularly rich in CE. RCT could therefore only occur through direct uptake in the hepatocyte.

1.8.6: HDL as a risk factor for the development of CHD

HDL is generally regarded as the most cardio-protective lipoprotein fraction because they promote cellular efflux of cholesterol (See section 1.8.2). HDL is a powerful predictor of CHD risk (Barter, 1995). Evidence that HDL₂ is a more powerful predictor of

CHD risk comes from a study by Miller, (1987) where it was demonstrated that HDL₂ levels were the most important predictor of CHD. Similar results were observed by Buring *et al.* (1991). When adjusted for age and sex, those suffering from CAD tended to have lower HDL cholesterol levels (35.0mg/dl) than did controls (43.3mg/dl) (Buring *et al.* 1991). A similar trend was observed in the HDL₂ subfraction of the same study group (12.1 mg/dl) in those with cases of CHD and (17.7 mg/dl) in control individuals. Therefore the HDL₂ subfraction seems to be the most discerning factor in terms of CHD risk assessment (Patsch, 1994). HDL₃ was found to be as important a predictor of CHD and exhibited the same cardioprotective effects as HDL₂ (Buring *et al.* 1991). Abnormalities in HDL metabolism are common in those at risk and/or suffering from CHD (Schaefer *et al.* 1989). All of these studies found significant correlation between low HDL levels and increased risk of CHD. Therefore individuals with a high risk of CHD have low concentrations of HDL, and lose the beneficial effects of the cardioprotective HDL. In a study by Genest *et al.* (1992), it was shown that up to 40 % of patients suffering from CHD had very low HDL levels. The NIH Consensus Development Panel provided actual data in relation to HDL concentration and the risk of CHD, where for every 0.03 mmol/l increase in HDL there was a 2 - 3 % reduction in CHD risk.

1.8.7: Conditions which predispose to low levels of HDL

Genetic conditions such as familial combined hyperlipidemia are an important contributor to the development of CHD. All individuals with this condition suffer from low HDL levels (Grundy *et al.* 1987). With Tangiers disease there are extremely low HDL levels due to the accelerated catabolism of HDL. Familial hypertriglyceridaemia results in the elevation of TAG levels with the consequential low HDL levels (Brunzell *et al.* 1983). Individuals suffering from non-insulin dependent diabetes mellitus show a tendency towards developing CAD (Howard, 1987) tend to have very low HDL cholesterol levels.

1.8.8: Protective role of HDL

The first mechanism by which HDL are protective is that they promote the efflux of cellular cholesterol from peripheral cells and transport cholesterol to the liver (Fielding *et al.* 1991). The mechanism by which this occurs has already been described previously

(See sections 1.8.2-1.8.3). HDL also prevents the oxidation of LDL which is common in the progression of atherogenesis (Witztum, 1991a). Oxidised LDL are formed as the result of the accumulation of LDL in the plasma. These oxidised LDL accumulate in the arterial wall, this process is further accelerated by oxidised LDL (Beisiegel, 1998). HDL serves to protect LDL from oxidation by transferring oxidised phospholipid from LDL to HDL (Parthasarathy *et al.* 1990). It has been demonstrated that the HDL₂ subfraction is the most cardioprotective fraction of HDL (Tall, 1990). Evidence for the antioxidative protective role for HDL comes from experiments by Parthasarathy *et al.* (1990) which demonstrated that HDL reduced LDL oxidation when induced by metal ions in endothelial cells *in vitro*. A reduction in the net negative charge on LDL surface resulted in reduced macrophage degradation of LDL.

The mechanism by which this is done is through the action of HDL associated hydrolytic enzymes, which are responsible for inactivating reactive species of oxidised lipids which form on LDL (Banka, 1996). One of these HDL associated enzymes, platelet activating factor acetylhydrolyase, exhibits substrate specificity for short chain peroxidised residues of phospholipid, which are produced as a consequence of oxidative damage (Stremmler *et al.* 1991). The second of these HDL associated enzymes is paroxonase which contribute to the antioxidant role of HDL by hydrolysing oxidised phospholipid (Watson *et al.* 1995). Most lipid peroxidation products are produced from fatty acids and cholesterol esters in the form of hydroperoxides (i.e. Cholesteryl ester hydroperoxides) are found in plasma HDL. HDL is thought to accept and neutralise these cholesteryl ester hyperperoxides, and other hydroperoxides through the action of paroxonases.

Christinson *et al.* (1995) have demonstrated that CETP is involved in the transfer of these hydroperoxides from LDL to HDL. HDL are susceptible to oxidation themselves and the result of this oxidation is impaired ability to promote cellular efflux of cholesterol, inhibited LCAT activity, and enhanced clearance of HDL from the circulation (Banka, 1996).

1.9.0: Low Density Lipoprotein (LDL)

LDL is the major cholesterol carrying lipoprotein fraction. They contain one apo B100 per particle which is a structural protein and makes up about 25% of the particle. The apo B100 is associated with phospholipid and free cholesterol in the

surface membrane of LDL. The rest of the particle (75%) is made up of lipid of which the majority of this is cholesterol ester (Gibbons, 1994). The polar surface coat of LDL is made up of phospholipid, free cholesterol and apo B100, which is a structural protein, which acts as a ligand for the cellular LDL receptor. It contains a high content of CE, which accounts for 50% of total LDL particle weight. In normolipidemics there is a preponderance of intermediate LDL which accounts for 50 % of total LDL mass. LDL is distributed over a density range of 1.019-1.063 g ml⁻¹, and may be divided into 3 major subclasses.

1.9.1: LDL subclasses

LDL may be resolved into 3 subclasses I, II, III using either density gradient ultracentrifugation or gel electrophoresis. Using ultracentrifugation, LDL subfractions may be isolated using the following density gradients, large LDL, d 1.02-1.03g. ml⁻¹, intermediate LDL, d 1.03-1.04 g. ml⁻¹ and small LDL, d 1.04-1.06g. ml⁻¹, which are protein rich lipid poor lipoproteins (Chapman *et al.* 1998). They differ in their lipid composition, size, and affinity for receptors. The lighter LDL subclasses have a higher TAG content and are excellent substrates for HL. LDL II is the principle carrier of CE. At high plasma TAG concentrations, there is increased production of LDL I and II. HL catabolises these LDL resulting in the production of LDL III which are atherogenic. Evidence for this comes for Goldberg *et al.* (1982) where LDL was incubated with HL and the overall LDL particle size was reduced. This is not as predominant in women as they have lower HL activity. The production of light LDL is positively associated with LPL activity and is used as a marker of efficient lipolysis (Karpe, 1993). Those deficient in LPL and normal HL activity, and CETP activity tend to get the formation of small dense LDL (Griffin, 1997). In normolipidaemics, there tends to be a preponderance of LDL II making up almost 50% of the overall LDL fraction.

1.9.2: Formation of LDL.

LDL are manufactured in the liver from the catabolism of VLDL particles which are catabolised to IDL and then to LDL from the action of LPL. LPL acts on chylomicrons and VLDL resulting in the formation of LDL. LDL formation occurs within the liver as a consequence of the action of hepatic lipase. IDL which is taken up by the liver does not contribute to the circulating LDL fraction (Fielding, 1997).

1.9.3: Catabolism of LDL

Two-thirds of LDL is removed from the plasma in the liver, while the rest is removed via extrahepatic tissue (Griffin *et al.* 1994). LDL acquires apo E, which is vital in liver uptake. Apo B100 is vital for receptor mediated removal of LDL. LDL II is removed most efficiently from the plasma, with 66 % being removed in the liver (Nigon *et al.* 1991). LDL, which is catabolised in cells, undergoes lysosomal degradation, releasing free cholesterol into the cytosol. Free cholesterol within the cell is important in the regulation of the LDL receptor gene. This cellular free cholesterol brings about the down regulation of the LDL receptor gene (Hobbs *et al.* 1992). Low levels of cellular cholesterol leads to the upregulation of this gene.

1.9.4: CETP activity and LDL

Considerable evidence exists to implicate CETP in the remodeling of the LDL subclasses and channeling of CE from HDL and large and small LDL into receptor active, less atherogenic LDL II. CETP has been implicated in remodeling of LDL subclasses, because in those who have CETP deficient plasma, an abnormal distribution of LDL subclasses occurs consisting of LDL I and LDL III, with a lack of LDL II, compared with controls (Sakai *et al.* 1991). A similar LDL subclass pattern was found in a study with alcoholic individuals, who are known to have a deficiency in CETP. Abstinence from alcohol in this patient group resulted in a normalisation of their LDL subclasses (Hirano *et al.* 1992). This data suggests that CETP is responsible for the shuttling of CE between LDL subgroups and is responsible for the generation of the more receptor active LDL II (Griffin, 1997). This suggests that CETP as well as being involved in the transfer of neutral lipids between VLDL, LDL and HDL, is also involved in the shuttling of CE from LDL I and LDL III into the LDL II subfraction LDL (Griffin *et al.* 1997). LDL II is the most active acceptor of CE from HDL (Griffin *et al.* 1997). The transfer of CE may occur independently between lipoprotein fractions without the reciprocal transfer of TG (Lagrost *et al.* 1995). With delayed clearance of TAG lipoproteins (chylomicrons and VLDL) which occurs with individuals with CAD evidence exists that CETP transfers TAG from chylomicrons and VLDL into LDL with the reciprocal exchange of CE into chylomicrons. These TAG rich LDL are susceptible to hydrolysis by HL which reduces the size of these lipoproteins resulting in a

preponderance of small dense atherogenic LDL (Austin *et al.* 1990). Increase TAG concentrations result in the production of dense type III LDL, which are not easily removed from the circulation. In normolipidaemics with a TAG < 1.5 mmol/l type II LDL distribution predominates, while with TAG concentrations > 1.5 mmol/l the production of the more atherogenic LDL III predominates. Elevated TRL concentrations result in the production of these LDL subclasses, which is mediated through the actions of CETP producing TAG rich LDL. LPL acting on these TAG rich LDL resulting in the formation of dense LDL. Tan *et al.* (1995) suggests that there is a significant relationship between TAG concentrations and the distribution of LDL subclasses. This relationship appears to be mediated through the actions of CETP.

1.9.5: LDL in RCT

LDL is the primary cholesterol carrying lipoprotein subfraction in plasma. During the process of RCT, cellular cholesterol is passed to HDL from where it is transferred to VLDL through the action of CETP and the VLDL are finally catabolised to LDL. LDL is the most receptor active as regards apo B mediated uptake by the liver. The majority (80%) of RCT occurs through this indirect pathway. LDL is the final step in RCT from where hepatic uptake of LDL occurs. The hepatic receptors recognise the apo B lipoprotein component of the LDL particle. This ultimately leads to the lysosomal degradation of the particle. Free cholesterol (FC) is released into the cell. This FC is important in regulating cellular metabolism as it leads to the down regulation of the apo B100 receptor gene leading to down regulation of receptor synthesis (Brown, *et al.* 1986). Therefore raising hepatic cellular cholesterol levels will result in the down regulation of the LDL receptor. The liver's ability to dispose of its cholesterol in the form of bile could, indirectly determine plasma LDL cholesterol levels.

1.9.6: LDL and CHD

The importance of elevated LDL cholesterol as a risk factor to CAD is indisputable (Levine *et al.* 1995). This is confirmed by findings of Austin *et al.* (1994) where preponderance of small dense LDL is more common in CAD cases than in controls. Accumulation of LDL in the plasma leads to the deposition of LDL in the arterial wall. This occurs as a result of the oxidation of LDL and as a result of lipolytic enzymes remodeling lipoprotein particles. LDL accumulation may occur as a result of

defects in LDL receptor (Hobbs *et al.* 1992) or in the apo protein on the LDL. There are many mutations within the LDL receptor gene are often the cause of hypercholesterolaemia. Down regulation of the LDL receptor gene as a result of cellular cholesterol levels also helps to raise LDL cholesterol levels. Mutations of the apo B100 component of the LDL results in inadequate binding to the LDL receptor resulting in hypercholesterolaemia (Vega and Grundy, 1986). Competition for LDL receptor occurs during postprandial lipaemia as a result of the production of VLDL, which also contain apo B100. These lipoproteins compete with LDL for receptor sites, thereby extending the residence time of LDL in the plasma. Distribution of LDL subclasses is also important in the contribution of LDL to CAD with the production of the less receptor active LDL I and III not being efficiently removed from plasma allowing them to be incorporated into endothelial tissues. Small dense LDL are a marker of CAD risk, with 40 –50 % of CAD patients having elevated concentrations of dense LDL (Stampfer *et al.* 1996)

The increase in cholesterol levels which occurs during the aging process, still remains a mystery but a potential cause could be the down regulation of LDL receptors with age. LDL can accumulate in plasma through one or more of the above pathways and are therefore susceptible to oxidative modification. Modified LDL are taken up by the macrophage scavenger receptor resulting in their transformation to foam cells. Foam cells are a major feature of lipid rich atherosclerotic plaques which secrete numerous factors which exhibit proatherogenic, proinflammatory and prothrombogenic activities. The quality of the LDL is important in determining if they are susceptible to oxidation and macrophage uptake resulting in atherosclerosis (Krauss, 1994; Superko and Krauss, 1994) with small dense LDL being more susceptible to oxidative modification (Tribble *et al.* 1992).

1.9.7: CETP in relation to lipoprotein metabolism

It is clear from this review on CETP metabolism that the enzyme plays a pivotal role in the transfer of lipids between lipoproteins. This increases during postprandial lipaemia, during which state the optimal modification of lipoproteins occurs. As a consequence of its activity, CE rich LDL are formed with the reduction of cholesterol in the HDL fraction. It is evident that CETP in conjunction with other enzymes may contribute to atherogenesis. Plasma cholesterol concentrations also seem to

correlate with CETP mass and activities. Whether this is as a result of a CETP genotype or as a result of plasma cholesterol itself remains to be confirmed as there are so many other dynamic processes including CETP activity occurring during lipaemia.

1.10.0: Plasma TAG as a risk factor for Coronary artery disease

Plasma cholesterol has been well established as a risk factor for CAD. There has been an ongoing debate as to the importance of plasma TAG concentration as a risk factor for the development of CAD. In an investigation by Bainton *et al.* (1992), an attempt to measure of the role of TAG and HDL₂ in predicting the occurrence of CHD in a population of 5000 middle aged men was carried out. When adjusted for plasma HDL cholesterol concentrations, plasma TAG concentration was as an important risk factor for CAD as plasma cholesterol concentrations. Simpson *et al.* (1990) studied the extent of postprandial lipaemia in 34 cases of CAD verses 18 controls. The findings were that the magnitude of postprandial lipaemia was elevated in cases of CAD. However the contribution of elevated postprandial lipaemia as a risk factor to CAD was not assessed in this investigation. Patsch *et al.* (1992) investigated the relationship between CAD and postprandial lipaemia in 62 angiographically verified male with 40 age-matched controls. The maximum TAG and 8 hour postprandial TAG concentrations were significantly higher in the CAD group, with the 6-8 hour TAG concentrations after a test meal being important in predicting the presence or absence of CAD. Meta-analysis by Austin *et al.* (1997) in a 12 population based prospective study evaluating a relationship between plasma TAG and the incidence of CAD events, revealed that plasma TAG concentration was a independent risk factor for CAD. Stampfer *et al.* (1996) & Miller *et al.* (1998) concluded that fasting plasma TAG is a strong independent risk factor for MI in individuals who had confirmed CAD. Postprandial TAG concentration is also regarded as a significant risk factor for CAD and is strongly correlated with fasting plasma TAG concentrations (Zilversmit, 1995). Any therapy, which would reduce postprandial lipaemia, would be considered to be anti-atherogenic.

1.10.1: Elevated plasma TAG and alters lipoprotein cholesterol concentrations

When the effect of TAG on lipoprotein metabolism is examined, it is apparent that TAG determines the lipid composition of HDL through the actions of CETP.

Evidence exist for the inverse association between HDL cholesterol concentrations and plasma TAG concentrations where high TAG concentrations resulted in low HDL cholesterol concentrations and increased coronary mortality (Crique *et al.* 1993). Analysis of the Framingham study showed that high plasma TAG concentrations in conjunction with low HDL cholesterol concentrations was associated with increased risk of CAD (Castelli *et al.* 1986). Hokanson & Austin (1996) assessed the risk of TAG concentrations on CAD by carrying out a meta-analysis of 17 studies carried out from 1965 to 1994. There is consistent evidence from numerous studies, of the negative association between plasma TAG and HDL cholesterol concentrations (Sprecher *et al.* 1994). This association may be mediated through the activities of CETP. Elevated plasma TAG results in the production of dense LDL which are much more atherogenic than less dense LDL. Dense LDL is a risk factor for CAD (Griffin *et al.* 1994) as they are prone to oxidation and macrophage uptake. TAG-rich VLDL, produced during postprandial lipaemia result in increased postprandial TAG concentrations. Prolonged postprandial lipaemia and delayed clearance of chylomicrons occur in individuals with CAD when compared with control subjects (Groot *et al.* 1991). Patsch *et al.* (1992) suggests that postprandial TAG concentrations may be a more important predictor of CAD risk than is fasting plasma TAG. These findings are confirmed by Karpe *et al.* (1994) where the production of apo B48 lipoproteins, particularly during postprandial lipaemia was directly related to the progression of CAD. Cholesterol rich atherogenic chylomicron remnants are produced when there is prolonged postprandial lipaemia, which are as atherogenic as LDL (Zilversmit *et al.* 1979).

Prolonged chylomicron residence in the plasma during lipaemia has a devastating effect on HDL cholesterol concentrations in particular HDL₂ cholesterol concentrations, with which the extent of postprandial lipaemia is inversely related to HDL₂ cholesterol levels. As a consequence of CETP activity which is increased during postprandial lipaemia (Tall *et al.* 1986) and raised in hyperlipidaemias (Bagdade *et al.* 1992a; Abbey *et al.* 1990) there is increased transfer of TAG from chylomicrons and in particular VLDL into HDL forming TAG rich HDL₂. A reciprocal transfer of CE into chylomicrons and VLDL occurs. CE rich VLDL and chylomicrons and TAG rich HDL₂ are produced. These cholesterol rich chylomicrons and VLDL are susceptible to oxidation and are rapidly incorporated into arterial wall ultimately form cholesterol deposition in arteries (Shaikh *et al.* 1991). HDL₂ which are rich in TAG are a

preferential substrate for HL which catabolises the HDL₂ into HDL₃ (Patsch *et al.* 1987 Patsch *et al.* 1984), resulting in a reduction of the cardioprotective HDL₂ fraction. There is also lipid exchange between TRL and LDL mediated by CETP in which TAG is transferred to LDL and CE to TRL. The LDL is catabolised by HL, which results in the formation of dense LDL (Karpe *et al.* 1993). It is evident that the presence of TAG rich lipoproteins in plasma has an impact on HDL and LDL lipid compositions. Delayed clearance of TRL by hepatic lipase during postprandial lipaemia impacts on LDL and HDL lipid compositions. This is mediated by CETP, resulting in the preponderance of atherogenic small LDL and HDL₃ an atherogenic lipoprotein profile as described by Austin *et al.* (1997). Any therapy which lowers plasma TAG concentration will improve overall lipoprotein profile regarding CAD risk due to the effects of plasma TAG concentrations have on HDL and LDL.

1.11.0: Effect of dietary fat intake on CETP

During alimentary lipaemia there is an increase in CE transfer between lipoproteins (Tall, 1986; Dullaart, *et al.* 1989). Many other studies have been carried out, which have shown that CETP activity increases 1.1 fold to 3 fold during postprandial lipaemia. Tall (1986) demonstrated that there is increased binding of CETP to HDL during postprandial lipaemia, due to an increase in phospholipid concentration. This result suggests that CETP is re-distributed between lipoproteins during postprandial lipaemia.

The effect of various types of dietary fats has also been investigated. Lottenberg *et al.* (1996) found that there was no significant change in CETP activity following dietary intervention with either polyunsaturated or saturated fat diets. They also observed that the postprandial increase in CETP activity was in proportion to the TAG postprandial response. In a study by Groener *et al.* (1991), similar results were observed following the administration of a polyunsaturated fat (PUFA) diet over a number of weeks as was found by Lottenberg *et al.* (1996a). They observed a decrease in CETP activity following a monounsaturated fat (MUFA) diet over same time course. Diets rich in trans-fatty acids show an increase in CETP activity (Abbey *et al.* 1994). It has been proposed that CETP exhibit substrate specificity for CE due to their different acyl chain composition. Clearly this will depend on the type of fat present in the diet. Severe alcohol consumption reduces CETP mass and activity by 27 % and 22 % respectively (Hannuksela *et al.* 1992). Upon abstention from alcohol these individuals were found to have CETP mass and activity higher than the controls. At a molecular level diet appears to result in the upregulation of the CETP gene as demonstrated by Quinet *et al.* (1990) in a rabbit model where a 2.5 fold increase in CETP mRNA and a 3.2 fold increase in activity was found in response to an atherogenic diet administered over 30 days.

1.11.1: Effect of dietary fatty acids on CETP

A number of studies have investigated the effects of acute and chronic fat intake on CETP mass and activity in normolipidaemic, hyperlipidaemic and NIDDM subjects (Abbey *et al.* 1990; Lottenberg *et al.* 1996b; van Tol *et al.* 1995; Bagdade *et al.* 1992a,b,c; Bagdade *et al.* 1996; Abbey *et al.* 1994; Kurushima *et al.* 1995; Groener *et al.* 1991; Jansen *et al.* 2000) (See table 1.1.1 and 1.1.2). Dietary fatty acids influence plasma TAG and cholesterol concentrations and changes in lipoprotein composition lipoprotein composition may be determined by CETP. CETP is associated with

increased risk of CAD (Bhatnagar *et al.* 1993). Therefore any alterations in CETP as a result of dietary intake and the consequent effects on CETP may prove to be either pro or anti-atherogenic.

Studies involving the administration of saturated fatty acids and their effects on CETP have shown that plasma and LDL cholesterol concentrations increase with a rise in CETP activity (Jansen *et al.* 2000). Increases in LDL cholesterol are thought to be attributed to the down regulation of hepatic LDL receptor (Wollet *et al.* 1992; Hajashi *et al.* 1993). Trans fatty acids also result in increases in CETP activity (van Tol *et al.* 1995). Trans fatty acids also raise LDL and total cholesterol concentrations similar to the effect of saturated diets. van Tol *et al.* (1995) demonstrated that an 18 % increase in CETP activity when 8 % trans fatty acid was administered for 3 weeks when compared to subjects consuming stearic or linoleic diets. In this study the increase in CETP activity was attributed to a rise in CETP mass, while the influence of increases in plasma cholesterol and TAG concentration on CETP activity using an endogenous CETP assay was not assessed. Similar increases in CETP activity in response to trans fatty acids were observed by Lagrost *et al.* (1992) and Abbey *et al.* (1994). These increases were attributed to a substrate effect.

Monounsaturated fatty acids, in contrast to saturated and trans fatty acids, appear to reduce CETP activity. Safflower oil (rich in linoleic acid) produced a decrease in CETP mass of 13 % and activity of 16 % (Abbey *et al.* 1990) and this decrease in activity has been attributed to a substrate effect. Van Tol *et al.* (1995) found that linoleic or stearic acid rich diets produced no change in CETP activity. The assay employed in this study was more an estimation of CETP mass rather than activity. Groener *et al.* (1991) found that a monounsaturated fatty acid diet reduced CETP by 10 %. A decrease in LDL and total cholesterol concentrations was also observed and the decrease in these correlated with the reduction in CETP, while a negative association with HDL cholesterol and CETP was noted. In normolipidaemics, monounsaturated fatty acids also induced a reduction in CETP activity with reductions in LDL and plasma cholesterol concentrations similar to the findings of (Abbey *et al.* 1994)

Using a hamster model Kurushima *et al.* (1995) demonstrated that oleic acid supplemented to an atherogenic diet induced a reduction in CETP, LDL and total cholesterol concentrations. A potential mechanism for the reduction in LDL cholesterol is revealed by animal studies (Kurushima *et al.* 1995) which suggest that MUFA intake

inhibited LDL receptor suppression. LDL receptor was upregulated in response to oleic acid while when palmitic acid was supplemented to diets in mice a down regulation of hepatic LDL receptor was noted (Kurushima *et al.* 1995).

Several studies have assessed the effects of n-3 PUFA on CETP and related plasma lipoproteins in normolipidaemics (Groener *et al.* 1995), hypercholesterolaemics (Abbey *et al.* 1990; Lottenberg *et al.* 1996; Bagdade *et al.* 1992c) and NIDDM individuals (Bagdade *et al.* 1996) and animals (Sugano *et al.* 1997). Sugano *et al.* (1997) found that increases in EPA in a rabbit model had no significant effect on CETP activity. Fish oil has a beneficial effects on plasma TAG concentrations and CETP mass and activity. A 23 % reduction in CETP activity and a 13 % reduction in mass in hypercholesterolaemic women was observed by Abbey *et al.* (1990). These reductions in CETP activity accounted for an increase in HDL₂/HDL₃ ratio. Bagdade *et al.* (1992c) supplemented hypercholesterolaemic IDDM sufferers with 6-g/day n-3 for 3 months and it was found that fish oil normalised CETP activity in these patients, and had similar findings with normolipidaemic IDDM patients (Bagdade *et al.* 1996). Groener *et al.* (1991) demonstrated that intake of fish oil produced a non-significant decrease in CETP activity of 3.1 %, compared to a monounsaturated diet which reduced CETP activity by 10 %. The decreases in CETP were associated with decreases in LDL cholesterol and total plasma cholesterol concentrations. Evidence exists that fish oil reduces CETP activity via a number of possible mechanisms. Polyunsaturated fatty acids are thought to maintain hepatic receptor mediated uptake of LDL unlike saturated fatty acids which down regulate hepatic LDL receptor mediated uptake (Kurushima *et al.* 1995). There is an increase in HDL CE EPA content in response to fish oil intake using a rabbit model. Abbey *et al.* (1990) demonstrated a four-fold increase in C20:5 (n-3) composition in HDL CE. Experiments by Morton *et al.* (1986) demonstrated that CETP exhibited substrate specificity towards saturated fatty acids. With increasing chain length and degree of unsaturation substrate specificity decreases. This provides a mechanism as to how HDL CE concentrations rise, as CETP is unable to efficiently remove CE from the HDL fraction. Finally the conclusions from many of the investigation into the effect of fatty acids on CETP was that CETP activity was substrate dependent (Lottenberg *et al.* 1996b; Fielding *et al.* 1995a; Tall *et al.* 1986; Castro and Fielding *et al.* 1985). Therefore decreases in plasma TAG concentrations associated with n-3 PUFA will reduce CETP activity as one of the primary substrates of CETP VLDL TAG is reduced

as a result of fish oil therefore reducing CETP activity and the potential atherogenic capability of CETP.

Table 1.1.1**Summary of the effects of dietary intake on CETP activity**

Study	Dose	Fatty acid	Duration	% change in activity	Patient type
Bagdade (1992)	6 g/d	n-3 PUFA	3 months	98 % ↓	Hypercholesterolaemic
Bagdade (1996)	4.6g/d	n-3 PUFA	2 months	58 % ↓	NIDDM
Groener (1991)	15.1 % en	Monounsaturated	5 weeks	11 % ↓	Normolipidaemic
	12.7 % en	Polyunsaturated	5 weeks	3.6 % ↓	Normolipidaemic
Lottenberg (1996)	45% total fat	Saturated fat	3 weeks	unchanged	Hypercholesterolaemic
	44% total fat	Polyunsaturated fat	3 weeks	unchanged	Hypercholesterolaemic
Abbey (1990)	9g/d	Safflower oil	6 weeks	7 % ↓	Hypercholesterolaemic
	14g/d	Linseed oil	6 weeks	16 % ↓	Hypercholesterolaemic
	3.8g/d	Fish oil	6 weeks	23 % ↓	Hypercholesterolaemic
Van Tol (1995)	8 % energy	Trans fatty acids	9 weeks	17-21 % ↑	Hypercholesterolaemic

Table 1.1.2
Summary of the effect of dietary lipids on CETP mass

Study	Dose	Fat type	Duration	% change in CETP mass	Patient type
Bagdade <i>et al.</i> 1992	6g/d	Fish oil	3 months	12 % ↓	Hypercholesterolaemic
Abbey <i>et al.</i> 1990 (CETP mass measured using indirectt assay)	3.8g/d	Safflower Linseed oil Fish oil	6 Week	12 % ↓ 2 % ↓ 13 % ↓	Hypercholesteroaemic
Jansen <i>et al.</i> 2000	20 total fat 38 % total fat 10 % total fat	Saturated MUFA Saturated	28 days	12 % ↑ No change No change	Normolipidaemics
Bagdade <i>et al.</i> 1996	4.6 g/d	n-3 PUFA	2 months	21 % ↑	NIDDM
Lottenberg <i>et al.</i> 1996	45 % total fat 44 % total fat	Saturated oil PUFA oil	21 days	Unchanged	Hypercholesterolaemics

1.12.0: Fish oil intake and Coronary Artery Disease

Epidemiological studies have shown a reduced rate of coronary incidence in populations with a high intake of fish oil i.e. Eskimos, compared to north American and northern European populations. Analysis of their diets revealed that a difference in n-3 fatty acid intake (Bang *et al.* 1976). The difference in n-3 fatty acid intake could be attributed to the higher fish intake, which is associated with increased n-3 polyunsaturated fatty acids intake and a low saturated fatty acid intake in Eskimo population. It is thought that this was responsible for their low death rate from CAD when compared with a northern European population (Kromann *et al.* 1980). There is evidence to suggest that the beneficial effects of fish oil intake on CAD can be attributed to eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) both of which are predominant fatty acids present in fish oil. EPA and DHA are very long chained polyunsaturated fatty acids with 20 and 22 carbons with 5 and 6 double bonds respectively. Beneficial effects of fish oils on coronary artery disease (see table 1.2) include the reduction of TAG concentrations, increased bleeding, decreased stickiness of platelets, and vasodilatation (Decaterina *et al.* 1990) and prevents fatal ischemia-induced cardiac arrhythmias in animal models (Kang and Leaf 2000). It would be beyond the realms of this thesis to review all literature on n-3 PUFA investigations therefore this review will concentrate only on the effects of n-3 PUFA on lipid metabolism.

1.12.1: Reduction of CAD relative risk in diseased patients attributed to Fish oil intake

Using rate of coronary events as a measurement of the benefit of consuming fish oil, Burr *et al.* (1989) found that when patients who had coronary artery diseases were asked to consume two portions of fish per week equating to 0.5 g/d, a 29 % reduction in death and recurrence of coronary artery disease events was noted in the follow up period when compared to the control group. Sisvock *et al.* (2000) indicated that the consumption of 1 fish meal per week provided a 50 % reduction in the risk of cardiac arrest. Bairati *et al.* (1992) found that upon consumption of 4.5g/d fish that there was a significant reduction (22 %) in the rate of restenosis in the fish oil group compared to the controls (40 %) after angioplasty. Analysis of data from the Western Electric study

by Dariglus *et al.* (1997) on the association of base line fish consumption over 30 years with risk of death from CAD, revealed that mortality from CAD was lowest in those who consumed the highest quantity of fish. Those who consumed 35 g fish /day had a 42 % lower death rate from non sudden death MI than did non-consumers. This data is consistent with data from Zutphen, (Kromhout *et al.* 1985), Rotterdam (Kromhout *et al.* 1995), Sweden (Norell *et al.* 1986) and the US (Albert *et al.* 1996) studies, which all demonstrated an inverse association between fish consumption and risk of death of CHD. Further analysis of the Western Electric Study (Shekelle *et al.* 1985) revealed that there was a graded response to fish oil intake and coronary events. Kromhout *et al.* (1985) suggests that 35g/d of fish results in 50 % reduction CHD mortality in risk populations. Studies carried out in Norway (Vollset *et al.* 1985) and Hawaii (Curb *et al.* 1985) found no association between fish consumption and CHD. Sacks *et al.* (1995) investigated the effects of fish oil intake at 6g/d for 28 months on the regression of coronary atherosclerosis in a group of angiographically documented coronary heart disease patients with normal plasma lipid levels. The findings of this study indicated that fish oil treatment did not produce a favourable change in atherosclerotic coronary arteries. It is important to note that this patient group was normolipidaemic (mean plasma cholesterol 4.83 mmol/l) yet they had suffered a coronary event. Although fish oil did improve the lipid profile in this population, non-lipid factors (possibly genetic) were probably more important in the cause of atherosclerosis in this population. Similar results were found in the Fish Oil Restenosis Trial where no difference in the rate of restenosis in patients after angioplasty was observed when either corn oil or fish oil was consumed. Discrepancies between the various long-term studies may be due to different dietary habits in populations, rate of follow up and differences in the categorisation of fish consumption. Although these studies provide somewhat conflicting results, on whether fish oil consumption reduces the rate of CAD incidents it is clear that risk factors such as plasma TAG concentrations, VLDL concentrations, thrombosis, endothelium dilatation of arteries symptom of CAD is reversed as a result of fish oil consumption (Nestel *et al.* 2000), all of which improves CAD prognosis.

Table 1.2: Summary of the beneficial of n-3 PUFA on indices associated with cardiovascular disease

Plasma Lipids

Reduce hepatic fatty acid synthetase
Reduce hepatic TG synthesis
Reduction of VLDL TG production
Apo B synthesis and secretion down regulated
Increases or conserves HDL cholesterol

Antithrombetic effects

Reduces TXA2 synthesis by platelets
Increase production of TXA3 a weak agonist
Decrease the ratio of TXA to PgI₂ and reduce platelet aggregability and vasoconstriction

Platelet

Decrease AA and increase EPA and DHA concentration
Decrease platelet aggregatability, adhesion
Decrease TXA2 synthesis

Macrophage

Reduce adhesion
Reduces chemotaxis
Reduce inflammatory reactions

Other effects

Enhances bleeding times
Reduces blood pressure
May increase antithrombin concentration
Improves Cardiac rhythm

1.12.2: Effect of fish oil on plasma TAG concentrations.

Evidence from numerous investigations exists on the effect of fish oil on plasma TAG concentrations. Harris *et al.* (1996) demonstrated that there was a 20-30 % reduction in fasting plasma TAG concentration as a result of fish oil intake. There appears to be a dose dependent relationship between the amount of fish oil intake and the percentage reduction in fasting TAG concentration (Sanders *et al.* 1989; Blonk *et al.* 1990; Schmidt *et al.* 1990). Long term supplementation also appears to be effective in TAG reduction where Roche *et al.* (1996) found a doubling in the expected decrease in TAG concentration with long term supplementation using 1 g/day fish. It has been suggested that long term supplementation may be more important in producing a hypotriglyceridaemic effect (Williams *et al.* 1992; Argen *et al.* 1996).

1.12.3: Dose response effect of fish oil supplementation on plasma TAG concentrations

Large doses of fish oil (24g/d for three weeks) (Harris *et al.* 1988a) result in alteration of plasma lipids in a very short time frame. The practicality of using these doses of fish oil long term is questionable. Roche & Gibney (1996) demonstrated that low dose fish oil over the longer term (1g/d for 16 weeks) was as effective at reducing plasma TAG concentrations as was found by Harris *et al.* (1988). Plasma TAG concentrations are reduced in a dose dependent manner (Bronk *et al.* 1990; Schmidt *et al.* 1990; Saunders *et al.* 1983). Below is an equation, which predicts the dose of fish oil and the expected reduction in plasma, TAG concentration (Roche and Gibney 2000).

$$Y = -7.67 - 3.05 X$$

where Y = % reduction in TAG concentration

and X = g/d of n-3 PUFA.

The reduction in relative risk of 1.32 for men and 1.76 for women is attributed to a 1 mmol/l reduction in plasma TAG concentrations as demonstrated by Hokanson & Austin (1996) and therefore a reduction in plasma TAG is important in reducing risk of CAD. Saynor & Gillott (1992) found that TAG concentration continued to decrease when using 4g/d n-3 PUFA for up to 4 years. A similar effect of n-3 PUFA in postprandial TAG concentrations was observed with 3.5 g/d n-3 PUFA reducing TAG

AUC by 54 % (Weintraub *et al.* 1988). Roche and Gibney (1996) observed a 32 % reduction in TAG AUC using 1g/d for 16 weeks similar to the reduction observed by Harris *et al.* (1988a) who used 28 g/d for 3 weeks.

Table 1.3: Summary of changes in fasting plasma TAG concentrations in n-3 PUFA intervention studies

Investigation	Dose	Duration	Fasting TAG concentration		
	n-3 PUFA		(mmol/l)		
	g/d	Weeks	Initial	Final	% change
Roche <i>et al.</i> 1996	1.0	16	0.82	0.65	- 21.2
Argen <i>et al.</i> 1996	2.3	15	1.21	0.89	- 26.5
Williams <i>et al.</i> 1992	2.7	6	1.05	0.79	- 24.8
Weintraub <i>et al.</i> 1996	7.0	3.5	0.63	0.63	- 42.7
Harris <i>et al.</i> 1988a	28.0	3	0.91	0.51	-43.7

1.12.4: Fish oils reduces plasma TAG concentrations - a mechanism

A mechanism as to how fish oil reduces fasting and postprandial plasma TAG concentrations is forthcoming from a study by Rustan & Drevon (1989). A rat hepatocyte model was used to elucidate a mechanism as to how EPA affects the production of TAG. There was a 50-80 % decreased secretion of TAG and CE by this cellular model in the presence of EPA compared to oleic acid. Analysis of VLDL indicated that there was a 50-60 % reduction in VLDL TAG and CE when cells were incubated with EPA compared to oleic acid. Illingworth *et al.* (1993) demonstrated that the reduction on VLDL production by n-3 PUFA was as a result of the suppression of acyl Co A 1,2 diacylglycerol acyl transferase a key step in the manufacture of TAG for VLDL (Marsh *et al.* 1987). The decreased activity of these enzymes provides a mechanism as to how fish oil consumption lowers VLDL production and VLDL TAG and CE concentrations. In human studies fish oil intake dramatically reduced hepatic TAG and VLDL output (Bordin *et al.* 1998). Lang *et al.* (1990) demonstrated that assembly and secretion of apo B TAG rich lipoproteins was inhibited by PUFA using rat hepatocyte model, while Kendrick *et al.* (1999) suggests as well as inhibiting assembly of apo B lipoproteins fish oil also increases the rate of degradation of apo B.

Postprandial TAG concentrations are reduced as a result of fish oil intake (Roche & Gibney, 1999b). This can be attributed to the inhibitory effect of n-3 PUFA on endogenous VLDL production as explained above. Both VLDL and chylomicrons compete for removal from circulation by LPL. The reduced VLDL output from the liver during lipaemia allows chylomicrons to compete more efficiently for LPL reducing the residence time of these lipoproteins in the circulation producing a less atherogenic lipoprotein profile. Recent evidence suggests that chylomicron remnants are reduced in the late postprandial phase as a result of fish oil intake (Westphal *et al.* 2000) which adds to the argument that LPL is more efficient at chylomicron removal as a consequence of fish oil intake. Investigations into the effect of n-3 PUFA intake on LPL activity have given mixed results with Harris *et al.* (1988c) finding no change in activity, while Kasim-Karakas *et al.* (1995) found a small but significant reduction in LPL activity after intervention with n-3 PUFA. However no conclusive evidence exist as to whether fish oil has any effect on LPL activity. Animal studies suggest that there is an upregulation of LPL mRNA as a result of n-3 PUFA intake (Murphy *et al.* 1993).

Further work needs to be carried out on this as investigations by Price *et al.* (2000) demonstrated the importance of n-3 PUFA on PPARs on lipid metabolism, work on the effect of these on LPL mRNA would be well worth investigating. PUFAs are a natural ligand for peroxisomal proliferator-activated receptors (PPARs) which regulate hepatic lipid metabolism at a cellular level. PUFAs suppress the transcription of hepatic genes encoding for lipogenic enzymes through the actions of PPARs (Clarke *et al.* 1996). PUFA interaction with PPAR can also induce the Beta-oxidation of fatty acids by the induction of the Beta-Co A oxidase gene (Gronn *et al.* 1992)

1.12.5: Fish oil and cholesterol metabolism

Animal studies indicate that cholesterol synthesis and HMG-CoA reductase activity are inhibited in animals fed fish oil. There is a reduction in cholesterol synthesis, yet there is an increase in LDL cholesterol concentration. A number of experiments which have tried to elucidate a potential mechanism accounting for this rise in LDL cholesterol, reveal that there is down-regulation of the LDL receptor in cultured hepatic cells as a result of n-3 PUFA administration (Lindsey *et al.* 1992), which ultimately reduces receptor mediated uptake of LDL. This mechanism may be attributable to a rise in LDL cholesterol concentration observed as a result of fish oil intake. Although there is an increase in LDL it is accounted for by larger less atherogenic LDL (Abbey *et al.* 1990; Suzukawa *et al.* 1995). One potentially adverse effect of fish oil on LDL cholesterol concentrations is increased susceptibility of LDL to oxidation and macrophage uptake (Suzukawa *et al.* 1995). However the effects of this increased potential for oxidation may be counteracted by antioxidants such as vitamin E.

Meta-analysis of 72 placebo controlled trials (Harris, 1996) lasting at least 2 weeks providing < 7g/day n-3 PUFA demonstrated no statistical clinical effect of n-3 PUFA supplementation on plasma total cholesterol. LDL cholesterol concentrations increased significantly (7 %) short term but returned to baseline with time in hyperlipidaemics, while a 3 % increase in LDL cholesterol concentrations were observed in normolipidaemics. It is thought that these increases are transient. Long-term supplementation with n-3 PUFA (28 months) by Sacks *et al.* (1995) reported an 8 % rise in LDL cholesterol as a result of fish oil supplementation with a 5 % increase in the placebo group resulting in no statistical difference between supplementation groups

in normolipidaemics. Leaf *et al.* (1994) reported 3 - 5 % decrease in LDL cholesterol concentrations with similar decreases in placebo groups as a result of n-3 PUFA intake for 6 and 4.5 months respectively. This indicates that with placebo controlled studies, no statistical change in LDL cholesterol was observed with respect to control groups as a result of long term intake of n-3 PUFA. Similar results were found in hyperlipidaemics with small changes in LDL cholesterol concentrations (Eirstland *et al.* 1994), while others found no changes in LDL cholesterol concentrations (Harris *et al.* 1991).

There is somewhat conflicting evidence from the literature as to the effect of fish oil on HDL cholesterol concentrations (Harris, 1996). Fish oil intake has a moderately positive effect on cholesterol metabolism with positive effect on HDL cholesterol concentrations. Pownall *et al.* (1999) observed that fish oil intake resulted in a reduction in TAG concentrations with an increase in LDL and HDL cholesterol concentrations. Several studies have shown increases in HDL cholesterol while others show no change in HDL cholesterol concentrations. Conclusions from Harris (1996), where meta-analysis of 73 placebo controlled studies, at an intake of < 7 g n-3 PUFA/day resulted in an increase in HDL cholesterol concentration in 3 % of studies while in the remaining studies HDL cholesterol concentrations remained unchanged. One would expect that such dramatic changes in plasma TAG would result in greater alterations in HDL concentrations due to the strong association between HDL cholesterol and plasma TAG. However although increases in HDL cholesterol concentrations may be modest as a result of fish oil intake, the actions of CETP activity are reduced as a result of fish oil intake (Abbey *et al.* 1990). The reduction of CETP activity results in a reduction in HDL TAG with cholesterol being retained in the HDL subfraction. This results in increases in HDL₂/HDL₃ (Abbey *et al.* 1990) cholesterol concentrations. Blonk *et al.* (1990) demonstrated that the control group had higher HDL₃ cholesterol concentrations than did those taking fish oils who had higher HDL₂ cholesterol concentrations. This relationship was dose dependent regarding fish oil intake. Blonk *et al.* (1990) also found a dose dependent relationship between fish oil intake and increases in HDL₂ cholesterol concentrations. The control group had higher HDL₃ cholesterol concentrations than did the fish oil group. HDL₂, which is high in cholesterol, is a less attractive substrate for

HL over HDL, which have a high TAG concentration. The consequence of fish oil intake seems to be that the cardioprotective HDL₂ fraction is conserved.

1.12.6: Fish oil and CETP mass and activity:

The importance of CETP on lipoprotein composition has been well documented. Changes in HDL concentrations as a result of fish oil intake may be attributed to alterations in CETP mass and activity. Investigations by Morton *et al.* (1986) have demonstrated the importance of substrate availability of CETP activity. The reduction in VLDL TAG concentrations as a result of fish oil intake may impact on CETP activity as VLDL TAG is a primary substrate in CETP neutral lipid exchange between VLDL and HDLs. Abbey *et al.* (1990) demonstrated that there was 13 % reduction in CETP activity in hypercholesterolaemics as a result of n-3 intake. Bagdade *et al.* (1992c) observed a 12 % reduction in CETP mass as a result of fish oil intake. Bagdade *et al.* (1996) demonstrated that 4.6 g/d n-3 fatty acid intake for 2 months produced a 14 % reduction in TAG and a 97 % reduction in CE transfer in a normolipidaemic IDDM population. The potential antiatherogenic effect of fish oils on HDL cholesterol (Harris *et al.* 1997) concentrations may be mediated through the activities of CETP. A potential mechanism by which CETP mediates this improvement in lipid profile could be attributed to the reduction in VLDL production as a result of fish oil intake. As there is reduced VLDL TAG concentrations to donate TG for CETP to transfer into HDL, CE is therefore retained in the HDL fraction as it is not transferred into VLDL. It is hypothesised that the reduction in CETP neutral lipid transfer can be attributed to a reduction in VLDL TAG concentrations. This hypothesis is confirmed by the findings of Lottenberg *et al.* (1996), where endogenous VLDL was used as an acceptor of CETP activity in a crossover study using a saturated fat and PUFA diets. There was less CE transfer to each subjects endogenous VLDL as a consequence of the PUFA diet. This was attributed to a reduction in the number of apo B lipoproteins and VLDL TAG concentrations as a result of fish intake available in plasma available for CE transfer. When CETP was incubated with equal concentrations of VLDL TAG no difference in CETP activity was observed pre and post trial indicating that a reduction in VLDL TAG concentrations were responsible for reduction in CETP activity (Lottenberg *et al.* 1996b).

Evidence exists that CE produced with n-3 PUFA are poor substrate for CETP as demonstrated in experiments by Morton *et al.* (1986) where CE with oleic and linoleic acid were transferred more readily. From the review of the investigations to date on the effect of fish oil on CETP and lipid metabolism, it appears that fish oil intake has a positive effect on CETP and lipoprotein metabolism, by reducing TAG concentrations (a independent risk factor of CAD (Stampfer *et al.* 1996)), conserving HDL cholesterol concentrations, which is probably mediated through a reduction in CETP activity. This may be as a result of reduced substrate availability, whether this occurs in normolipidaemic healthy population remains to be investigated although evidence (Harris 1996) suggests that as a result of n-3 intake HDL is conserved and even rises in normolipidaemics as a result of fish oil intake.

1.12.7: Antithrombotic effects of fish oil

Numerous studies have demonstrated that fish oil has an effect on thrombosis. This phenomena was first observed by Dyreberg & Bang (1979) who showed that there was a prolonged bleeding time in Eskimo populations,. Many other studies since then have demonstrated the antithrombotic effects of fish oil through either reduction in platelet aggregation or prolonged bleeding times (Saunders *et al.* 1983; Thorngren *et al.* 1981; Schmidt *et al.* 1990). Dietary supplementation using n-3 PUFA results in the incorporation of EPA and DHA into platelet phospholipids at the expense of arachidonic acid (Leaf *et al.* 1998). The incorporation of these fatty acids into platelet phospholipids is responsible for the antithrombotic properties associated with fish oil.

Upon platelet activation a substantial amount of free arachidonic acid is released. Arachidonic acid is a precursor for the eicosinoids which are produced by platelets and the vascular endothelium which controls the clotting process. A prothrombotic and vasoconstrictory factor known as thromboxane A₂ is manufactured from arachidonic acid. EPA competes with arachidonic acid and results in the production of thromboxane A₃, which doesn't have the same prothrombotic power as thromboxane A₂ (Needelman *et al.* 1979). Saturated fatty acids result in an increase in platelet arachidonic acid levels and therefore these fatty acids are deemed to be prothrombotic (Heemsherk *et al.* 1991). Therefore EPA and DHA replace arachidonic inhibit cyclooxygenases and lipoxygenase and reduce eicosanoid synthesis, in particular

thromboxane 2 and other signalling agents such as leukotriene B4 produced by platelets and macrophage. Reduction in thromboxane A2 results in a reduction of the thrombotic tendency of platelets, attributed to the intake of n-3 PUFA.

1.12.8: Incorporation of n-3 PUFA into platelet and plasma phospholipids - an index of dietary compliance

There is a large body of scientific evidence which has shown that the intake of n-3 PUFA is reflected by the incorporation of eicosapentaenoic acid (EPA; C20:5n-3) and docosahexaenoic acid (DHA; C22:6n-3) into platelets (Dougherty *et al.* 1987; Riboli *et al.* 1987; Prisco *et al.* 1996) and plasma (Sadou *et al.* 1995; Zuijdgeest van Leeuwen *et al.* 1999) phospholipids. These fatty acids are essential fatty acids, which come only from dietary sources.

Prisco *et al.* (1995) and Vidgren *et al.* (1997) have found significant increases in platelet phospholipid EPA and DHA as a result of n-3 PUFA intake. Similarly Hjartaker *et al.* (1997) demonstrated that the incorporation of EPA and DHA into plasma phospholipids was a good indicator of dietary fish and fish oil intake when compared with dietary analysis. Incorporation of EPA and DHA increases for up to four months during dietary supplementation with n-3 PUFA after which maximal incorporation of EPA and DHA in phospholipids is reached. It appears that the phospholipid fraction is most responsive to dietary changes in EPA and DHA with both FAs being incorporated. EPA is incorporated preferentially over DHA into phospholipids. EPA and DHA are both incorporated preferentially into the phosphatidylcholine and phosphatidylethanolamine fractions of phospholipid (Prisco *et al.* 1996). EPA is incorporated into cholesterol esters, while DHA is not (Zuijdgeest van Leeuwen *et al.* 1999). DHA is preferentially incorporated into plasma TAG, therefore high concentrations of DHA are observed in adipose tissue (Kasim-Karakas *et al.* 1995).

EPA and DHA are incorporated into platelets at the expense of arachidonic acid (C20:4 n-6) plasma (Prisco *et al.* 1996) and platelet phospholipid (Mori *et al.* 2000; Goodnight *et al.* 1982; van Schanck *et al.* 1985). This has antithrombotic potential as a reduction in arachidonic acid results in a reduction in the production of thromboxane A2 which is pivotal in the promotion of platelet aggregation as described in section 1.12.7.

Incorporation of EPA and DHA into platelets occurs dose dependently (Saunders *et al.* 1989). In response to 4g/d EPA and DHA platelet concentrations of EPA and DHA increase by 3.5-6.5 times and 1.5-2.6 times baseline values respectively at 2 months and by 4.0-5.5 times and 1.2-1.8 times at 4 months supplementation (Prisco *et al.* 1996). Blonk *et al.* (1990) found there was no significant difference in the incorporation of n-3 fatty acids into platelet phospholipids when either 3g/d or 6g/d fish oil was administered. At doses < 3g/d there was a significant dose related incorporation of n-3 fatty acids. It appears that maximal incorporation of EPA and DHA occurs by about two months of supplementation (Carthwright *et al.* 1985; Popp- Snijder *et al.* 1984). Three months withdrawal from DHA and EPA supplementation results in a normalisation of platelet phospholipid composition (Prisco *et al.* 1996). As a result of the properties of EPA and DHA incorporation into plasma and platelet phospholipids, gas chromatographic measurement of EPA and DHA are used in administered.

1.13.0: Conjugated Linoleic Acid

In recent years the interest in conjugated linoleic acid (CLA) has increased due to findings that CLA has anti-carcinogenic (Banni *et al.* 1999), anti-atherogenic (Nicolosi *et al.* 1997; Lee *et al.* 1994) and anti-inflammatory properties.

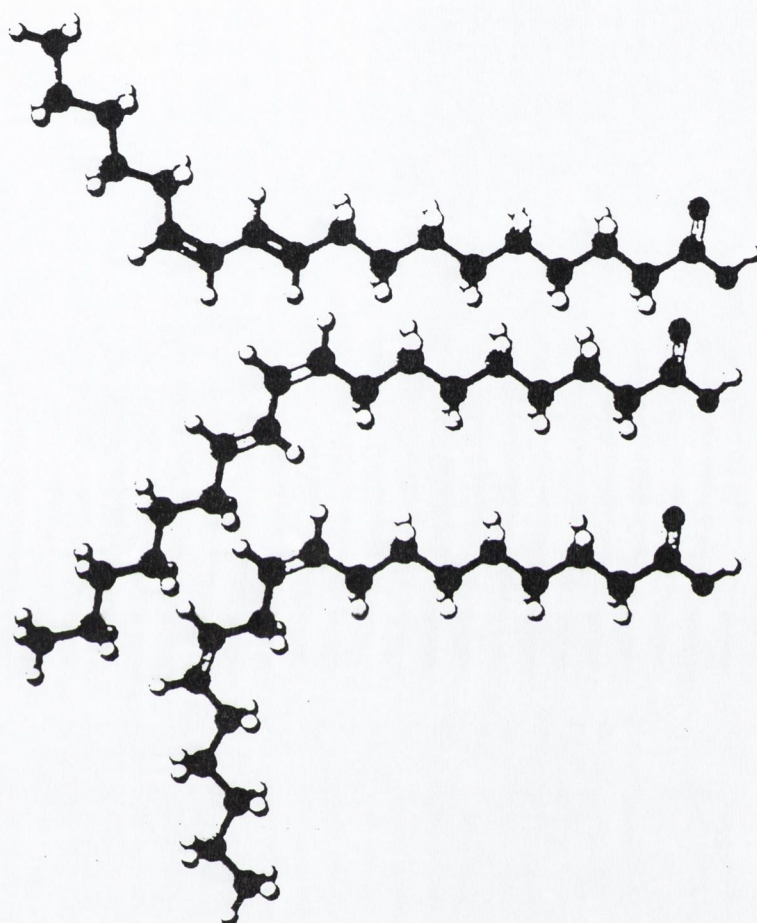
CLA is a collective term which describes a mixture of naturally occurring positional and geometric isomers of linoleic acid having conjugated double bonds which may be of either cis or trans configuration (See figure 1.3). The most predominant isomer of CLA is the *cis*-9 (18:2 *cis*-9, *trans*-11) making up 80-90 % of all naturally occurring CLA (Banni & Martin, (1998); Dormany & Wickens, (1987)). The main source of CLA in humans is from dairy fats. CLA is formed in the rumen of ruminants by intestinal flora known as *Butyrivibrio fibrisolvens* (Kepler *et al.* 1970). Isomerisation of linoleic acid by the enzyme linoleate isomerase present in *Butyrivibrio fibrisolvens* results in the formation of the various isomers of CLA.

Adolf *et al.* (2000) demonstrated that a metabolic pathway may exist in humans for the endogenous synthesis of CLA. 11 *trans*-octadecenoate fed as TAG was converted by $\Delta 9$ desaturase to CLA (*cis*-9, *trans*-11 18:2) or other CLA isomers possibly by bacteria in the human intestine. This pathway was investigated in one subject only and therefore more extensive research needs to be carried out before these findings can be confirmed. Recent evidence suggests that CLA may be formed by the conversion from dietary *trans* fatty acids (C18:1 *trans* fatty acid) in humans (Salminen *et al.* 1998).

1.13.1: Dietary sources of CLA.

Animal products are the principle sources of CLA in the diet, with dairy products being a particularly rich source of CLA. The CLA composition of milk fat and beef fat varies depending on the time of year, and the breed of the animal. The CLA content in beef ranges from 2.9-4.3 mg/g of fat, lamb 5.6 mg/g fat, turkey 2.5 mg/g fat, and seafood values ranged from 0.3-0.6 mg/g fat (Chin *et al.* 1992). The CLA content of various foods is presented in table 1.5. CLA is stable and its concentration does not change as a result of storage or processing. Daily consumption of CLA by humans is estimated to be around 160 mg/day. However due to the promotion of low fat products CLA intake today is probably decreasing.

Figure 1.3: Structure of isomers of conjugated linoleic acid and linoleic acid



Structure of (top) *trans*-10, *cis*-12, (middle) *cis*-9, *trans*-11 CLA, and (bottom) linoleic acid. Reprinted from Pariza, P.W. (2000)

Table 1.4: Dietary sources of Conjugated Linoleic Acid

Foodstuff	Total CLA (mg/g fat)	<i>cis</i>-9, <i>trans</i>-11 (% total CLA)	<i>trans</i>-10,<i>cis</i>-12 (% total CLA)
Meat (raw)			
Fresh ground beef	4.3 ± 0.13	85	
Lamb	5.6 ± 0.29	84	
Pork	0.6 ± 0.06	82	
Chicken	0.9 ± 0.02	84	
Cheese			
Cream	3.8 ± 0.08	90	
Cheddar	4.1 ± 0.14	80	
Cottage	4.5 ± 0.13	83	
Swiss	6.7 ± 0.56	90	
Blue	5.7 ± 0.18	90	
Dairy Products			
Homogenised Milk	5.5 ± 0.30	92	
Condensed milk	7.0 ± 0.29	82	
Butter	4.7 ± 0.36	88	
Ice cream	3.6 ± 0.10	86	
Low fat yogurt	4.4 ± 0.21	86	
Plain yogurt	4.8 ± 0.26	84	
Commercial oil			
Safflower	0.7 ± 0.14	44	41
Sunflower	0.4 ± 0.02	38	37
Vegetable	0.3 ± 0.02	41	39
Corn	0.2 ± 0.01	39	37
Olive	0.2 ± 0.01	47	40

Source Chin *et al.* (1992)

1.13.2: Conjugated Linoleic Acid and Coronary Artery Disease

A number of investigations in animals on the effects of CLA on CAD and plasma lipids, carcinogenesis and immune function have been carried out in the past number of years. For the purpose of this literature review, the focus will be only on the effects of CLA on coronary artery disease and plasma lipids and body composition. To date no literature has been published on the effects of CLA on CAD and lipoprotein metabolism in humans. Evidence from the literature published in recent years suggests that CLA may have beneficial effects on coronary artery disease and lipid metabolism in animals.

1.13.3: Effect of plasma TAG concentrations

Investigations to date on the effects of dietary supplementation with CLA on plasma TAG concentrations using animal models has been somewhat mixed. Lee *et al.* (1994) investigated the effects of supplementation of an atherogenic diet with CLA (0.5g/day) in rabbits for 22 weeks. There was an increase in plasma TAG concentrations as would be expected in response to an atherogenic diet. The plasma TAG concentrations in the CLA group were lower than those of the controls. However, this difference between the groups was not significant. There was a non-significant increase in VLDL TAG concentration as a result of supplementation of the diets of pigs with CLA at 1 % of total dietary fat (Stangl *et al.* 1999). In contrast to the findings of these two studies others have had positive findings in terms of reducing TAG concentrations as a consequence of CLA intake. Significant reduction in plasma TAG concentrations ($P < 0.01$) were found in C57BL/6 mice fed 5g/kg CLA in conjunction with an atherogenic diet when compare with controls after 15 weeks (Munday *et al.* 1999). Conjugated linoleic acid intake ranging from 0.06, 0.11 and 1.1 % total dietary fat intake in hamsters resulted in a significant reduction in plasma TAG concentrations of between 28 % and 36 % when compared to controls (Nicolosi *et al.* 1997). Most recently Stangl (2000) demonstrated that rats fed CLA at 3 % total energy intake resulted in a significant reduction in VLDL TAG concentrations. Gavino *et al.* (2000) demonstrated that an isomeric mixture of CLA lowered plasma TAG concentration, while pure *cis*-9, *trans*-11 did not alter plasma TAG relative to the control group suggesting that isomers other than *cis*-9, *trans*-11 18:2 are responsible for the reduction of TAG concentrations. Yotsumoto *et al.* (1999) found that *trans*-10, *cis*-12 CLA isomer reduced hepatic TAG synthesis and apo B secretion in vitro suggesting that CLA not only reduces hepatic TAG synthesis but may also reduce VLDL lipoprotein

numbers in the plasma due to the inhibitory effects of *trans*-10, *cis*-12 CLA isomer on apo B secretion.

A potential mechanism as to how conjugated linoleic acid has an effect on plasma and VLDL TAG concentrations can be hypothesised through the effect that CLA has on peroxisome proliferator-activated receptors (PPARs). PPAR α is expressed in brown adipose tissue, kidney, liver and skeletal muscle. CLA may elicit its effects on lipid metabolism in a similar manner to fibrates. Fibrates are known to reduce increase LPL concentrations and reduce apo C III an inhibitor of LPL activity (Levy *et al.* 1993; Tikkanen *et al.* 1992). Alterations in the gene expression of LPL and apo C III are mediated through the interaction of fibrates with peroxisome proliferator activated receptors (PPARs), where there is increased expression of LPL mRNA and reduced expression of apo C III mRNA (Schoonjans *et al.* 1996a,b).

Moya-Camarena *et al.* (1999a) demonstrated that CLA is a high affinity ligand of PPAR α and is responsible for the regulation of genes involved in lipid metabolism. Several isomers of CLA are activators of PPAR α (Moya-Camarena *et al.* 1999a) with the *cis*-9, *trans*-11 being most potent. PUFA induce an increase in β -oxidation in rat liver, which is mediated through PPAR (Flatmark *et al.* 1988). Moya-Carmarena (1999a) demonstrated CLA elicits a similar response in mice. CLA is a ligand for PPAR γ which is responsible for the regulation of genes controlling lipid metabolism in the adipocyte such as LPL, acyl-Co-A synthase and the fatty acid binding protein (Houseknecht *et al.* 1998). Therefore if CLA increases the expression of LPL through PPAR γ in the adipocyte, enhanced clearance of triglyceride rich lipoproteins would occur resulting in the hypotriglyceridaemic effect attributed to CLA.

In cultured 3T3-L1 adipocytes, the *trans*-10, *cis*-12 isomer reduced both lipoprotein lipase activity and intracellular TAG and enhanced the rate of glycerol release into the medium (Park *et al.* 1999a; Park *et al.* 1999b). Depending on the concentration of CLA administered, adipocyte LPL activity in vitro may be increased or decreased (Park *et al.* 1997). It is clear there exists a molecular pathway whereby CLA results in the regulation of genes controlling lipid metabolism.

1.13.4: Effect of CLA on cholesterol concentrations

CLA intake has a lowering effect on cholesterol metabolism from the evidence of animal studies (Lee *et al.* 1994; Munday *et al.* 1999; Nicolosi *et al.* 1997). LDL cholesterol concentrations and LDL:HDL cholesterol ratios were significantly reduced

($P < 0.05$) in rabbits fed CLA at 0.5 g/day in conjunction with an atherogenic diet (Lee *et al.* 1994). There was no change in LDL or HDL cholesterol concentrations using a mouse a model, fed an atherogenic diet, supplemented with CLA. However the HDL:LDL cholesterol ratio was significantly increased (Munday *et al.* 1999). Gavino *et al.* (2000) found that a mixture of CLA isomers reduced total cholesterol concentrations in male Golden Syrian hamsters, but a pure *cis*-9, *trans*-11 isomer of CLA did not. Neither *cis*-9, *trans*-11 18:2 or a CLA isomeric mixture had any effect on HDL cholesterol concentrations in male Golden Syrian hamsters. Nicolosi *et al.* (1997) demonstrated that a CLA intake of 0.06 - 0.11 % total energy intake significantly reduced plasma cholesterol by 21-26 % and non HDL cholesterol concentrations by between 9 - 13 % in hamsters. Dietary supplementation of CLA in swine resulted in an increase in LDL: HDL cholesterol ratio (Stangl *et al.* 1999). In contrast a rat model supplemented with 3g CLA /100g total fat, CLA exhibited no effect on HDL or LDL cholesterol concentrations (Stangl G., 1999). Nicolosi *et al.* (1997) demonstrated that CLA supplementation exhibited no effect on HDL cholesterol in hamsters.

An investigation by de Deckere *et al.* (1999) into the effects of isomeric blends of CLA on plasma lipids in hamsters showed that the *trans*-10, *cis*-12 isomer was the cholesterol lowering agent, with total cholesterol concentrations decreasing by 10 %. The *trans*-10, *cis*-12 isomer is also the active isomer in lowering apolipoprotein B concentrations (Pariza *et al.* 1996). Recent evidence from Yotsumoto *et al.* (1999) has suggested that the *trans*-10, *cis*-12 CLA isomer suppress cholesterol ester synthesis and apo B secretion in hepG2 cells. This could be a potential mechanism whereby CLA reduces VLDL synthesis and cholesterol concentrations resulting in reduced VLDL cholesterol concentrations and ultimately LDL cholesterol concentration in plasma.

It appears from the literature that CLA has mixed effects on plasma lipoprotein cholesterol composition depending on the animal models used, with the lowering of LDL cholesterol concentrations in observed some studies. In most investigations HDL cholesterol concentrations remain unchanged leading to an increase in the HDL: LDL ratios which is known to exhibit cardio protective effects.

1.13.5: Effect of CLA on the progression of atherosclerosis

There have been three papers in recent years investigating the effect of CLA on the rate of atherosclerotic plaque development (Lee *et al.* 1994; Nicolosi *et al.* 1997; Munday *et al.* 1999). Lee *et al.* (1994) demonstrated that feeding CLA at 0.5g /day resulted in a 30 % less cholesterol deposition in the aorta of rabbits which had been fed

an atherogenic diet supplemented with CLA at 0.5 g/day compared to controls (18.8 ± 4.6 mg/g control group and 13.2 ± 2.5 mg/g in CLA supplemented group). The total aortic surface covered by fatty lesions was also lower in the CLA group (43 ± 11 %) when compared with (55 ± 14 %) the control group but this difference between groups was not statistically significant. Similarly, Nicolosi *et al.* (1997) found that CLA administered at low dose 0.05 % fat intake for ten weeks in hamsters reduced aortic streak formation by 26 % when compared to control group. Nicolosi *et al.* (1997) had similar findings regarding plaque formation in hamsters. Munday *et al.* (1999) demonstrated that CLA administered to C57BL/6 mice at a concentration of 2.5 and 5 g/kg, in conjunction with an atherogenic diet developed a significantly greater area of fatty streaks than did controls receiving linoleic acid. The type of mouse model could also have an influence on the outcome of this study, as no indirect reverse cholesterol pathway is evident in this model as they are CETP knockouts. It appears that in response to atherogenic diet CLA administration may reduce atherosclerotic, due to its positive effects on plasma lipids and its effect on plaque formation (Lee *et al.* 1994; Nicolosi *et al.* 1997). However aortic plaque formation was only significantly reduced in the investigation by Lee *et al.* (1994).

1.13.6: Effect of CLA on body weight

A number of studies have addressed the effect of CLA on weight in animals and humans. Sprague-Dawley rats fed a CLA supplemented diet at 3g/100g for 3 weeks, underwent food restriction for 18 days. The CLA fed rat had lower body weights and increased lean/fat ratios compared with controls (Stangl, 2000). The CLA fed group had 27 % carcass fat with protein enhanced by 11 % which was equivalent to a 45 % higher protein fat ratio. Similar modulation of body composition was found in mice, pigs and chicks when diets were supplemented with CLA where lean body mass increased by 5-14 % (Dugan *et al.* 1997; Pariza *et al.* 1996; Park *et al.* 1997; Park *et al.* 1999a,b). Nicolosi *et al.* (1997) demonstrated that CLA supplementation had no effect on body weight of hamsters supplemented with CLA at either 0.06 %, 0.11 % and 1.1 % of energy after 8 weeks dietary supplementation, but significant reduction in body weight were noted by week 11 when compared to controls.

In humans CLA supplementation at 2.7 g/day induced a 20 % reduction in body fat in non-obese subjects but exhibited no effect in obese individuals (Atkinson *et al.* 1999). In moderately obese human subjects (BMI >27 kg/m²), CLA supplementation at 7.2 g/day for 12 weeks resulted in a significant reduction in body fat mass and a

significant increase in lean body mass (Gudmundsun *et al.* 1999). Evidence from experiments by Gavino *et al.* (2000) indicated that the *cis*-9, *trans*-11 isomer is not responsible for body partitioning in Golden Syrian hamsters where energy intake was matched in all groups, were fed pure *cis*-9, *trans*-11 isomer did not differ in body fat accumulation when compared with controls taking linoleic acid. However those hamsters fed a mixture of CLA isomers exhibited significantly less body fat accumulation and weight gain suggesting perhaps that the *trans*-10, *cis*-12 isomer may be active in reducing body fat accumulation.

Much research remains to be carried out to elucidate a mechanism as to how CLA induces partitioning of lean and fat body ratios, but it is hypothesised that CLA may inhibit adipocyte proliferation and differentiation and increase B-oxidation in skeletal muscle cells and adipocytes. A mechanism as to how CLA induces body partitioning is mediated through CLA interaction PPAR α (Moya-Carmarena *et al.* 1999a) which results in the induction of the acyl co A oxidase, enoyl-Co-A hydratase/dehydrogenase and keto-acyl-CoA thiolase genes, all of which are important in determining the rate of peroxisomal B oxidation (Lee *et al.* 1995; Zhang *et al.* 1993). Evidence for upregulation of these genes comes for experiments in mice where a 6 to 9 fold increase in mRNA of acyl Co A oxidase in the hepatocyte in response to CLA supplemented in the diet at concentrations varying from 0.5% to 1.5% (Belury & van den Heuvel, 1997). West *et al.* (1998) found that feeding CLA increased energy expenditure by 1 % in mice. Carnitine palmitoyl transferase activity is increased by CLA supplementation (Park *et al.* 1997), resulting in increased β oxidation of lipids. Uncoupling proteins are also implicated in the regulation of fat as an energy source in muscle tissue. The upregulation of these proteins increases lipid oxidation by muscle (Boss *et al.* 1998; Miller *et al.* 1997) particularly during the starved state. CLA may have an effect on uncoupling proteins. An increase fat oxidation as was demonstrated by the partitioning effect of CLA on fat / lean body composition in rats which underwent food restriction (Stangl G.I., 2000). Park *et al.* (1999a) demonstrated that *trans*-10, *cis*-12 CLA isomer was the active isomer in altering body composition in mice. Increased muscle in mice was maintained up to 8 weeks after withdrawal from CLA supplementation (Park *et al.* 1999b). Numerous investigations have shown that fat depots are reduced by dietary CLA (Delany *et al.* 1999; Park *et al.* 1999a; Park *et al.* 1999b), this is attributed to a reduction in adipocyte LPL as CLA reduces LPL activity in 3T3-Li adipocyte cells in vitro (Park *et al.* 1997).

1.13.7: Antithrombotic effects of CLA

Belury and Kemp-Steczko (1997) demonstrated that CLA is a substrate for microsomal $\Delta 6$ desaturase. This enzyme is responsible catalysis of linoleic acid to arachidonic acid in vitro. CLA appears to reduce the concentration of arachidonic acid and to increase oleic acid in phospholipid in the hepatocyte. It is possible that this may also happen in platelet phospholipid as a result of CLA supplementation. If so the decrease in arachidonic acid in platelet phospholipid composition would lead to reduced thromboxane A₂ (which promotes platelet aggregation). Truitt *et al.* (1999) demonstrated that CLA may possess antithrombotic properties. A mixture of CLA isomers *cis*-9, *trans*-11 and *trans*-10, *cis*-12 inhibited arachidonic acid and collagen induced platelet aggregation, with both isomers appearing to have similar antithrombotic activities. Linoleic acid didn't possess any properties regarding inhibition of platelet aggregation.

1.13.8: CLA and inflammatory component of atherosclerosis

Macrophage uptake of LDL and resultant incorporation into arterial walls in the form of foam cells is a component of atherosclerosis which appears to be reduced by CLA supplementation (Lee *et al.* 1994). Recent evidence by Chew *et al.* (1997) has demonstrated that supplementation of CLA to an in-vitro macrophage model reduced the phagocytic activity of macrophage. Contradictory to these findings, Cook *et al.* (1994) noted an enhancement of the phagocytic capacity of macrophage in rats fed 0.5 % CLA for 4 weeks, with Miller *et al.* (1993) demonstrating a similar effect in mice.

A number of investigations have noted reductions in cytokines and eicosanoids which stimulate macrophage activity. PGE₂ is a stimulant of macrophage activity. Using *invitro* tissue models it has been revealed that CLA supplementation suppresses PGE₂ production in bone cultures (Li *et al.* 1988), rat sera (Sugano *et al.* 1998), and mouse epidermis (Kavanagh *et al.* 1999). CLA also reduces TNF α and IL 6, two cytokines implicated in the progression of atherosclerosis (Ross *et al.* 1993), in Male Sprague Dawely rats. The in-vitro evidence implicates that CLA may reduce macrophage activity and cytokines associated with the progression of atherosclerosis. Lee *et al.* (1994) demonstrated that CLA supplementation in mice reduced plaque formation in arterial walls. These findings suggest that CLA may reduce the inflammatory component of atherosclerosis.

1.13.9: CLA incorporation into plasma lipids

Gas Chromatography analysis has revealed that *cis*-9, *trans*-11 18:2 is the predominant isomer found incorporated into the phospholipid fraction of animal tissue where a mixture of isomers of CLA are fed (Ha *et al.* 1990; Ip *et al.* 1991). It is present in the phospholipid of all serum lipoproteins (Harrison *et al.* 1985; Iverson *et al.* 1985). Analysis of the various isomers of CLA is difficult. However GC is sufficient for analysis of the fatty acid methyl esters of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers of CLA using a highly polar 50-m capillary column. Analysis of the other positional and geometric isomers of CLA cannot be separated using GC. Therefore silver nitrate high-performance liquid chromatography of methyl esters of CLA is employed, and some investigators have used a series of columns to achieve better separation. Transmethylation at high temperatures results in excessive formation of *trans/trans* isoforms of CLA. Therefore transmethylation techniques using lower temperatures are used in the transmethylation of biological samples (Jiang *et al.* 1999).

The main dietary sources of CLA are the fat of meat and dairy products. Diet is thought to be the main source of CLA in humans with an average intake of about 160mg/day coming from meat and dairy products. The average amount of *cis*-9, *trans*-10 18:2 present in human adipose tissue is 0.5 % w/w with a range between 0.27 and 0.72 % (Jiang *et al.* 1999). The CLA content in serum ranges from 0.12 % to 0.50 %, which is about ½ those present in adipose tissue (Fogerty *et al.* 1988). Concentrations of CLA in and plasma correlates with intake of dairy products (Fogerty *et al.* 1988). Evidence for these findings exists from the data of Jiang *et al.* (1999) where the % increase in CLA correlates with milk intake. Similarly Hwang *et al.* (1994) demonstrated that humans fed cheese over a number of weeks resulted in a 19-27 % increase in the concentration of CLA in total fatty acid composition.

CLA is present in the phospholipid of serum lipoproteins (Harrison *et al.* 1985; Iverson *et al.* 1985). Belury *et al.* (1997b) found that not only was CLA present in phospholipid but also TAG. The *cis*-9, *trans*-11 isomer is the only isomer which may be detected in the phospholipid fraction of animal tissue, even after a mixture of isomers has been fed (Ha *et al.* 1990; Ip *et al.* 1991). The *cis*-9, *trans*-11 isomer of CLA is incorporated into the total fatty acid lipid composition at the expense of linoleate in the phospholipid fraction in Sencar mice (Burley *et al.* 1997b). Similarly Gavino *et al.* (1999) found that using GC analysis, the *cis*-9, *trans*-11 isomer was incorporated into total fatty acid composition at the expense of linoleic acid using a hamster model. Stangl *et al.* (2000) suggest that CLA may be incorporated into plasma lipids at the

expense of 18:2 n-6, 18:3 n-6, 20:3 n-6 and 20:4 n-6 when CLA is supplemented in the diet of swine.

The dietary intake of trans fatty acids (C18:1 trans fatty acid) results in an increase in the concentration of CLA in serum suggesting that there is a pathway in humans for the conversion of dietary trans fatty acids to CLA (Salminen *et al.* 1998). Pollard *et al.* (1980) demonstrated that rat liver microsomes are capable of manufacturing CLA. Both these investigations suggest that a pathway may exist in animals for the endogenous synthesis of CLA. The *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers of CLA are metabolised to 20:4 Δ 5,8,11,13 and 20:4 Δ 5,8,12,14 or 20:3 Δ 8,12,14 respectively via desaturation and elongation pathways. Sebedio *et al.* (1997) found that there is a greater quantity of 20:4 Δ 5,8,11,12 in total liver lipid in rats, suggesting the *trans*-10, *cis*-12 isomer may be more rapidly metabolised. This may explain why many investigators have found trouble detecting the *trans*-10, *cis*-12 isomer of CLA in tissue and plasma. Because of these findings by Sebedio *et al.* (1997), and the fact that the *cis*-9, *trans*-11 isomer is present in plasma and tissue it is thought that the *cis*-9, *trans*-11 isomer is the one, which is biologically active.

CLA administered at 0.1 % of total fat in rats equates to about 0.015g CLA per day. It is clear from this literature review some of the beneficial effects of administering CLA at these doses in animals. When doses administered in animal studies at 0.1 % are extrapolated to the human situation CLA intake of 0.1 % total dietary fat would be equivalent to about 3.5 g/day CLA per day for a 70 kg man. One would expect that at this dose of CLA would be effective in elucidating a response in lipid metabolism in humans.

14.0: Objectives of this thesis

The aims of this thesis are

- 1) To investigate the effects of a fat-rich test meal on cholesteryl ester transfer protein (CETP) mass and activity during postprandial lipaemia in normolipidaemic individuals. The present study, with a relatively large study group, a detailed analysis of plasma lipids and HDL subfractions was performed in order to examine the complex relationship between CETP and lipid metabolism and coronary artery disease risk factors in fasting and postprandial states.
- 2) An investigation into the effects of moderately elevated cholesterol concentration on CETP mass and activity when compared to subjects with normal plasma cholesterol concentrations was made in the fasted and postprandial states. An assessment was made as to whether differences in CETP mass and activity between groups was responsible for difference in lipoprotein profile between the groups.
- 3) An assessment of the frequency of a known polymorphism of the CETP gene was made in a normolipidaemic population. Questions were asked as to whether this polymorphism exhibits an influence on CETP mass and activity and related plasma lipoproteins in normolipidaemics. An assessment as to whether the presence of absence of this polymorphism of CETP is associated with increased or decreased risk factors for the development coronary artery disease is made.
- 4) The effects of dietary intervention using fish oil and a novel fatty acid known as conjugated linoleic acid on plasma lipoprotein metabolism and CETP mass and activity and CAD risk factors was investigated. CETP mass and activity were measured to determine if alterations in plasma lipoprotein profile as a result of dietary intervention using fatty acids or were caused by changes in CETP mass and activity.

Chapter 2

Methods

2.0 Methods

2.1: Installation of Quality Control system on RA-XT clinical chemistry analyser

All colorimetric methods were carried out on a RA-XT clinical chemistry analyser. Each assay was installed on the RA-XT clinical chemistry analyser using an individual calibrator for each assay described in the following sections. The calibrator had an assigned value established by the manufacturer, from which the method being used on the auto-analyser could be standardised. The calibrator was reconstituted as detailed on package insert. An internal quality control system for each biochemical parameter was established using quality control plasma. Setting up of the internal quality control system involved assaying a batch of quality control plasma 20 times. This analysis was then carried on a second batch of QC samples, and then another batch of samples were assayed again on each of the following four days. From this a within batch and between batch variation of the assay was obtained. A quality control chart was set up which allowed a parameter to be within two standard deviations of the expected mean. If a result for a parameter was greater than two standard deviations from the mean, the method was regarded as being out of control and the batch of analytes were rejected. During analysis of samples internal quality control samples were run every fifteenth sample. All methods installed on the RA-XT autoanalyser had a coefficient of variation of $< 5\%$.

2.2: Determination of Cholesterol concentration

Total plasma cholesterol concentration was determined using Biomerieux PAP 1200 total cholesterol kit (Cat. # 61 226). The principle of the method is that cholesterol ester is converted to free cholesterol and fatty acids by cholesterol esterase. The cholesterol is then oxidised by the action of cholesterol oxidase forming cholestene-3-one and hydrogen peroxide. Hydrogen peroxide combines with phenol and 4-ammino antipyrine. This is acted on by a peroxidase enzyme resulting in the formation of quinoneimine and water. The quinoneimine produced is in direct proportion to the amount of cholesterol present in the sample. Quinoneimine is then quantified spectrophotometrically at 500 nm. Biomerieux calimat (Cat. # 62 321) was used as calibrator for the installation of total cholesterol assay on the RA-XT clinical chemistry analyser. Biomerieux Lyotrol N (Cat. # 62 373) and Bayer control sera 2 were used as quality control material for the cholesterol assay.

2.3: Determination of Triacylglycerol concentrations

TAG analysis was carried out using Biomerieux PAP 1200 total triglyceride enzymatic kit (Cat. # 61 238). The principle of the assay was as follows, TAG was broken down by lipase to glycerol and fatty acids. The glycerol component is then converted to glycerol-3-phosphate using glycerokinase and ATP. Glycerol-3-phosphate is oxidised by glycerol 3-phosphate oxidase to dihydroxyacetone phosphatase and hydrogen peroxide. The substrates parachlorophenol and amino-4-antipyrine in the presence of peroxide is then converted to quinoneimine in the presence of peroxidase. Quinoneimine is quantified spectrophotometrically at 505nm. The TAG assay was calibrated on RA-XT chemistry analyser using Biomerieux Calimat (Cat. # 62321). The quality control plasma used for the TAG assay was Biomerieux Lyotrol N (Cat. # 62 373) and Bayer control sera 2.

2.4: Determination of Non Esterified Fatty Acid (NEFA) concentration

Plasma NEFA analysis was carried out using Randox NEFA kit (Cat # FA 115). This enzymatic assay involves the conversion of NEFA to acyl CoA by the addition of ATP and CoA, through the action of Acyl CoA Synthetase. Acyl CoA oxidase oxidises Acyl CoA to 2,3,-trans-Enoyl-CoA and H_2O_2 . 4-aminoantipyrine and N-ethyl-N-(2hydroxy-3-sulphopropyl) m-toluidine is coupled with hydrogen peroxide. This is then acted on by a peroxidase enzyme resulting in the production of purple adduct which can be read spectrophotometrically at 505nm and water. NEFA assay was calibrated on a Technicon RA-XT clinical chemistry analyser using NEFA 1 mmol/l standard, supplied with the Randox NEFA kit. The NEFA quality control plasma were supplied by Wako chemicals (Cat # 416-00201).

2.5: Determination of Phospholipid concentration

Phospholipid analysis was carried out using Biomerieux PAP 150 phospholipid kit (Cat. # 61 491). This enzymatic assay involves the enzymatic breakdown of phospholipid by the enzyme phospholipase to choline. Choline oxidase converts choline to betaine and hydrogen peroxide. The hydrogen peroxide in the presence of phenol and amino-4 antipyrine is enzymatically acted upon by peroxidase resulting in the formation of quinoneimine and water. Quinoneimine is quantified at a maximum absorption of 505nm. Phospholipid assay was calibrated on RA-XT chemistry analyser

using Biomerieux Calimat (Cat. # 62321). The quality control plasma used for the phospholipid assay was Biomerieux Lyotrol N (Cat. # 62 373) and Bayer control sera 2.

2.6: Determination of Apo A1 concentration

Apolipoprotein A1 analysis was performed using Biomerieux apo A1 assay kit (Cat. # 62 032). The principle of the apo A1 method is based on the reaction of the plasma sample apolipoprotein A1 with specific antiserum in the apo A1 assay. This results in the formation of an insoluble complex which can be measured turbidimetrically at 340 nm. By constructing a standard curve the absorbance of standards can be determined. From this standard curve, the concentration of plasma samples is calculated. Calibration was performed using Biomerieux apo calibrator (Cat. # 62 042). Chemistry parameters for this assay were programmed into a Technicon RA-XT clinical chemistry analyser and calculations were performed automatically. Quality control was carried out using precipnorm and precipath quality control serum supplied by Boehringer Mannheim (Cat. # 781 827).

2.7: Determination of apo B concentration

Apo B analysis was performed using a Biomerieux apo B assay kit (Cat # 62 002). This is an immunochemical reaction using antiserum to human LDL which was manufactured from injection of goats with human LDL. The antigen-antibody complex formed in this assay leads to the formation of an insoluble complex, whose turbidity increases with increasing apo B concentration. The assay is read at 340 nm. Concentrations were calculated from a calibration curve using apo protein calibrator (Biomerieux, France, Cat # 62 042) which had been preprogrammed on a Technicon RA-XT. Precinorm and precipath (Boehringer Mannheim Inc.) were used as quality control standards.

2.8: Determination of Glucose concentration

Glucose analysis was performed using Biomerieux glucose enzymatic assay kit (Cat. # 61 273). This assay is based on the glucose oxidase method where glucose oxidase reacts with glucose and oxygen to produce gluconic acid and hydrogen peroxide. Hydrogen peroxide in the presence of phenol and amino-4 antipyrine is enzymatically acted on by peroxidase resulting in the formation of quinoneimine which

shows maximal absorption at 505nm. The quantity of quinoneimine is in direct proportion to the concentration of glucose present in the sample. Glucose assay was calibrated on RA-XT chemistry analyser using Biomerieux calimat (Cat. # 62321). Biomerieux Lyotrol N (Cat. # 62 373) and Bayer control serum 2 were used as quality control samples for the glucose assay.

2.9: Isolation of the HDL subfractions from plasma

Plasma total HDL and HDL₃ subclass were separated using Quantolip reagent A (Ref. # 8251015) and Quantolip reagent B (Ref. # 8252015). These reagents contain polyethylene glycol (PEG) of different concentrations and pH, dissolved in 0.1 mmol phosphate buffer, and 0.9g/l sodium azide. PEG reagent of different concentrations and pH results in the selective precipitation of different lipoprotein fractions. Reagent A precipitates VLDL and LDL subfractions, while reagent B precipitates both of those subfractions along with total HDL. The HDL precipitation reagent was added to plasma samples in a ratio of 2:1. This mixture was then vortexed and incubated at room temperature for 10 minutes. The samples were centrifuged at 4500 rpm for 15 minutes. The resultant supernatants were removed carefully ensuring that pellet formed at the bottom of the tubes were not disturbed. With every batch of samples precipitated for HDL analysis an immunolip control sample was also precipitated. This precipitation control sample was then assayed with HDL samples to ensure that HDL precipitation was successful.

2.10: Analysis of HDL subfractions

The supernatant from HDL fractions were then assayed on RA-XT automated analyser for cholesterol, phospholipid, triacylglycerol and apolipoprotein A1 using chemistry program specific for assaying these lipids/protein in the HDL sub-fractions. All results were expressed in mmol/l. Composition of the HDL₂ sub-fractions was calculated by subtracting the assay value for each of the analytes in the total HDL from that assay value in the HDL₃ sub-fraction.

2.11: Analysis of the lipid composition of the HDL subfractions

The same assay techniques were used to analyse HDL lipid and protein concentrations as was used for the analysis of plasma samples. The calibrator and quality control sera were precipitated in the same fashion described above (Section 2.10) to isolate the HDL fraction of the calibrator and quality control sera. The calibrator serum was precipitated with HDL reagent A and the RA-XT chemistry analyser was calibrated accordingly was treated in the same fashion as the HDL samples. Quality control of each HDL lipid parameters was ensured using Lyotrol N, precinorm and precipath (Boehringer Mannheim) quality control plasma, which was precipitated with Immuno HDL reagent A. HDL apo A1 quality control was ensured using precinorm and precipath quality control sera (Boehringer Mannheim; Cat. # 781 827) which was precipitated by Immuno quantolip reagent A to isolate the HDL subfraction.

2.12: Isolation and measurement of LDL cholesterol concentrations.

LDL was precipitated using a Biomeriux LDL precipitation reagent. 1 ml of LDL precipitating reagent and 50 µl of plasma were vortex and incubated at 4 °C for 25 minutes. Samples were then centrifuged at 4000 rpm for 25 minutes. Supernatant was removed and pellet was well drained. Pellet was then resuspended using 0.5 ml a pellet resuspension agent supplied in LDL precipitation kit. Samples were then stored at – 20°C until LDL cholesterol analysis could be performed using a Technicon RA-XT clinical chemistry analyser. An immunolip (Immuno, Austria) control sample was precipitated with every fifth plasma LDL isolations to ensure that LDL precipitation reagents were working properly. Precinorm and precipath (Boehringer Mannheim) were used as LDL cholesterol controls. These control sera were precipitate in the same manner as plasma samples, but were precipitated in batches, frozen and run as daily controls when LDL cholesterol were been run of RA-XT clinical chemistry analyser.

2.13.0: ELISA assay for the determination of CETP mass

The capture monoclonal antibody (2F8) to identify the epitope of CETP was obtained from Pfizer Pharmaceuticals Inc. The 2F8 antibody was added to Costar High Binding 8-well strip plates at a concentration of 1.0µg protein/100µl PBS (pH 7.8) per well. The plates were sealed and incubated over night at 4° C. The free antibody was

removed by washing plates 4 times with 250 μ l of 0.05% Tween 20/PBS (pH7.8). Then 350 μ l of block solution (0.05 Tween 20/1% BSA/PBS) was added. The plates were incubated at room temperature for 3 hours. The block solution was removed, wells were then rinsed once with 250 μ l/well Tween/PBS.

A standard curve was constructed using CETP standard (2.57 μ g/ml) which was supplied from Pfizer Pharmaceuticals Inc. A standard curve in the range of 10 ng/100 μ l - 0.08 ng/100 μ l was constructed from CETP standard by diluting in block solution. Samples were diluted 1/100 with block solution. All assays were carried out in triplicate. Quality control was maintained using plasma standard (2.0 μ g/ml) which was supplied by Pfizer pharmaceuticals Inc. This was diluted to within the range of the standard curve. On each ELISA plate a standard curve was constructed, blanks, and control samples run on each ELISA plate.

Samples and standards were diluted appropriately in block solution and 100 μ l of diluted sample was placed in each well. The plates were incubated at room temperature for 2.5 hours. The ELISA plates were washed 4 times with 250 μ l Tween/PBS. 100 μ l block solution containing 2E7 detection monoclonal antibody (approximately 0.4 μ g 2E7 protein per 100 μ l) (Pfizer Pharmaceuticals) conjugated to alkaline phosphatase (See section 2.15.1 for manufacture of this conjugate) was added to each well. The samples were incubated at room temperature for 1.25 hrs. The detection antibody was removed, by rinsing 3 times with 250 μ l per well Tween /PBS, followed by a single rinse with 4.6 mM Na₂CO₃, 19 mM NaHCO₃, 2 mM MgCl₂, pH 9.5. The ELISA plates were sealed and the colour was allowed to develop overnight at room temperature. Absorbance was read at 405 nm.

2.13.1: Conjugation of Alkaline Phosphatase to 2E7 anti-CETP monoclonal antibody

Sigma alkaline phosphatase (Cat. #P-5521) was centrifuged at 4500 rpm for 5 minutes. The pellet was resuspended in PBS, pH 7.0, to a concentration of 2 - 4 mg/ml. Alkaline phosphatase and 2E7 Mab were combined in a ratio of 1:4 giving a final protein concentration of 1 - 2 mg/ml. This was dialysed for 24 - 48 hrs, against three changes of PBS, pH 7.0 at 4^o C. 25% glutaraldehyde (Sigma Cat. # G-5882) was added for a final concentration of 0.2%. This was incubated at room temperature for 2 hrs and dialysed against two changes of PBS/0.02% NaN₃, pH 7.0, and then two changes of 50 mM tris/0.02% NaN₃, pH 8.0 at 4^o C over 48 hrs.

2.13.2: Calculation of results

Absorbance value of samples were measured using a Microtitre Elisa plate reader. Concentrations of each sample were calculated using a CETP standard curve (See figure 1) which was run on each Elisa plate with plasma samples and standards. Results were expressed CETP mass $\mu\text{g/ml}$. An intra assay coefficient of variation of $< 4\%$ and inter assay coefficient of variation of $< 6\%$ for CETP mass assay was achieved.

2.14.0: Determination of cholesteryl ester transfer protein activity using fluorescence

Combine 10 μl of NBD-CE Donor, 10 μl VLDL acceptor particle (Wak Chemi Medical, Bad Soden, Germany) with 3 μl plasma sample, in 500 μl of 10 mM tris, 150 mM NaCl, 2mM EDTA, pH 7.4. Incubate for 3 hours at 37°C . Fluorescence of samples was measured in a RF-1501 Shimadzu Spectrofluorophotometer at excitation wavelength of 464 nm and emission wavelength of 535 nm.

2.14.1: Construction of standard curve

The molecular weight and concentration of fluorescent substrate is printed on each CETP activity kit (Wak Chemi Medical, Bad Soden, Germany). A standard curve was constructed to determine the relationship between fluorescence intensity and mass transfer using the NBD-CE donor particle diluted in isopropanol. Take 5 μl of donor particle and dilute in 2ml of isopropanol, and vortexed thoroughly to ensure that NBD-CE was unquenched by measuring fluorescence in fluorimeter. If intensity was slowly increasing inadequate dispersion of NBD-CE had occurred. When the fluorescence intensity stabilised the top working standard (TWS) was diluted four fold in a serial dilution. NBD-CE was expressed in pmol/ml. A standard curve was constructed by plotting fluorescent intensity against NBD-CE concentration. The fluorescent intensity of samples were read from the standard curve and expressed in pmol/ml. A new standard curve was constructed with each new batch of samples being assayed.

CETP plasma standard (2.0 $\mu\text{g/ml}$) supplied by Pfizer Pharmaceuticals was used as a quality control sample for all CETP activity assays. The intra-assay coefficient of variation was $< 8\%$ and the inter-assay coefficient of variation was $< 12\%$ for CETP activity assay. In sample blank water replaced plasma, otherwise blanks were treated the same as plasma samples.

Fluorescence values for blanks were subtracted from fluorescence values for plasma samples, these were then read from the standard curve. Rate of cholesteryl ester transfer was calculated by converting fluorescent values into pmol NBD-CE transferred / 3 hours using the standard curve.

2.15.0: Measurement of CETP activity using radiolabelled CE

2.15.1: Preparation of radiolabelled HDL₃

Radiolabelled HDL₃ was prepared by a method derived from that of Tollefson *et al.* (1988). HDL₃ was isolated from 20 ml of fasting human plasma by adjusting its density to 1.13 g/ml using solid KBr. 2.1 ml of plasma placed in Beckman optiseal tube and overlaid with saline solution of $d = 1.13$ g/ml. HDL fraction $d > 1.13$ g/ml was recovered via tube slicing, and was dialysed for 24 hours in TBS pH 7.4. A quantity of 10 nmol of (1,2, (n)-³H- cholesterol (specific activity, 46.3 Ci/mmol) was evaporated to dryness using a vortex evaporator. This was redissolved in 50 μ l of ethanol. The plasma fraction of $d > 1.13$ g/ml was then added to the radiolabeled cholesterol solution under gentle stirring. The mixture was incubated for 24 hours at 37 °C in a shaking water bath to allow esterification and incorporation of ³H-cholesterol into HDL₃. After incubation the density of the solution was adjusted to 1.13g/ml and subjected to ultracentrifugation as describe above. The resulting supernatant was adjusted to $d < 1.21$ g/ml and fraction $d < 1.21$ g/ml with HDL₃ was recovered. The HDL₃ was subject to another ultracentrifugation at 250000 x g with a TLA 100.4 rotor for 10 hours. The HDL₃ preparation was dialysed against TBS pH7.4 for 24 hours. The labeled preparation of HDL₃ had a specific activity of about 43,000 cpm/nmol of cholesterol using TLC. More than 96 % of radioactivity was associated with the CE fraction.

2.15.2: Measurement of cholesteryl ester transfer activity using radiolabelled CE.

Using this assay, CETP activity of a sample was measured on its ability to promote the transfer of radiolabelled CE from HDL₃ to endogenous apo B lipoproteins (Abbey *et al.* 1994). 20 μ l of HDL₃ containing 2.5 nmol cholesterol (40000 CPM/30 μ l) was added to 300 μ l plasma. 1 μ l of DNTB (90 mmol/l) was added to this mixture to inhibit LCAT activity. Samples were incubated for 3 hr at 37 °C. After the incubation apo B lipoproteins were precipitated using Manganese chloride/ heparin as outline in section 2.15.3. Precipitant was washed with 1ml of 0.5M NaCl. Pellets were then

resuspended in 10 W/V NaCl. Radioactivity of supernatant and pellet was measured using 5-ml scintillation fluid. The percentage radioactivity transferred to apo B lipoproteins was determined (Abbey *et al.* 1994). All assays were carried out in triplicate. Coefficient of variation for this assay was 4.8 % (n = 15).

2.15.3: Precipitation of apo B lipoproteins.

250 µl of plasma placed in glass centrifuge tube. 10 µl of heparin solution (5000 units /ml and 12.5 µl of 1 M MnCl₂ are added to plasma and mixture is vortexed. Samples are allowed to stand in a water bath for 30 minutes. Samples then centrifuged at 1500 x g for 30 minutes at 4 °C.

2.16.0: Ultracentrifugation techniques

2.16.1: Isolation of chylomicrons:

Procedure for the isolation of chylomicrons similar to that of Grundy and Mok (1976) with some modifications. 1.6 ml of plasma was carefully overlaid with 1.6 ml of saline solution in a 3.2-ml Beckman ultracentrifuge tube. Saline solution was prepared by dissolving 9.6 g NaCl in 1l of distilled water (d = 1.006). The density of saline solution was checked using a hydrometer. Tubes were centrifuged at 300,000 x g using a TLA 100.4 rotor for 20 minutes in a Beckman TLX Ultracentrifuge. Subsequent to ultracentrifugation chylomicrons were removed within a 0.5 ml using a pasteur pipette. Chylomicrons and supernatant were put into aliquots, stored and analysed for cholesterol and TAG concentrations using procedures described in sections 2.4 and 2.5.

2.16.2: Isolation of VLDL

VLDL was isolated using ultracentrifugation techniques. Density of serum was adjusted by the addition of a certain volume of a stock density solution (d = 1.34 g/ml). This stock solution was prepared by addition of 153 g NaCl and 354 g KBr made up to 1l with distilled water. Saline solutions of lower densities were prepared a second stock solution consisting of 0.15 M NaCl (d = 1.005). Solutions of lower densities were prepared by dilution of stock solution with saline solution (d = 1.005). The following equation was used to calculate dilutions.

$$A \times 1.005 + B \times 1.34 = (A + B)X$$

A = volume of saline (d = 1.005)

B = volume of stock solution

X = desired density of solution

Density of serum and infernatents were adjusted using the following equation

$$A \times Y + B \times Z = (A + B) X$$

A = volume of serum or infernatent

B = volume of salt solution

Y = density of serum or infernatent whose density is to be raised

Z = density of salt solution

X = density desired

VLDL was isolated at a density of 1.02 g/ml. Density of plasma was adjusted to 1.02 g/ml by the addition of an appropriate quantity density solution d = 1.34 g/ml to plasma. 1.6 ml of plasma was carefully overlaid with 1.6 ml of saline solution of density 1.02 g/ml and centrifuged at 250000 x g for 3 hr. VLDL was removed within 0.5 ml using a Pasteur pipette. VLDL was stored at -70°C until TAG and Cholesterol concentrations could be determined. The infernatent was analysed for TAG and cholesterol concentrations.

2.17: DNA analysis

Genomic DNA was isolated from whole blood by heamolysing the red blood cells. White blood cells were pelleted and washed with Tris EDTA pH 7.5 until no trace of red blood corpuscles was evident. The white blood cells were treated with proteinase K for 45 minutes, after which it was denatured. Amplification of a 213 base pair fragment of the CETP gene containing the Taq 1B was carried out using PCR. The forward (GGT CCT AGC TGC ATT GCA AAC) and reverse (GAT GGA GCC TCC GTC GTC ACC TGA) primers were synthesized on a 391 DNA synthesizer (Applied Biosystems). The PCR reaction consisted of a 7 minute denaturation at 94°C, then 35 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute 12 seconds and finally a 5 minute extension at 72°C. This PCR program was carried out on 25 µl reactions containing 1.25mM dATP, dCTP, dGTP, dTTP, 6 % 20mM Mg²⁺ buffer, 0.6

units and 1 µl DNA in 15mM Mg2 buffer, plus the forward and reverse primers. 5 µl of the PCR product was digested with 1 unit Taq 1B (Boehringer Mannheim) for 4 hours at 65°C. Digestion products were run on 3 % agarose gel. Individuals who did not have restriction site had a band at 213 bp (B-B-), heterozygotes had bands at 213, 142 and 71 bp (B+B-), while individuals who cut had bands at 142 bp and 71 bp (B+B+).

2.18.0: Gas Chromatography Analysis

2.18.1: Isolation of platelet and plasma phospholipids

Platelet phospholipids were isolated as follows. 400 µl of plasma or 1ml of a platelet pellet suspended in 1ml of Tris/EDTA was placed in a Boroscillate 16 ml tube. 2.5 ml of chloroform:methanol (2:1) with 0.01 % w/v BHT was added. Samples were vortexed for one minute and then centrifuged at 2500 rpm for 10 minutes. The supernatants were removed using a glass pasteur pipette and transferred to a 12x75mm boroscillate tube. 2 ml of chloroform with 0.01 % BHT was added to sample, vortexed and centrifuged 2500 rpm for 5 minutes. Supernatant was removed with a Pasteur pipette and pooled with the first lipid extract. Lipid present in organic phase was dried using a vortex evaporator (AGB Scientific, Dublin). When samples were dried, they were flushed with N₂ and sealed to prevent lipid oxidation and stored at - 20 °C.

2.18.2: Isolation of phospholipids and cholesterol esters

Total phospholipid and cholesterol esters were isolated from plasma and phospholipid samples using Thin Layer Chromatography (TLC). The following solvent system was used 80 % v/v Petroleum ether, 20 % v/v Diethyl ether and 2 % v/v Formic acid. The solvent system set up in the TLC tank with two sheets of filter paper. The TLC tank was allowed to equilibrate for 1 hour. Silica Whatman plates were activated by heating to 120 °C for one hour. Lipid extracts were reconstituted in 30 µl of chloroform. Sample was vortexed and applied to the TLC plate in 5 µl aliquots. 30 µl of chloroform was again added to lipids sample and applied to TLC plate to ensure total lipid recovery. Phospholipid and cholesterol ester standards were run on each TLC plate. TLC plate was placed in TLC tank and allowed to develop for 35 minutes. The plate was removed from the tank and allowed to dry in the fume hood for 30 minutes. Lipid fractions were identified by developing TLC plate by spraying plate with a 0.1 % solution of 2,7 dichlorofluorescein in methanol and then viewed under an UV lamp.

Lipid fractions were identified by comparing how far they migrate compared with lipid standards. Desired lipid fraction was scraped off TLC plate and placed into a borosillate tube. Lipids were extracted from silica by the addition of 3 ml of chloroform:methanol (2:1). Samples were vortexed for 60 seconds. Silica was precipitated by centrifugation at 2500 rpm for 5 minutes. Solvent fraction was removed and placed in a 12 x 75 mm borosillate tube. Lipid extraction was carried out X2 times to ensure maximal lipid recovery. Pooled lipid extracts were dried in a vortex evaporator. Dried samples were flushed with nitrogen, sealed and stored at -20°C .

2.18.3: Generation of methyl esters of platelet phospholipids

Plasma and platelets phospholipids were transesterified, in order to allow gas chromatographic analysis of phospholipid fatty acids. 0.5 ml Boron trifluoride methanol (BF_3) was added to each dried phospholipid sample and vortexed for 30 seconds. Samples were then flushed with N_2 , sealed and incubated on a heating block at 75°C for 30 minutes. Methyl esters were then extracted by adding 0.5 ml hexane to samples and vortexing for 60 seconds. Supernatant was then removed using Pasteur pipette and transferred to 10x75 mm borosillate tube. This extraction procedure was repeated twice more. Methyl esters were placed in vortex evaporator and dried. Once dried samples were flushed with N_2 , sealed and stored at -20°C .

2.18.4: Generation of methyl esters of cholesterol esters for gas chromatography analysis

Cholesterol ester were transmethyalted by the addition of 2.0 ml of 0.1M NaOH in methanol to samples. Samples were then placed on heating block at 75°C for 15 minutes. 0.5 ml of BF_3 was then added, samples were flushed with N_2 and placed on heating block for 15 minutes. Methyl esters were then extracted from samples as described previously.

2.18.5: Generation of methyl esters in total plasma lipids for CLA analysis

Methyl esters of total plasma lipids were prepared by adding 0.5 ml of 0.01M NaOH in dry methanol. Samples were vortexed and flushed with N₂ and then placed in a heating block at 60°C for 15 minutes. 0.75 ml of BF₃ was then added to mixture, samples were vortexed and incubated at 60°C for 15 minutes. Lipids were extracted with 0.5 ml of hexane X3 as described in section 2.18.1. Samples were dried in a vortex evaporator, and stored under N₂ at -20 °C until analysis.

2.18.6: Gas chromatography conditions for the analysis of the fatty acid methyl esters of platelet phospholipid and plasma cholesterol esters

Fatty acid methyl esters of phospholipids and cholesterol esters were analysed using a Shimadzu GC-14A gas liquid chromatograph (Mason Technology, Dublin) fitted with a Shimadzu C-R6A integrator. A 25 M BP 21 polar aluminum silica column (Scientific Glass Engineering Ltd, Milton Keynes, England) was fitted. Oven conditions consisted of two temperature ramps from 120°C to 200°C at 4 °C / min. Held at 200°C for 12 minutes and then increased to 220°C at 4 °C/min. Column was then held at 220°C for 12 minutes. Detector and injector temperatures were 260°C and 250°C respectively. Fatty acids were identified by comparing the relative retention times of fatty acids with a FAME standard (Sigma, Chemical Co. St. Louis, MO, USA). Fatty acid compositions were calculated as a percentage of total fatty acids of the standard.

2.18.7: Analysis of methyl ester of CLA in total plasma lipids using Gas chromatography.

The methyl ester composition of total plasma lipids for CLA were analysed using a Shimadzu GC-14A Gas Liquid Chromatograph (Mason Technologies, Dublin) which was fitted with a Shimadzu C-16A integrator. A CP Sil 88 fused silica column (50m x 0.22 mm, 0.2 film thickness; Chrompack Ltd, Middelburg, The Netherlands) was fitted. Nitrogen was used as a carrier gas. Oven temperature conditions for each run were an initial column temperature of 120°C which increase at 8°C/min to 180°C. Column temperature was held at 180°C for 40 minutes. Column temperature was increased at 4°C/min to 220°C and was held at 220°C for 15 minutes. Peaks were identified using a FAME standard spiked with known concentrations of the cis-9, trans-11 and trans-10, cis-12 isomer of CLA. Fatty acids were identified with retention times

compared to standard and fatty acid compositions were calculated as a percentage of the total fatty acids.

Chapter 3

The effect of postprandial lipaemia and Taq 1B polymorphism of the cholesteryl ester transfer protein (CETP) gene on CETP mass, activity, associated lipoproteins and plasma lipids

3.0: Introduction:

Increases in cholesteryl ester transfer protein (CETP) activity during postprandial lipaemia have been reported previously (Tall *et al.* 1986) where a 2-3-fold increase in cholesteryl ester (CE) transfer was reported. As a consequence of CETP activity, there is a transfer of cholesteryl ester (CE) from high-density lipoprotein (HDL) particles to very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) particles with a reciprocal transfer of triacylglycerol (TAG). Alterations in HDL size and composition occur as a result of CETP activity. Precursors to HDL are pre Beta HDL, which contain apo A1 proteins with a small amount of phospholipid. These Pre Beta HDL can bind to cells promoting efflux of cellular cholesterol (Fielding and Fielding 1995). They may combine with chylomicron remnants produced during postprandial lipaemia and accumulate CE and phospholipids. This results in the formation of mature HDL, which is facilitated through the actions of LCAT (Skinner *et al.* 1994). Mature HDL can be divided into HDL₂ and HDL₃ based of their lipid and lipoprotein composition. HDL subfractions are dynamic and constantly change from more dense HDL₃ to less dense HDL₂ particularly during postprandial lipaemia through the activities of CETP. Triglyceride rich HDL₂ is catabolised by hepatic lipase resulting in the formation of HDL₃. VLDL and IDL accept CE from HDL with a reciprocal exchange of TAG into HDL as a result of CETP activities. VLDL and IDL are catabolised to form LDL. A number of publications suggest that CETP activity may be pro-atherogenic, because it results in the reduction of HDL cholesterol, increased production of dense atherogenic LDL and increased catabolism of TG rich HDL by hepatic lipase (Lagrost *et al.* 1994c; Tall, AR 1993). The increase in CETP mass, activity and the redistribution of CE from HDL to VLDL and LDL lipoprotein fractions during postprandial lipaemia, suggests the pro-atherogenic effect of CETP may be elevated during lipaemia. Cholesterol intake (Fielding & Fielding 1995c; Quinet *et al.* 1990), dietary trans fatty acid consumption (Abbey *et al.* 1994) alcohol consumption (Savolainen *et al.* 1990) and genetic variations, all account for differences in CETP mass and activities in normolipidaemics. Where there is genetic variants of CETP, for example the absence of Taq 1B polymorphism (Bernard *et al.* 1998), or splicing defects which are common in the Japanese population (Inazo *et al.* 1990; Koizumi *et al.* 1991), there is increased HDL cholesterol concentrations.

A number of previous studies have investigated the effect and frequency of the Taq 1B polymorphism of the CETP gene (Kuivenhoven *et al.* 1997; Hannuksula *et al.* 1994; Kuivenhoven *et al.* 1998; Freeman *et al.* 1993). Kuivenhoven *et al.* (1998) noted

that the frequency of Taq 1B polymorphism in a group of patients with coronary atherosclerosis was B+ B+, 35 %, B+ B-, 49 % and B- B- 16 %. Data from many of these previous studies indicate those who show the absence of the Taq 1B cutting site (B- B-) have lower CETP mass and higher HDL cholesterol concentrations (Kondo *et al.* 1989; Freeman *et al.* 1990). It is hypothesized that an altered HDL cholesterol concentration in these individuals is a direct result of the presence or absence of the Taq 1B polymorphism on the CETP gene. However a mechanism as to how this occurs is not forth coming.

The present investigation was designed to address whether there is an increase in CETP mass and activity in response to a test meal containing a typical amount of fat (40g) in a large normolipidaemic study group. Although the effects of acute fat ingestion on CETP mass and activity have been previously studied, the majority have measured either CETP mass or activity but not both. Many of the previous studies on CETP have concentrated on CETP mass and activity in hyperlipidaemic subjects in order to ascertain if their condition results in altered CETP. In this study, a large number of lipid parameters were analysed to try and ascertain if CETP activities could be attributed to greater coronary risk factors in a normolipidaemic population. Finally an investigation into the effects of the Taq 1B polymorphism on CETP mass or activity and associated lipids in fasting and postprandial states was carried out.

3.2.0: Methods

3.2.1: Study subjects

The study was conducted in the nutrition laboratory at the Trinity Centre, St. James Hospital, Dublin 8. Sixty-three non-smoking healthy volunteers were recruited from the personnel of Trinity College Dublin and St. James's Hospital, Dublin. Biochemical inclusion criteria included fasting plasma cholesterol < 7.0 mmol/l, plasma triglyceride < 2.0 mmol/l, glucose < 110 mg/dl, gamma-glutamyltransferase < 60 IU/l, haemoglobin > 12 g/dl and a BMI < 30. Each study day began between 07:30 and 08:00 am, following a 12 h overnight fast. Subjects were asked to abstain from alcohol and exercise 24 h before a postprandial day. During postprandial investigations each subject was asked to abstain from food and drink with the exception of water, decaffeinated coffee and diet drinks. The test meal was prepared each day. Each subject received 40g fat which was high in monounsaturated fatty acids (Flora Sun Flower Oil), 150 g of skimmed milk and 5 g of orange marmalade flavour system (Scientific Hospital Supplies, UK Limited) which was mixed with water.

A 21 gauge 32mm venous catheter (Abbott Ireland Ltd., Dublin, Ireland) was inserted into the antecubital vein of the non-dominant forearm. Blood samples for CETP analysis were collected in 5 ml citrated tubes ((0.106M-sodium citrate) (Starstead monovette). Blood samples for lipid analysis were collected into 10 ml heparinised vacutainers. All blood samples were drawn before the test meal was administered. The test meal was consumed within 15 minutes. A further blood sample was drawn 6 h after the test meal. All samples were centrifuged (3000 rpm for 15 min) at room temperature. Plasma was removed, mixed and aliquots of 0.5 ml fractions were made. Samples for CETP analysis were snap frozen under liquid N₂ and stored (-70 °C) until CETP mass and activity was assayed. Plasma for lipid analysis was stored at -20 °C.

3.2.2: Laboratory methods

Analysis of plasma TAG (TAG PAP Biomerieux, France), cholesterol (Biomerieux PAP), NEFA (Acyl Co A synthetase-acyl Co A oxidase) Wako Chemicals, GmbH, Germany), phospholipid (Biomerieux PAP), apo A1 (Biomerieux) were measured. HDL cholesterol, TAG, phospholipid and apo A1 were measured using the methods mentioned previously following precipitation of HDL with Immuno Quantolip HDL (Immuno AG, Vienna) precipitating reagent. All determinations were performed on a Technicon RA-Xt Chemistry Analyser (Technicon Inc., Tarrytown, NY., U.S.A.)

CETP activity in plasma was determined using a fluorescent transfer method (WAK-Chemi Medical, Bad Soden, Germany). A fluorescent labelled cholesterol linoleate is sequestered within a donor particle in a quenched state. CETP facilitates the transfer of cholesterol ester to an acceptor particle where the fluorescence is unquenched. The increase in fluorescence is proportional to the rate of transfer activity. Total CETP mass in plasma was determined using an alkaline phosphatase based sandwich ELISA assay as described previously by Clarke *et al.* (1995).

3.2.3: DNA analysis

Genomic DNA was isolated from whole blood by heamolysing the red blood cells. White blood cells were pelleted and washed with Tris EDTA pH 7.5 until no trace of red blood corpuscles was evident. The white blood cells were treated with proteinase K for 45 minutes, after which it was denatured. Amplification of a 213 base pair fragment of the CETP gene containing the Taq 1B was carried out using PCR. The forward (GGT CCT AGC TGC ATT GCA AAC) and reverse (GAT GGA GCC TCC GTC GTC ACC TGA) primers were synthesized on a 391 DNA synthesizer (Applied Biosystems). The PCR reaction consisted of a 7 minute denaturation at 94°C, then 35 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute 12 seconds and finally a 5 minute extension at 72°C. This PCR program was carried out on 25 µl reactions containing 1.25mM dATP, dCTP, dGTP, dTTP, 6 % 20mM Mg²⁺ buffer, 0.6 units and 1µl DNA in 15mM Mg² buffer, plus the forward and reverse primers. 5 µl of the PCR product was digested with 1 unit Taq 1B (Boehringer Mannheim) for 4 hours at 65°C. Digestion products were run on 3 % agarose gel. Individuals who did not have restriction site had a band at 213 bp (B-B-), heterozygotes had bands at 213, 142 and 71 bp (B+B-), while individuals who cut had bands at 142 bp and 71 bp (B+B+).

3.2.4: Data Analysis

All statistical analysis was carried out on an apple Macintosh statistical package Data Desk 4.1 (Data Description Inc., NY., U.S.A). The distribution of the data for each variable was assessed. Some of the variables were transformed to normalise the distribution of some of the data sets ($\sqrt{\text{HDL}}$, log of CETP data). Paired student t-test was used to analyse the difference in variables between fasting and 6 h postprandial samples. Pearson product moment correlation analysis was used to investigate the relationship between plasma lipid and lipoprotein concentrations and CETP metabolism. Stepwise multiple regression analysis investigated the factors, which determined CETP

mass, and activity in the fasted and 6 h postprandially. One way ANOVA was used to compare significant difference in CETP mass, activity and plasma lipids according to Taq 1B polymorphism grouping.

3.3.0: Results

3.3.1: Plasma lipids and CETP during Postprandial Lipaemia

Sixty-three subjects, 39 males and 24 females, participated in this study. The total study group had a mean age of 32.4 (SD 9.6) years, a mean weight of 73.48 (SD 13.65) kg and a mean body mass index (BMI; kg/m²) of 24.14 (SD 2.62). The effect of postprandial lipaemia on CETP was investigated at 2 hourly (h) intervals over eight hours. Maximal CETP mass and activity occurred at 6 h, 2 h after peak triglyceride production (See figures 3.3.1.1 and 3.3.1.2). Therefore 6 h time point was used to access the effect of lipaemia on CETP in this investigation. Fasting and 6 h postprandial CETP mass, activity and lipid concentrations are given in table 3.3.1.1. There was a significant increase in CETP mass ($P = 0.001$) and activity ($P \leq 0.0001$) from the fasted state to 6 h after ingestion of test meal. Plasma TAG and plasma NEFA were significantly increased ($P \leq 0.0001$) during postprandial lipaemia at 6 h, while LDL cholesterol concentrations were significantly ($P = 0.001$) reduced. The compositions of the HDL subfractions are given in table 3.3.1.2. Total phospholipid, apolipoprotein A1 and triglyceride concentrations were significantly increased ($P \leq 0.0001$) at 6 h. Total HDL cholesterol did not increase significantly. HDL₃ apo A1 and TAG concentrations were significantly ($P < 0.05$) increased 6 h postprandially, whilst cholesterol and phospholipid concentrations did not change significantly. All of the lipids of the HDL₂ subfraction increased significantly 6 h after ingestion of test meal, with the increase in HDL₂ TAG being the most significant ($P \leq 0.0001$). There was a strong positive relationship between CETP mass and activity in the fasted and postprandial states for the total study group ($r = 0.470$; $P \leq 0.0001$).

3.3.2: Plasma lipids and CETP metabolism

Fasting CETP mass was significantly correlated with age ($r = 0.310$, $P = 0.01$). Postprandial CETP mass (6 h) was significantly correlated with BMI ($r = 0.270$, $P = 0.03$) and body weight ($r = 0.265$, $P = 0.03$). Postprandial CETP activity was not significantly associated with age, weight and BMI. Stepwise multiple regression analysis of fasting CETP mass demonstrated that fasting plasma cholesterol was the most important factor ($\beta = 0.354$; $P = 0.0003$), followed by age ($\beta = 0.023$; $P = 0.01$). Stepwise multiple regression analysis of CETP mass at 6 h showed that fasting CETP mass was the most important determinant ($\beta = 0.019$; $P = 0.0001$), followed by fasting LDL cholesterol concentration ($\beta = 0.047$; $P = 0.003$), HDL₃ TAG concentration ($\beta =$

0.898; $P = 0.0101$) and HDL₃ apo A1 concentration ($\beta = -0.240$; $P = 0.01$). Step wise multiple regression analysis of fasting CETP activity showed that fasting CETP mass was the principle determinant ($\beta = 26.746$; $P = 0.0001$), while fasting plasma NEFA had a small effect ($\beta = 43.002$; $P = 0.06$). Multiple stepwise regression analysis of 6 h CETP activity showed that fasting cholesterol was the principle determinant ($\beta = 0.055$; $P = 0.0025$) followed by TRL TAG ($\beta = -0.013$; $P = 0.005$), and mass at 6 h ($\beta = 0.254$; $P = 0.007$).

3.3.3: Taq 1B genotype

The distribution of CETP Taq 1B alleles within this study group was 36.5 % B+ B+, 53.9 % B+ B- and 9.52 % B- B-. Analysis of variance was used on the comparison of the lipid concentrations of the B+ B+, B+ B- and B- B- polymorphisms to see if Taq 1B restriction site exhibited any influence on the lipid profile of subjects grouped according to particular polymorphism results are shown in table 3.3.3. Those individuals who were homozygous for the absence of the Taq 1B polymorphism (B- B-) had a significantly ($P < 0.05$) lower fasting CETP mass, higher fasting HDL, HDL₂ cholesterol and HDL phospholipid concentration than did those individuals who were heterozygous (B+ B-) or homozygous (B+ B+) for the presence of Taq 1B polymorphism (See table 3.3.3.). A similar trend was noted when 6 h CETP and 6 h plasma lipids were compared according to polymorphism grouping. However significant statistical association was not achieved.

Table 3.3.1.1: Mean values and standard deviations for plasma CETP mass and activity and for plasma lipids in the fasted state and at 6 hours following an acute fat load in healthy normolipidaemic subjects (n = 63).

	Time			
	0 hours		6 hours	
	Mean	SD	Mean	SD
CETP mass (µg/ml)	2.040	0.686	2.314*	0.985
CETP activity (pmol/ NBD-CE transfer/ 3hr)	29.851	7.232	39.011†	12.45
Triglyceride (mmol/l)	0.987	0.467	1.071†	0.477
NEFA (mmol/l)	0.482	0.185	0.616*	0.199
Cholesterol (mmol/l)	4.982	1.048	4.926*	0.989
LDL cholesterol (mmol/l)	3.621	0.785	3.582*	0.653

Values reported represent mean and standard deviation, of total study group (n = 63). Significant difference in mass and activity between 0 hours and 6 hours * $P < 0.001$; † $P < 0.0001$.

Table 3.3.1.2: Mean values and standard deviations for the concentrations of cholesterol, phospholipid, triacylglycerol (TAG) in the HDL subfractions in the fasted and postprandial states following an acute fat load in healthy normolipidaemic subjects (n = 63).

	Total HDL				HDL ₃				HDL ₂			
	<u>0 hours</u>		<u>6 hours</u>		<u>0 hours</u>		<u>6 hours</u>		<u>0 hours</u>		<u>6 hours</u>	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Cholesterol (mmol/l)	1.162	0.310	1.174	0.358	0.917	0.254	0.887	0.285	0.248	0.221	0.332†	0.254
Phospholipid (mmol/l)	1.047	0.230	1.168§	0.296	0.856	0.205	0.875	0.278	0.213	0.174	0.335†	0.279
Apo A1 (g/l)	1.74	0.669	2.18§	0.318	1.482	0.636	1.71*	0.528	0.378	0.357	0.561*	0.513
TAG (mmol/l)	0.159	0.057	0.210§	0.09	0.135	0.051	0.165*	0.081	0.033	0.033	0.063§	0.057

Values reported represent mean and standard deviation, of total study group (n = 63).

Significant difference in HDL composition between 0 hours and 6 hours. * $P < 0.05$; † $P < 0.005$; § $P \leq 0.0001$.

Table 3.3.3: Mean values and standard deviations for fasting levels of plasma CETP mass, activity, total HDL and HDL₂ cholesterol and HDL phospholipid concentrations according to CETP Taq 1 B genotype in 63 healthy normolipidaemic subjects.

	Genotype						<i>P</i> value
	<u>B- B- (n = 6)</u>		<u>B- B+ (n = 34)</u>		<u>B+ B+ (n = 23)</u>		
	Mean	SD	Mean	SD	Mean	SD	
CETP mass (µg/ml)	1.342	0.773	2.119	0.623	2.105	0.674	0.002
CETP activity (pmol/NBD-CE transfer/min)	26.116	7.229	29.72	10.130	31.042	9.075	N.S
HDL Cholesterol (mmol/l)	1.506	0.598	1.114	0.237	1.140	0.292	0.017
HDL ₂ Cholesterol (mmol/l)	0.526	0.405	0.200	0.173	0.245	0.173	0.05
HDL Phospholipid (mmol/l)	1.321	0.290	1.011	0.212	1.027	0.197	0.002

P values Indicates significant difference between B+ B+, B+ B- Taq 1B groupings and B- B- Taq 1B grouping using one way analysis of variance

Figure 3.3.1.1
Increases in CETP mass during postprandial lipaemia (n 18)

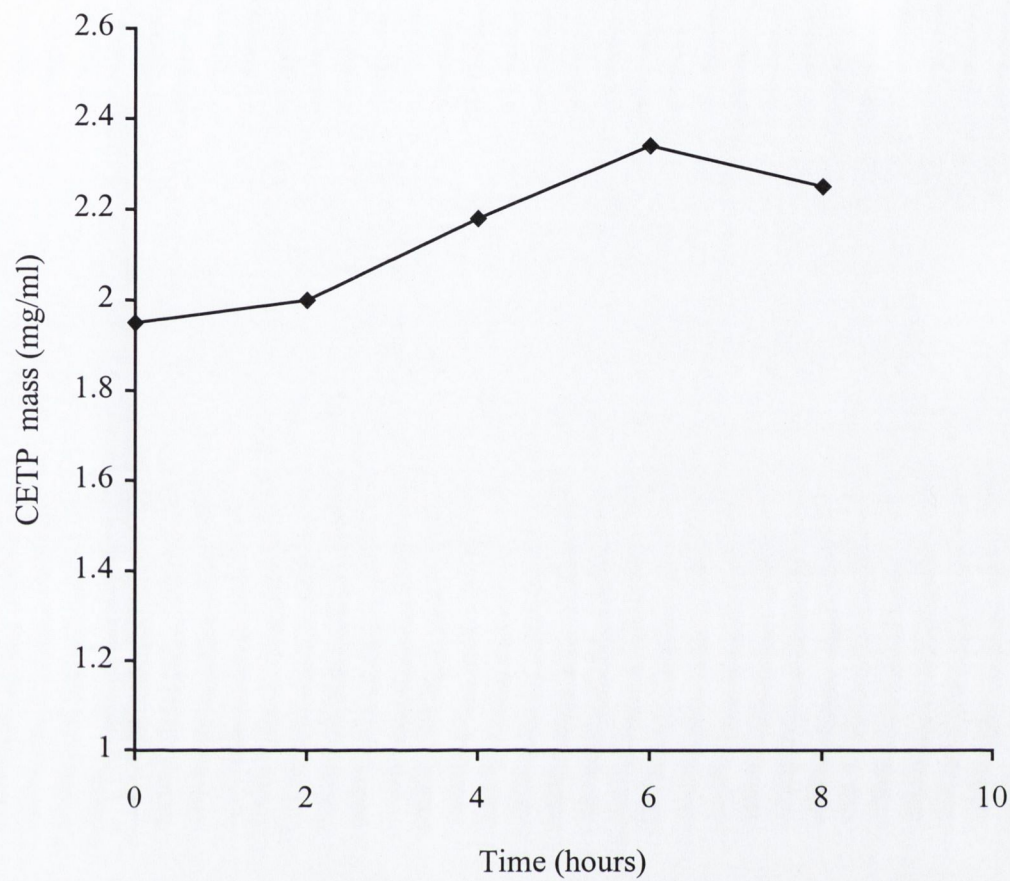
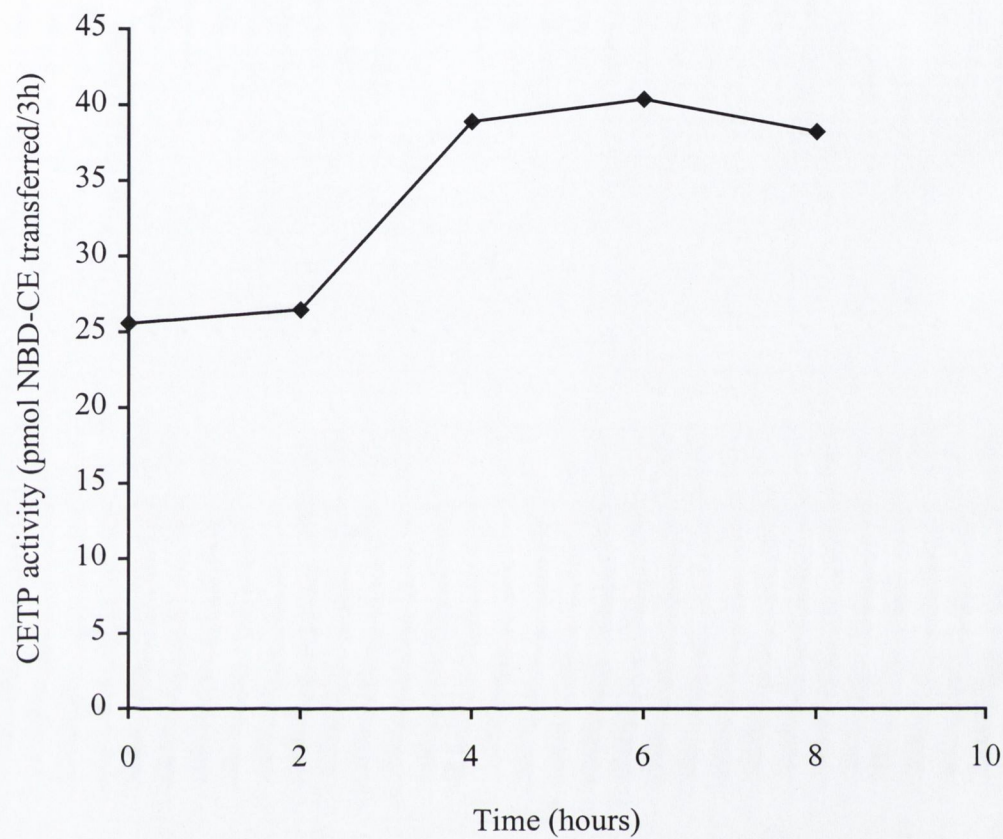


Figure 3.3.1.2

Increase in CETP activity during postprandial lipaemia (n 18)



3.4.1: Discussion

In a study by Tall *et al.* (1986) there was a 1.1 - 1.7 fold increase in CE transfer in response to a 135g fat rich test meal. In the present study there was an increase of 1.3 fold in CETP activity ($P = 0.0001$), and 1.13 fold increase in CETP mass ($P = 0.0001$) during postprandial lipaemia in response to a 40 g fat test meal. An investigation of the rate of production of CETP during postprandial in 18 subjects in the fasted state and at 2 hourly intervals for eight hours demonstrated that maximal CETP mass and activity were found at 6 h after ingestion of test meal similar to the findings of Tall *et al.* (1986). The 6 h samples were used as the time point for CETP and lipid analysis to see the effect of maximal CETP production on plasma lipids after ingestion of the test meal. Castro & Fielding, (1985) demonstrated that CE transfer doubled during postprandial lipaemia. Previous studies show that increases in CETP mass during postprandial lipaemia in type II diabetics and normal control subjects were significant (Lottenberg *et al.* 1996b). Discrepancies as to increases in CETP activity during postprandial lipaemia between studies may be explained by fat dose and composition of test meals administered to subjects. A MUFA test meal (which was administered in this study) tends to produce a lower postprandial response than does a saturated fat meal (de Bruin *et al.* 1993; Groener *et al.* 1991) as would the dose of fat administered in the test meal. Lottenberg *et al.* (1996a) demonstrated that increases in CETP activity were also accounted for by increases in substrate availability during lipaemia.

In the present study analysis of HDL subfractions showed the HDL₂ subfraction had the greatest increase in lipid composition 6 h postprandially, with HDL₂ TAG showing the most significant increase (Patsch *et al.* 1987; Patsch *et al.* 1984), followed by HDL₂ cholesterol, phospholipid and apo A1 concentrations. Total HDL cholesterol did not change significantly 6 h postprandially in this study as was demonstrated previously by Karpe *et al.* (1993). The HDL₃ subfraction showed the lowest change in composition during postprandial lipaemia, with significant ($P < 0.05$) increases in HDL₃ TAG and apo A1 being significant ($P < 0.05$).

The finding of the present study that fasting plasma cholesterol is the most important determinant of fasting CETP mass is supported by the study of Kahri *et al.* (1994) and Tato *et al.* (1995a). In other studies, this association failed to attain statistical significance (Jones *et al.* 1996; Tato *et al.* 1997). Tato *et al.* (1995a) also observed an association between postprandial CETP mass and both BMI and weight, but failed to reach statistical significance. The present study did find these associations

to be statistically significant, possibly because of a normolipidaemic study group used in this investigation compared to the study of Tato *et al.* (1995a) where the study group was hypercholesterolaemic.

Multiple stepwise regression analysis demonstrated that fasting plasma cholesterol was the primary determinant of CETP mass followed closely by age which confirms a link between CETP, plasma cholesterol and age, suggesting that plasma cholesterol may determine CETP mass. This data suggests that high CETP levels may be a consequence of elevated plasma cholesterol levels (Inazu *et al.* 1992; Bagdade *et al.* 1992a). Multiple stepwise regression analysis showed that the predictors of 6 h postprandial CETP mass were LDL cholesterol ($\beta = 0.047$), HDL₃ TAG ($\beta = 0.898$; $P = 0.01$) both of which are functional indicators of CETP activity and HDL₃ apo A1 ($\beta = -0.240$; $P = 0.015$) which is important in regulation of CETP activity (Guyard-Dangremont *et al.* 1994) and binding of CETP to HDL (MacPhearson *et al.* 1996).

Regression analysis showed that fasting CETP activity was associated with CETP mass. This has been shown in other studies (Clarke *et al.* 1995; Jones *et al.* 1996; Tato *et al.* 1995a). CETP activity was determined using a new quick fluorimetric assay. Rapid analysis of samples was possible and use of radioactive substrates was excluded. When fluorimetric CETP activity assay was compared with an established CETP mass assay (Clarke *et al.* 1995), satisfactory correlation was achieved considering that plasma used for the CETP activity assay contained many of endogenous plasma proteins which inhibit or activate CETP activity unlike many previous method of measuring CETP activity (Groener *et al.* 1986). This is one of the first investigations to carry out a comparison of this new method for the measurement of CETP activity with an established method for the measurement of CETP mass.

The association between plasma NEFA concentration and CETP activity approached significance ($P = 0.06$) as was demonstrated by Largrost *et al.* (1995). Postprandial CETP activity at 6 h was associated with plasma cholesterol ($P = 0.002$), TRL TAG ($P = 0.005$) and mass ($P = 0.007$) at 6 h. This association demonstrates that CETP activity in postprandial lipaemia is perhaps influenced by the availability of CE and TAG. Lottenberg *et al.* (1996a), Lassel *et al.* (1998) and Mann *et al.* (1991) have all demonstrated the importance of substrate availability in the regulation of CETP activity.

The frequency of the Taq 1B polymorphism of CETP was similar in our study group to that found in other studies (Kuivenhoven *et al.* 1998; Bernard *et al.* 1998). The relationship between the Taq 1B polymorphism and CETP mass, activity, and plasma lipids was investigated. The presence of the Taq 1B restriction site resulted in significantly higher ($P < 0.002$) fasting CETP and lower HDL cholesterol concentrations as has been demonstrated by Hannuksela *et al.* (1994); Kahri *et al.* (1998) and Kuivenhoven *et al.* (1998) over those individuals who were homozygous for the absence of the polymorphism. We also found a significant effect ($P < 0.05$) of Taq 1B RFLP on HDL₂ cholesterol concentrations (Freeman *et al.* 1993). The absence of Taq 1B restriction (B- B-) cutting site resulted in increased total HDL phospholipid concentrations which up to now has not been reported in any previous studies. This data seems to indicate that different alleles of CETP gene in a normolipidaemic population have an effect on CETP mass, HDL cholesterol and phospholipid concentrations. There is a lower concentration of CETP in those with B- B- allele. It could be hypothesized that there is less transfer of CE from HDL resulting in a greater concentration of CE being retained in the HDL fraction due to a lower concentration of CETP. There was a non-significant difference in CETP activity between the genotypes. These results conflict with previous studies (Freeman *et al.* 1993) where it has been suggested that association of HDL cholesterol concentrations with Taq 1B was only identified in extreme situations i.e. smokers, alcoholics, or alpha-lipoproteinemia but not in normolipidaemics. From the data in this study it would seem that in the normal lipidaemic population depending on their Taq 1B genotype, there is either an increased risk or decreased risk of being susceptible to coronary heart disease as a result of the CETP genotype. There was no statically significant association between the presence and absence of Taq 1B polymorphism and 6 h CETP, HDL cholesterol and phospholipid concentrations. This lack of association may be as a result of increased lipid concentration during lipaemia masking the effect Taq 1B polymorphism on plasma CETP and lipoprotein composition.

In summary the findings of this study were that there was a significant increase in CETP mass, activity, plasma TAG, and HDL₂ lipids in response to a test meal. CETP concentrations were associated with plasma cholesterol concentrations. Elevated CETP resulted in high LDL to HDL ratios demonstrating the proatherogenic effect of CETP in normolipidaemics. Increased neutral lipid exchange between lipoproteins as a result of CETP activity occurred during lipaemia suggesting the proatherogenic effects

of CETP may be increased during postprandial lipaemia. Polymorphisms of CETP were also important in determining basal CETP and HDL cholesterol and phospholipid concentrations indicating that there is a genetic predisposition to either anti or proatherogenic effects of CETP (elevated CETP concentration's resulting in low HDL cholesterol). The data in this study implicates that CETP may be proatherogenic at certain concentrations in a normolipidaemic population and that this effect may be exacerbated during lipaemia.

Chapter 4

Effect of plasma cholesterol concentrations on CETP mass, activity and related plasma lipoproteins

4.1: Introduction

Postprandial lipid metabolism is known to be important in relation to the risk of CAD. CETP activities are also known to affect CAD risk factors. This chapter will focus on the effect of plasma cholesterol concentrations on the interaction between CETP and postprandial lipoprotein metabolism. CETP plays an important role in lipoprotein metabolism by facilitating the exchange of CE and TG between HDL and apo B lipoproteins (Tall *et al.* 1986). CE is synthesised by LCAT during postprandial lipaemia (Castro & Fielding 1985). There is an increase in chylomicron and VLDL TAG concentrations during postprandial lipaemia, both of which contribute to increased activity of CETP. Therefore the potentially atherogenic capability of CETP activity may be exacerbated during postprandial lipaemia due to the increased availability of substrate (Fielding & Havel 1996). The consequence of elevated CETP activity is an increase in the production of atherogenic apo B lipoproteins and a reduction of HDL cholesterol concentrations. Plasma HDL cholesterol concentrations are inversely associated with development of coronary artery disease and CETP concentrations. The Framingham heart study indicates that low HDL cholesterol concentrations in association with elevated TAG concentrations are a risk factor for increased CAD development.

Several studies have examined the association between elevated CETP mass and activity and hyperlipidaemias. Bhatnagar *et al.* (1995) noted that NIDDM with hypercholesterolaemia (n 31) treated with pravastatin for 8 weeks in a double blind placebo controlled study resulted in the normalisation of CETP activity. Several investigations have demonstrated that hypercholesterolaemics have increased CETP concentrations over normal lipidaemics, with Moulin *et al.* (1992) reporting significantly higher CETP concentration in patients with nephrotic syndrome when compared to normolipidaemics. Tato *et al.* (1995a) noted that hypercholesterolaemics (n 113) had a 42 % higher CETP activity than did normolipidaemics (n 50), with Inazu *et al.* (1992) having similar findings. Bagdade *et al.* (1991) demonstrated that in hypercholesterolaemic patients (n 13) there was a significant difference in postprandial CETP activity at 1h, 2h, 4h, when compared to controls. The findings of these studies indicate that increased CETP activity in these patients contributes to increase CAD risk, due to increase CE concentrations in apo B lipoproteins.

Numerous investigations have been carried out on the effects of postprandial lipaemia on CETP mass and activity (Tall *et al.* 1986; Castro & Fielding 1988;

Lottenberg *et al.* 1994). These studies have shown increases in CETP mass and activity during the postprandial state. At a molecular level animal studies have shown there is an increase in the synthesis of CETP mRNA in response to atherogenic diets in rabbits (Quintet *et al.* 1990) demonstrating that CETP gene expression is responsive to dietary lipid intake. Tall *et al.* (1986) and Sutherland *et al.* (1998) have demonstrated that increasing chain length of dietary fatty acids in meals and production of apo B lipoproteins during postprandial lipaemia are important in determining postprandial CETP mass and activity. Groener *et al.* (1991) demonstrated that habitual fat consumption was important in determining CETP activity during postprandial lipaemia with habitual saturated fat diet producing a greater increase in CETP activity than habitual MUFA or PUFA diets regardless of meal composition used to induce postprandial lipaemia.

Previous work on the effects of postprandial lipaemia on CETP mass and activity has involved small subject numbers, and a comprehensive investigation into the relationship between CETP and plasma lipids and lipoproteins in a normolipidaemic (4.0 mmol/l total cholesterol) and moderately hypercholesterolaemic (6.01 mmol/l total cholesterol) population has not been carried out. The objective of this study was to investigate the effects of normal cholesterol and a moderately elevated cholesterol concentrations and normal TAG concentrations with no symptoms of CAD on CETP and related lipoproteins and risk factors for CAD in the fasted and postprandial states in a large population group. Many previous studies have only focused on the effects of hyperlipidaemias and diseased states on CETP mass and or activity. We also wanted to make a comprehensive investigation to assess the determinants of fasting and postprandial CETP mass and activity in subjects with normal and moderately elevated plasma cholesterol concentrations. In previous studies CETP activity was measured using the rate of transfer of a radiolabelled CE from HDL into apo B lipoproteins. In this study CETP activity was measured using a novel fluorescence technique. This method is simple to perform and did not require the separation of lipoprotein subfractions. The assay was validated by comparing it with more established method for the measurement of CETP mass (Clarke *et al.* 1995).

4.2: Methodology

4.2.1: Study design

This study was approved by the Ethics Committee of the Federated Dublin Voluntary Hospitals. This trial was conducted on an outpatient basis and subjects gave a written informed consent before participating in the trial. Sixty-three non-smoking healthy volunteers, 39 males and 24 females, between the ages of 18 and 50 years participated in the study. A screening blood sample was taken from each of the subjects to ensure that they adhered to the following exclusion criteria.

Inclusion criteria

Age 18 –60 years

BMI < 25 Kg/m²

Plasma Cholesterol < 7.5 mmol/l

Plasma TAG < 2 mmol/l

Gammaglutamyl transferase < 60 IU

Heamoglobin > 11mg/dl

Not habitual consumers of n-3 PUFA

> 60 minutes strenuous exercise per week

Each subject completed one postprandial investigation. This postprandial investigation required an overnight fast for 12 hours. Each postprandial day began between 8.00am and 8.30am. A cannula was inserted into the non-dominant arm. A fasting blood sample was then drawn. A test meal which was composed of 40g of fat (Flora Sunflower oil), 150g of skimmed milk and 5g of module orange flavour which was added to improve the palatability of the test meal. Postprandial blood samples were drawn every two hours for eight hours following the administration of the test meal. For the purpose of this study the fasting and 6 h blood samples were assayed. Subjects were asked to abstain from all other food and drink with the exception of tea, decaffeinated coffee, diet soft drinks and water.

4.2.2: Handling of blood samples

Blood samples for lipid analysis was collected in 10 ml Beckton and Dickinson heparin vacutainer tubes. Blood samples for CETP analysis were collected into 5 ml Starstead (Wexford, Ireland) monovette citrate tubes. Blood samples were immediately

centrifuged for 10 minutes at 3000 rpm. Plasma for lipid analysis was aliquoted into 0.5ml fractions and stored immediately at -20 °C. 2 ml of plasma was aliquoted for HDL isolation as described in section 2.9. Plasma for CETP analysis was aliquoted into 0.5 ml fractions, and were immediately snap frozen using liquid nitrogen to arrest all protease activity in the plasma samples. These samples were then stored at -80 °C until lipid analysis could be carried out.

4.2.3: Laboratory methods

Analysis of plasma TAG (TAG PAP Biomerieux, France), cholesterol (Cholesterol PAP, Biomerieux, France), NEFA (Acyl Co A synthetase-acyl Co A oxidase) Wako Chemicals, Gmbh, Germany), phospholipid (Phospholipid PAP Biomerieux, France), Apo A1 (Biomerieux, France) were carried out. HDL cholesterol, TAG, phospholipid and apo A1 were measured using the methods mentioned previously in chapter 2 following precipitation of HDL with Immuno Quantolip HDL (Immuno AG, Vienna, Austria) precipitating reagent. All Plasma lipids and proteins were assayed on a Technicon RA-XT Chemistry Analyser (Technicon Inc., Tarrytown, NY., U.S.A.) as detailed in chapter 2.

CETP activity was measured using a novel fluorescent transfer method (WAK-Chemi Medical, Bad Soden, Germany). A fluorescent labelled cholesterol linoleate is sequestered within a donor particle in a quenched state. CETP facilitated the transfer of cholesterol ester into an acceptor particle where the fluorescence is unquenched. The increase in fluorescence is proportional to the rate of transfer activity. Details of the assay are described in section 2.14.0. CETP mass was determined using an alkaline phosphatase based sandwich ELISA as developed by Clarke *et al.* (1995) and described in section 2.13.0.

4.2.4: Statistical Methods

The distribution of the data for each variable was assessed. Some of the variables were transformed to normalise the distribution of some of the data sets ($\sqrt{\text{HDL}}$, log of CETP data). Paired student t-test were used to analyse the difference in variables between fasting and 6 hours postprandially. Pearson product moment correlation analysis was used to investigate the relationship between plasma lipid and lipoprotein concentrations and CETP metabolism.

The study cohort was divided according to fasting plasma cholesterol concentrations to investigate differences in CETP and HDL metabolism in those with higher (moderately elevated) fasting cholesterol concentrations (> 5.2 mmol/l) compared to those with lower (normal) fasting cholesterol concentrations (< 5.2 mmol/l). A pooled t-test was used to investigate the significant differences in plasma lipid and lipoprotein concentrations and CETP metabolism between the high and low cholesterol groups, while paired t-test and Pearson product moment correlation analysis were used to investigate the relationship between lipid and CETP within both groups.

4.3.0: Results

4.3.1 CETP mass, activity and plasma lipids

The moderately elevated cholesterol group was significantly older ($P \leq 0.0001$) and had a greater body weight ($P = 0.07$) and BMI ($P = 0.0002$) than those with normal plasma cholesterol concentrations as shown in Table 4.3.1.1. Fasting and 6 h CETP mass, activity and plasma lipids of the moderately elevated and normal plasma cholesterol groups are presented in table 4.3.1.2. In the normal cholesterol group there was a non-significant increase in CETP mass ($P = 0.08$) from 0h to 6h, while a significant increase in CETP activity ($P = 0.001$) was observed during postprandial lipaemia. Plasma TAG concentrations increased significantly ($P = 0.01$) at the 6 h postprandial time point. There was no significant change in plasma cholesterol concentrations, LDL cholesterol concentration were reduced non-significantly six hours postprandially, compared to fasting values. In the moderately elevated cholesterol group both CETP mass and activity increased significantly ($P = 0.005$; $P \leq 0.0001$) following a test meal. Increases in 6 h TAG concentrations approached significance ($P = 0.07$). A non-significant decrease in plasma and LDL cholesterol concentrations were noted 6 h after ingestion of test meal.

Plasma and LDL cholesterol concentrations were significantly ($P \leq 0.0001$) different between the groups as would be expected. Fasting levels of CETP mass or activity were not significantly different between groups, but CETP mass and activity at 6 h were significantly ($P = 0.01$) higher in the moderately elevated cholesterol group.

Table 4.3.1.1: Age and body weights of the moderately elevated (> 5.2 mmol/l) and normal (< 5.2 mmol/l) plasma cholesterol groups.

	< 5.2 mmol/l total cholesterol (n = 39)	> 5.2 mmol/l total cholesterol (n = 24)
Age Years	28.66 (8.10)	38.54* (8.83)
Body weight Kg	71.80 (13.49)	78.48† (13.14)
BMI Kg/m ²	23.54 (2.22)	25.66§ (2.74)

Values reported represent the mean with standard deviation in parenthesis of the < 5.2 mmol/l total cholesterol study group.

Significance difference between groups *P ≤ 0.0001, †P = 0.07, §P = 0.0002.

4.3.1.2: CETP mass, activity and plasma lipid concentrations of the moderately elevated (> 5.2 mmol/l) and normal (< 5.2 mmol/l) total cholesterol groups.

	< 5.2 mmol/l total cholesterol (n = 39)		> 5.2 mmol/l total cholesterol (n = 24)	
	0 hours	6 hours	0 hours	6 hours
CETP mass µg/ml	1.96 (0.61)	2.09 (0.71)	2.22 (0.81)	2.67§‡ (0.75)
CETP activity pmol NBD-CE transferred/3hr.	145.51 (30.66)	177.50† (59.82)	156.50 (43.87)	223.67§‡ (75.08)
Cholesterol mmol/l	4.42 (0.54)	4.38 (0.56)	6.01* (0.63)	5.94§* (0.75)
LDL cholesterol mmol/l	3.141 (0.54)	3.007 (0.66)	4.611* (0.57)	4.506* (0.82)
TAG mmol/l	0.91 (0.40)	1.02§ (0.46)	1.12 (0.36)	1.17 (0.49)

Values reported represent mean with standard deviation in parenthesis of the < > 5.2 mmol/l total cholesterol study group.

Significant difference between 0 h and 6 h, †P ≤ 0.001, §P ≤ 0.01.

Significant difference between groups *P ≤ 0.0001 ‡P ≤ 0.01

4.3.2: HDL analysis

Pooled t-tests were completed to investigate whether HDL composition between 0 h and 6 h was different between the moderately elevated and normal plasma cholesterol groups. This analysis showed that HDL₃ TAG was the only parameter in which the change was significantly different ($P \leq 0.0001$).

The composition of total HDL and HDL subfractions for the low ($< 5.2\text{mmol/l}$ total cholesterol) and the moderately elevated ($> 5.2\text{ mmol/l}$ total cholesterol) plasma cholesterol groups in the fasted and postprandial states are shown in tables 4.3.2.1 and 4.3.2.2 respectively. During postprandial lipaemia in the low cholesterol group, there were significant increases in total HDL phospholipid ($P = 0.002$), apo A1 ($P \leq 0.0001$) and TAG ($P = 0.0009$) concentrations. In the HDL₃ fraction, apo A1 was significantly increased ($P = 0.004$) whereas the concentrations of HDL₃ cholesterol, phospholipid and TAG did not change significantly. The concentration of all the components of the HDL₂ fraction were significantly ($P \leq 0.0003$) increased during the postprandial response.

In the moderately elevated plasma cholesterol group, total HDL phospholipids increased significantly ($P = 0.0002$) following the test meal. Total HDL apo A1 and TAG concentrations increased between 0 and 6 hours, but the change did not reach statistical significance ($P = 0.09$). There were no significant changes in the compositions of the HDL₂ or HDL₃ subfractions between 0 and 6 hours postprandially. There was no significant difference in the lipid composition of the HDL subfractions in either the normal or moderately elevated plasma cholesterol groups

Table 4.3.2.1: Lipid and apo A1 concentrations of HDL subfractions of the low (< 5.2 mmol/l) plasma cholesterol group (n = 39) in fasting and postprandial states.

	Total HDL		HDL ₃		HDL ₂	
	0 hours	6 hours	0 hours	6 hours	0 hours	6 hours
Cholesterol (mmol/l)	1.111 (0.276)	1.156 (0.375)	0.906 (0.254)	0.859 (0.248)	0.225 (0.183)	0.357** (0.286)
Phospholipid (mmol/l)	1.007 (0.204)	1.134§ (0.298)	0.853 (0.18)	0.858 (0.236)	0.183 (0.142)	0.330** (0.254)
Apo A1 (g/l)	1.631 (0.591)	2.172‡ (0.357)	1.392 (0.558)	1.761† (0.534)	0.273 (0.210)	0.561** (0.33)
Triacylglyceride (mmol/l)	0.15 (0.057)	0.207† (0.090)	0.132 (0.048)	0.156 (0.075)	0.030 (0.03)	0.066‡ (0.03)

Values reported represent mean with (Standard deviation) in parenthesis, of the < 5.2 mmol/l total cholesterol group (n = 39).
Significant difference in HDL composition between 0 hours and 6 hours. §P < 0.002; †P < 0.004; ‡P ≤ 0.0001; **P < 0.0003.

Table 4.3.2.2: Lipid and apo A1 concentrations of the HDL subfractions of the moderately elevated (> 5.2 mmol/l) plasma cholesterol group (n = 24) in fasting and postprandial states.

	Total HDL		HDL ₃		HDL ₂	
	0 hours	6 hours	0 hours	6 hours	0 hours	6 hours
Cholesterol (mmol/l)	1.178 (0.241)	1.212 (0.358)	0.937 (0.245)	0.948 (0.359)	0.243 (0.205)	0.290 (0.200)
Phospholipid (mmol/l)	1.083 (0.213)	1.248* (0.300)	0.903 (0.238)	0.924 (0.357)	0.231 (0.176)	0.267 (0.193)
Apo A1 (g/l)	1.959 (0.789)	2.19 (0.267)	1.56 (0.720)	1.601 (0.570)	0.48 (0.384)	0.591 (0.609)
Triglyceride (mmol/l)	0.171 (0.063)	0.204 (0.078)	0.141 (0.06)	0.15 (0.087)	0.036 (0.03)	0.066 (0.045)

Values reported represent mean with (Standard deviation) in parenthesis, of the ≥ 5.2 mmol/l total cholesterol group (n = 24).

Significant difference in HDL composition between 0 hours and 6 hours. *P < 0.0002.

4.3.3: Chylomicron and triglyceride poor lipoprotein fraction lipid analysis

Cholesterol and TAG concentrations of the chylomicron and triglyceride poor lipoprotein fractions of the low and moderately elevated plasma cholesterol groups are presented in tables 4.3.3.1 and 4.3.3.2. There was a significant ($P \leq 0.05$) increase in chylomicron TAG concentration in the low cholesterol group 6 hr after ingestion of test meal, with a non-significant increase in 6 hr chylomicron TAG concentration in the moderately elevated cholesterol group 6 h after ingestion of test meal. A significant ($P \leq 0.05$) reduction in chylomicron cholesterol concentration was noted in both cholesterol groupings 6 h after ingestion of test meal.

The moderately elevated plasma cholesterol group (> 5.2 mmol/l) had significantly ($P \leq 0.05$) higher fasting chylomicron TAG and cholesterol concentrations and significantly ($P = 0.01$) higher 6 h chylomicron cholesterol concentrations than did the low cholesterol group (< 5.2 mmol/l). The moderately elevated cholesterol group had significantly ($P \leq 0.0001$) higher triglyceride poor lipoprotein fraction cholesterol concentrations in the fasted and 6 h after ingestion of test meal than did the low cholesterol group. The moderately elevated plasma cholesterol group also had a significantly ($P = 0.004$) higher fasting TPL TAG concentration than the low cholesterol group.

4.3.4: Correlation analysis of the high and low cholesterol groups.

In the low cholesterol group (< 5.2 mmol/l total cholesterol group), no significant correlation between fasting CETP mass and activity with fasting plasma lipid and lipoprotein concentrations was observed. Six hour CETP mass was positively correlated with 6 h total plasma cholesterol concentrations ($r = 0.327$; $P = 0.04$) and 6 h HDL₃ apo A1 concentrations ($r = 0.338$; $P = 0.03$). Postprandial CETP activity (6 h) was significantly correlated with 6 h plasma cholesterol ($r = 0.318$; $P = 0.05$) and 6 h LDL cholesterol concentrations ($r = 0.319$; $P = 0.05$). Postprandial CETP activity was positively but not significantly associated with 6 h HDL₃ apo A-1 concentrations ($r = 0.280$; $P = 0.08$) and negatively correlated with 6 h HDL₂ apo A1 concentration ($r = -0.414$; $P = 0.009$).

In the moderately elevated plasma cholesterol group (> 5.2 mmol/l) fasting CETP mass was positively correlated with fasting plasma cholesterol ($r = 0.539$; $P = 0.008$), fasting LDL cholesterol ($r = 0.443$; $P = 0.045$), fasting total HDL apo A1 ($r =$

0.563; $P = 0.0079$) and fasting HDL₃ apo A1 ($r = 0.513$; $P = 0.0175$) concentrations. Fasting CETP activity was not significantly correlated with any plasma lipid or lipoprotein concentration in the fasted state. There was a positive association between fasting CETP activity and fasting HDL₂ phospholipid concentration, but this did not reach significance ($r = 0.404$; $P = 0.06$). In the postprandial state there was no significant correlation between 6 h CETP mass or activity with plasma lipid and lipoprotein concentrations at 6 h. 6 h CETP activity was non-significantly ($r = 0.322$; $P = 0.06$) associated with 6 h TRL cholesterol concentrations

Table 4.3.3.1: Lipid content of the chylomicron fraction of the moderately elevated and normal plasma cholesterol groups in fasted and 6 h postprandial states

	< 5.2 mmol/l (n = 39)		> 5.2 mmol/l (n = 21)	
	0 hr	6 hr	0 hr	6 hr
Triglyceride (mmol/l)	0.51 (0.18)	0.75* (0.52)	0.76† (0.29)	0.82 (0.46)
Cholesterol (mmol/l)	0.18 (0.12)	0.15* (0.13)	0.27‡ (0.13)	0.18‡* (0.14)

Values represent mean and standard deviations in parenthesis

Significant difference between 0 h and 6 h *P ≤ 0.05

Significant difference between groups †P = 0.003; ‡P ≤ 0.01

Table 4.3.3.2: Lipid content of triglyceride poor lipoprotein fraction of the moderately elevated and normal plasma cholesterol groups in the fasted and 6 h postprandial states

	< 5.2 mmol/l (n = 39)		> 5.2 mmol/l (n = 21)	
	0 hr	6 hr	0 hr	6 hr
Triglyceride (mmol/l)	0.36 (0.12)	0.37 (0.13)	0.45* (0.10)	0.42 (0.10)
Cholesterol (mmol/l)	2.89 (0.66)	2.92 (0.68)	3.81* (0.47)	3.78† (0.51)

Values represent mean and standard deviations in parenthesis

Significant difference between 0 h and 6 h

Significant difference between groups *P = 0.004; †P ≤ 0.0001

4.3.5: CETP mass and activity correlation analysis

The relationship between CETP mass and activity in the fasted and postprandial states for the high and low cholesterol groups are presented in Figures 4.3.5.1, 4.3.5.2, 4.3.5.3, and 4.3.5.4. These show a significant and positive association between mass and activity in both groups. The strength of these associations are much greater in the moderately elevated plasma cholesterol group.

Figure 4.3.5.1

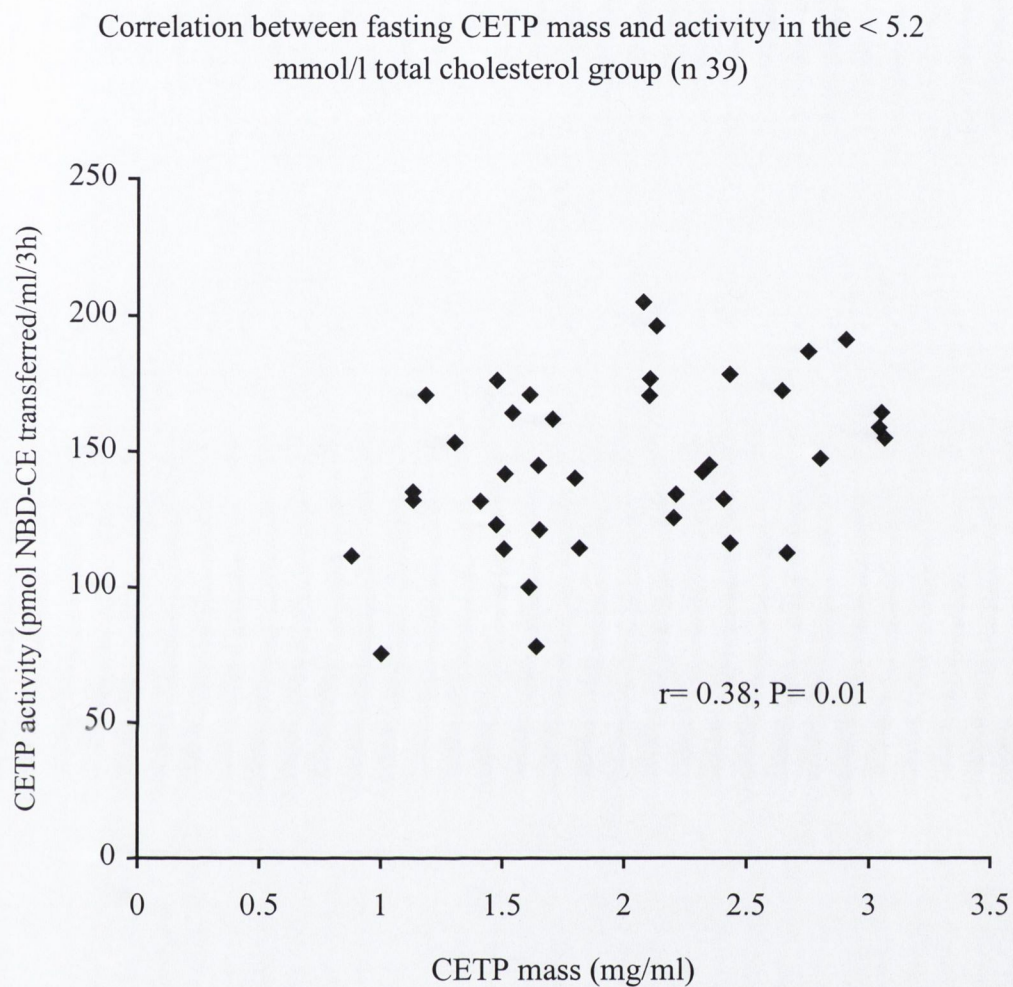


Figure 4.3.5.2

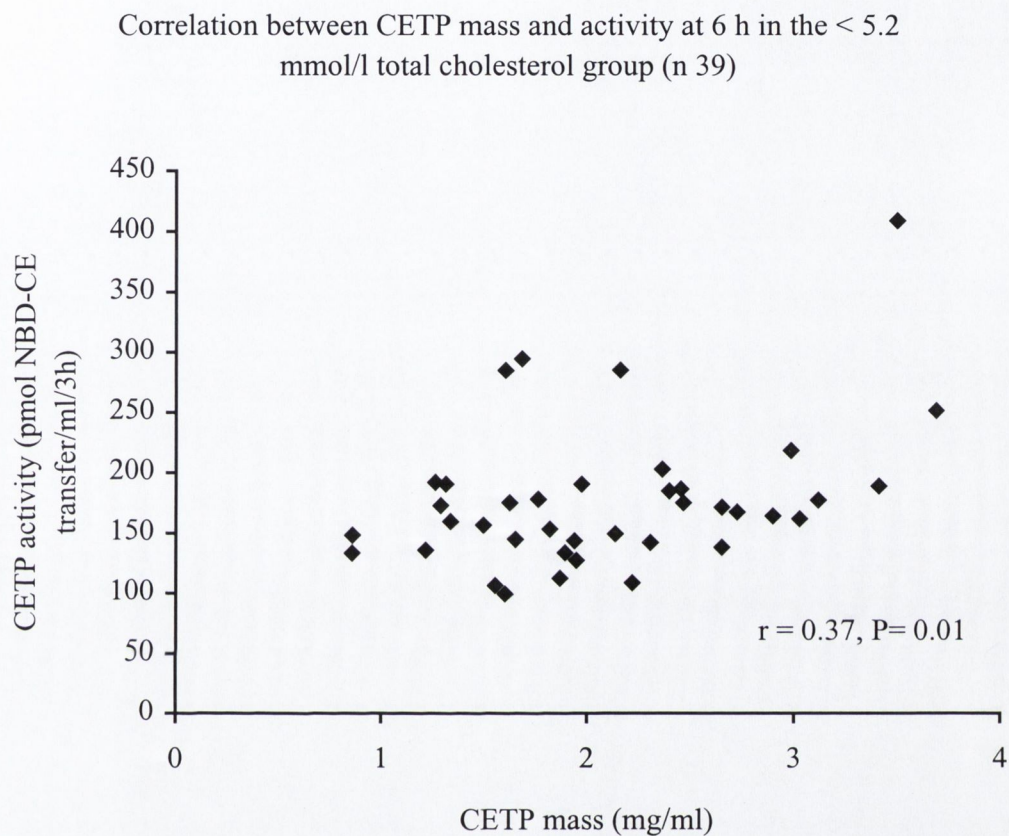


Figure 4.3.5.3

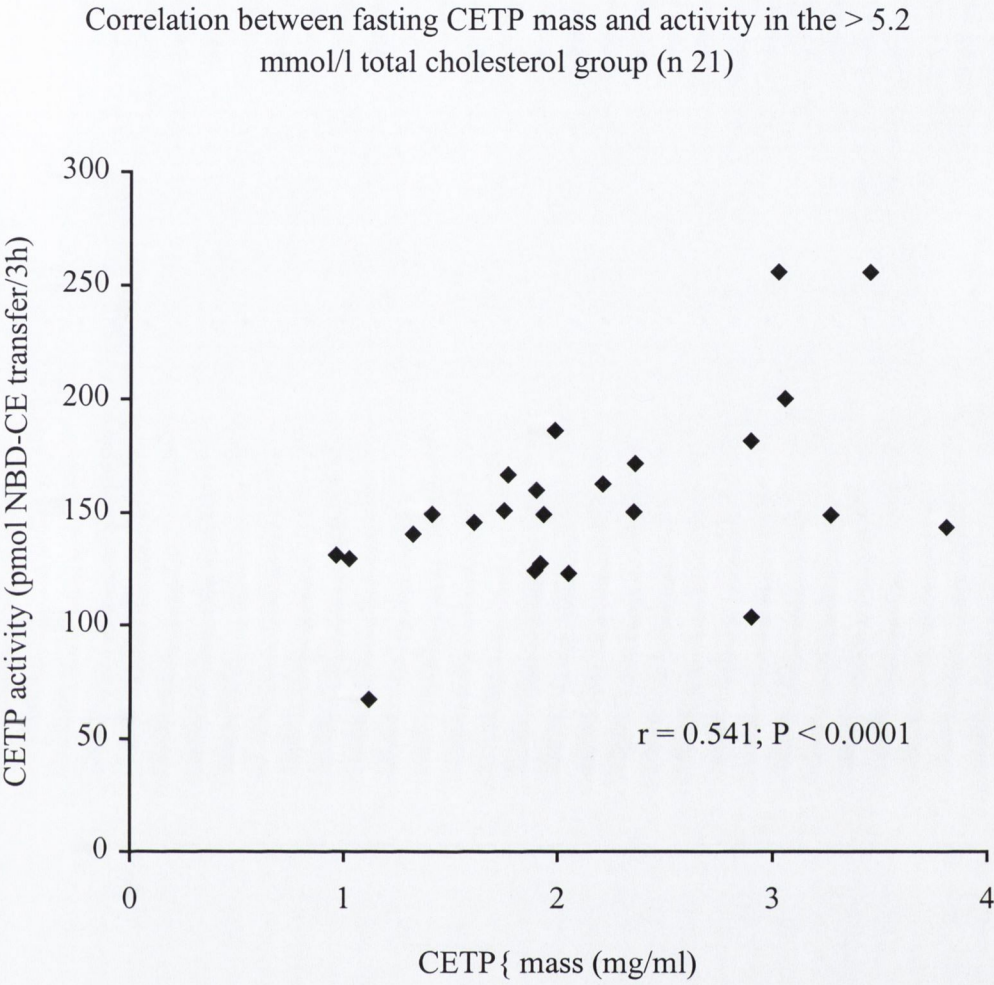
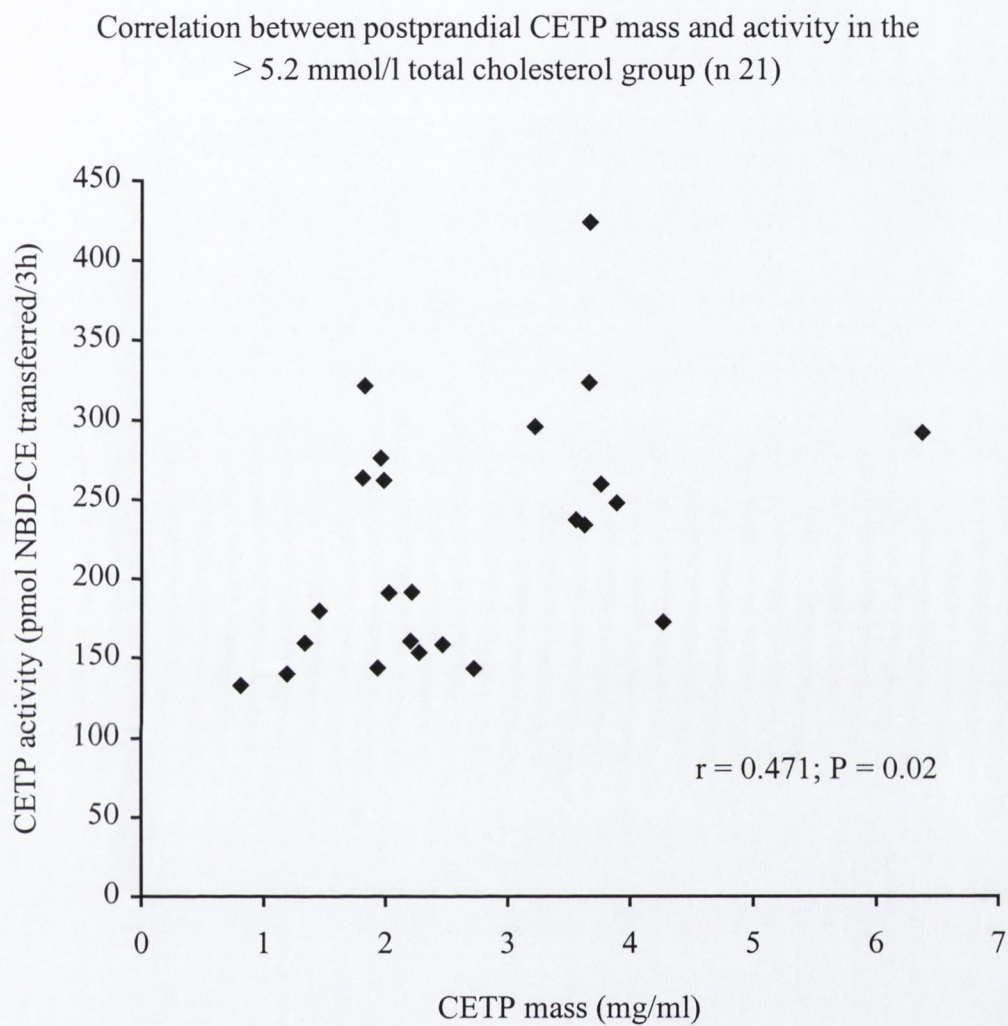


Figure 4.3.5.4



4.4.0: Discussion

4.4.1: CETP mass, activity and plasma lipid analysis

In order to determine the effect of plasma cholesterol concentration on CETP metabolism the study cohort was divided into two cholesterol groups of comparable sample numbers and similar fasting plasma TAG concentrations. The moderately elevated (high) plasma cholesterol group had significantly higher plasma and LDL cholesterol concentration than the low cholesterol group as would be expected. The moderately elevated plasma cholesterol group were significantly older and heavier suggesting a relationship between age, weight and plasma cholesterol. In the elevated plasma cholesterol group fasting CETP mass was 13 % higher and fasting CETP activity was 7 % higher compared with the low cholesterol group. This confirms findings of studies by Bagdade *et al.* (1991); Inazu *et al.* (1992); Tall A.R. (1987) Moulin *et al.* (1992) who demonstrated that subjects with elevated plasma cholesterol concentrations had elevated CETP concentrations. Furthermore the high-cholesterol group also had significantly higher CETP mass and activity 6 h after ingestion of test meal. The low cholesterol group displayed a 9.6 % increase in CETP mass and a 22 % increase in CETP activity 6 h after test meal, while the high cholesterol group demonstrated a 20 % increase in CETP mass and 42 % increase in CETP activity in response to the same test meal at 6 h. These are interesting findings in regards to contrasting responses depending on cholesterol grouping in CETP mass and activity following ingestion of a test meal. These results clearly indicate that the high cholesterol group exhibits a greater transfer potential of CE into apo B lipoproteins during lipaemia when compared to those with low cholesterol, considering that the fat load administered to both groups was the same. This has important implications as regards CAD risk given that elevated CETP activity is considered to be potentially atherogenic.

The moderately elevated plasma-cholesterol group had a 35 % higher fasting cholesterol and 43 % higher LDL cholesterol concentration than did the low-cholesterol group. A publication by the adult treatment panel of the National Cholesterol Education Program developed in 1988 identified an LDL cholesterol of between 3.36-4.14 mmol/l as a border line risk factor for the development of CHD, while those who also had HDL cholesterol level of less than 0.91 mmol/l were deemed to be at very high risk. The moderately elevated plasma cholesterol group had a LDL cholesterol concentration at

the high end of LDL cholesterol concentrations set out by the NCEP at border line risk of CAD. The high cholesterol group in this study showed a definite increased risk of developing CHD over that of the low cholesterol group. This appears to be attributed to the exaggerated CETP activity particularly during postprandial lipaemia in this group when compared to the low cholesterol group and also because of the strong association between LDL cholesterol concentration and CETP mass in this group. Evidence that there is a genetic predisposition to increased CETP activity as demonstrated by Kuivenhoven *et al.* (1998) and the fact that exaggerated CETP activity results in the production of atherogenic apo B lipoproteins suggests that CETP may cause the low HDL/LDL ratios noted in the moderately elevated cholesterol group in this study. Although the contribution of substrate availability such as plasma TAG especially if postprandial lipaemia is prolonged due to sluggish clearance of TAG rich lipoproteins and CE concentrations to the elevation of CETP activity cannot be ignored.

In the moderately elevated plasma cholesterol group there was a significant correlation between CETP mass and activity in the fasted ($r = 0.541$; $P \leq 0.0001$) and at the 6 h postprandial states ($r = 0.471$; $P = 0.02$), with similar findings in the low cholesterol group and have been reported by other investigators (Clarke *et al.* 1995; Tato *et al.* 1995a).

4.4.2: Lipoprotein analysis

The changes in HDL composition as a result of meal ingestion were significantly different between the moderately elevated and low plasma cholesterol groups. In the low-cholesterol group, there was a significant increase ($P < 0.004$) in total HDL apo A1, TAG and phospholipid concentrations at 6 h while there was a non significant increase in HDL cholesterol as demonstrated by Tall *et al.* (1982) and Groot *et al.* (1984) during the late postprandial phase. The increase in these lipids and apo A1 concentrations was accounted for in the HDL₂ fraction. There was a significant increase in 6 h HDL₂ TAG concentrations similar to the finding of Patsch, (1987). HDL₂ phospholipid concentration also increased, similar to the findings by Ihm *et al.* (1982). Significant increases in 6 h HDL₂ cholesterol concentrations were observed in this group which is beneficial in terms of reducing CAD risk due to the cardioprotective properties of HDL₂ (Tall 1990). These findings are in contrast to the observations noted in postprandial HDL₂ lipids in the moderately elevated plasma cholesterol group where no significant

increases were noted. Increases in lipid concentrations of the HDL₃ sub-fraction during postprandial lipaemia did not reach significance similar to the findings of Taskinen *et al.* (1986). Increases in the HDL₂ apo A1 concentration during postprandial lipaemia in the low cholesterol group suggests that as well as an increase in the size, the HDL particles during postprandial lipaemia (formation of lipid rich less dense HDL₂) also increase in number as apo A1 is a structural protein with between 2-4 apo A1 molecules per HDL lipoprotein. These changes in the lipid composition of the HDL₂ subfraction indicate that RCT process was increased during postprandial lipaemia in this group.

In the elevated plasma cholesterol group, there was an increase in total HDL cholesterol, phospholipid TAG and apo A1 concentrations 6 h after ingestion of test meal with increases in total HDL phospholipid concentration only being statistically significant. There were non-significant increases in HDL₂ lipid and apo A1 concentrations with Patsch, *et al.* (1984) noting similar observations. An explanation for the low increase in the HDL composition, in particular HDL₂ in the high-cholesterol group at 6 h may be evident from the findings of Patsch *et al.* (1984). They observed that the higher the fasting HDL concentration, in particular fasting HDL₂ concentration the lower the increase in HDL lipid composition in response to postprandial lipaemia. This was observed in the moderately elevated plasma cholesterol group in the current study.

The moderately elevated plasma cholesterol group had a higher CETP activity and so there was a greater transfer of CE into apo B lipoproteins, increasing LDL cholesterol concentrations, while in the low plasma cholesterol group there was lower CETP activity so CE was retained in the HDL subfraction. This appeared to be confirmed by analysis of the ratios of HDL and LDL cholesterol (See section 4.4.3).

There was an expected increase in chylomicron TAG concentration in both groups as a result of ingestion of test meal. The high cholesterol group had a significantly higher fasting chylomicron TAG, cholesterol and 6 h cholesterol concentration when compared with the low cholesterol group. Griffin (1997) hypothesised that CETP facilitated the exchange of CE into chylomicrons with reciprocal exchange of TAG, resulting in the formation of small dense LDL. Karpe *et al.* (1997) has demonstrated that chylomicrons are taken up by aortic tissue using in vitro models in a similar manner to LDL. Increased chylomicron cholesterol concentrations which are potentially atherogenic are possibly caused by CETP activity.

This appears to be confirmed by the findings of this study where increased CETP activity was non-significantly associated with increased chylomicron cholesterol concentrations.

4.4.3: Ratio of LDL to HDL in the moderately elevated and low-cholesterol groups.

The low-cholesterol group had a lower level of LDL cholesterol than did the high cholesterol group. When analysis of the ratio of LDL cholesterol to HDL cholesterol was carried out on both groups, the low-cholesterol group had a LDL:HDL ratio of 2.82:1 while the high-cholesterol group had a LDL:HDL ratio of 3.94:1. This data shows that those individuals who had higher plasma cholesterol and CETP also had elevated LDL to HDL cholesterol ratio, demonstrating an elevated risk of this group for CHD (Stampfer *et al.* 1996). Variations in CETP activity may be responsible for the different HDL to LDL cholesterol ratios between the study groups as there was a strong association between CETP and LDL cholesterol concentrations in this study.

4.4.4: Correlation of CETP and plasma lipids in the high and low cholesterol groups.

In the low-cholesterol group there was no significant correlation between fasting CETP mass and activity or any plasma lipids. This was probably due to low levels of CETP enzyme production or substrate availability during the fasted state. During postprandial lipaemia there was positive association between CETP mass and plasma cholesterol. Postprandial CETP activity was positively correlated with plasma cholesterol ($P \leq 0.05$) and LDL cholesterol ($P \leq 0.05$). Similar results were observed by Tato *et al.* (1995a). This demonstrates that CETP activity in this group may be regulated by substrate (CE) availability.

In the high-cholesterol (moderately elevated) group fasting CETP mass was significantly correlated with plasma cholesterol ($P \leq 0.008$) and LDL cholesterol ($P \leq 0.05$). Mac Pherson *et al.* (1991) and Tato *et al.* (1995) have previously demonstrated this relationship. Fasting CETP activity only approached significance with these lipids. A possible explanation for this finding maybe that in the fasting state the protein was not saturated with substrate and operating at its catalytic maximum. The cholesterol composition of lipoprotein is dynamic, constantly changing depending on fed or fasted states, therefore associations between CETP and cholesterol concentrations may be subject to some variability depending on whether one is in a fed or fasted state.

However this data demonstrates that CETP mass appears to be determined by plasma and LDL cholesterol levels in both study groups similar to observations by Moulin *et al.* (1992), Bagdade *et al.* (1991) and Inazu *et al.* (1992). This data suggests that plasma cholesterol concentrations may result in increased production of CETP mRNA (Fielding and Havel 1996). During postprandial lipaemia no correlation between CETP mass and activity and plasma lipids was found, possibly because there was an excess of substrate for CETP during lipaemia in this study group due to their greater TAG concentrations during postprandial lipaemia. Substrate saturation of enzyme may occur during lipaemia. The only way to confirm these findings would be to perform supplementation experiments on CETP with varying concentrations of substrate.

In the low-cholesterol group there was a significant correlation between fasting CETP mass and activity and HDL₃ apo A1. Similar results were found by Jones *et al.* (1996) in diabetic patients. During postprandial lipaemia correlation was observed between CETP activity and HDL₃ apo A1 but this was found to be non significant. A significant negative correlation was found between HDL₂ apo A1 and postprandial CETP activity. While these results may seem contradictory they can be explained through the complex system through which apo A1 plays a regulatory role on CETP as demonstrated by Guyard-Dangermount *et al.* (1994) where apo A1 and other apo proteins as suggested by Lagrost *et al.* (1994a) and Vadiveloo *et al.* (1990) above or below certain concentrations plays an inhibitory or activating role on CETP activity. Apo A1 is also thought to facilitate the binding of CETP to HDL which further complicates the relationship between CETP and apo A1 (MacPherson *et al.* 1996).

In the moderately elevated plasma cholesterol group fasting CETP mass correlated with total apo A1 which upon further analysis of the HDL sub-fractions was found to be primarily in the HDL₃ sub-fraction. No correlation was found with activity. These findings are similar to those observed in the low cholesterol group and maybe explained through the effects apo A1 has on CETP as explained in the previous paragraph, and demonstrated by Guyard-Dangermount *et al.* (1994), Lagrost *et al.* (1994a), Vadiveloo *et al.* (1990) and Mac Pherson *et al.* (1996).

4.4.5: Principle findings in this study

There was a very significant increase in CETP mass, activity plasma TAG and HDL₂ lipid in response to a MUFA test meal in both cholesterol groups. There was a

dramatic difference in increases in CETP mass and activity as a result of administration of a fat rich test meal, depending on plasma cholesterol grouping. The increased CETP activity during postprandial lipaemia in the moderately elevated plasma cholesterol group over that of the normal plasma cholesterol group, contributes to the increased risk factors for the development of CAD in this group. Elevated CETP is associated with high LDL to HDL ratios. Positive correlation was observed between CETP mass and activity in fasting and postprandial states. There was significant correlation between fasting CETP mass and fasting plasma cholesterol and LDL cholesterol in the high cholesterol group, while postprandial CETP activity correlated with postprandial plasma and LDL cholesterol in the low cholesterol group. Apo A1 seemed to show significant correlation with CETP mass and activity in either the fasting or postprandial states depending on the cholesterol grouping indicating that it plays some sort of regulatory role in CETP metabolism in this study group. Clearly moderately elevated plasma cholesterol results in greater risk factors for the development of CAD, evidence from this study suggest that increased CETP mass and activity particularly at 6h in response to a fat rich test meal in this group may be responsible for the development of these risk factors.

Chapter 5

Effects of low dose fish oil supplementation (0.9 g/d) over 16 weeks on Cholesteryl Ester Transfer Protein mass, activity, plasma lipids and lipoproteins

5.1: Introduction

CETP mediates the transfer of CE from HDL to VLDL and LDL with the reciprocal transfer of TAG into HDL. It is possible that CETP activity may be atherogenic due to its ability to lower HDL cholesterol concentrations and increase cholesterol composition of apo B lipoproteins. Conditions resulting in increased CETP activity such as hyperlipidemias (Tall *et al.* 1987; Bagdade *et al.* 1991; Bagdade *et al.* 1992a; Tato *et al.* 1995), diabetes (Bagdade *et al.* 1991), nephrotic syndrome (Moulin *et al.* 1992) and genetic variants of the CETP gene (Kuivenhoven *et al.* 1998) all result in increased risk factors for the development of CAD.

Several studies involving dietary intervention in animals and humans demonstrate that the administration of dietary fatty acids has an effect on CETP and related plasma lipoproteins. Diets rich in saturated fat result in an increase in CETP mass and activity in humans (Tall *et al.* 1993) and in animals (Quintet *et al.* 1990). *Trans*-fatty acids increased CETP mass and activity (van Tol *et al.* 1995; Abbey *et al.* 1994), while oleic acid (Abbey *et al.* 1994) and n-3 PUFA (Lottenberg *et al.* 1996a; Abbey *et al.* 1990; Bagdade *et al.* 1992c) reduced CETP activity in hyperlipidemics. A recent investigation by Jansen *et al.* (2000) demonstrated that monounsaturated fatty acid (MUFA) intake in conjunction with a low fat diet reduced CETP activity in normolipidemics.

Interest in the potential beneficial effects of dietary supplementation with fish oil and its benefits on coronary artery disease has come from the observations of Bang *et al.* (1976) who found that Eskimo populations had a significantly lower incidence of CAD than did industrialised nations. This was attributed to a high intake of n-3 PUFA in this population. Since then epidemiological studies have demonstrated an inverse association between mortality from CAD and fish oil intake (Kromhout *et al.* 1985; Kromhout *et al.* 1995; Albert *et al.* 1996).

Very few studies have investigated whether dietary supplementation with fish oil has any effect on CETP and reverse cholesterol transport (RCT) in normolipidaemics. Abbey *et al.* (1990) demonstrated that hypercholesterolaemics administered 3.8g/d fish oil for 6 weeks resulted in a 23 % reduction in CETP activity. Bagdade *et al.* (1992c) administered 6g/day fish oil to 12 hypercholesterolemic subjects for 6 weeks resulted in a normalisation of fasting CETP activity and a 10 % reduction in fasting CETP mass. Lottenberg *et al.* (1996a) demonstrated that supplementation of n-3 PUFA as 44% of

total fat intake (n 10) for 3 weeks resulted in no difference in fasting CETP mass or activity, with a 10 % non-significant increase in postprandial CETP mass as a result of the PUFA diet.

HDL is an integral lipoprotein in CETP metabolism and RCT, however there is conflicting data as to the effect of fish oil on HDL cholesterol concentrations. The dose of fish oil intake is important with an intake of greater than 12g / day of fish oil resulting in reductions in HDL cholesterol concentrations (Nestel *et al* 1986). Studies using lower doses of fish oil have resulted in HDL cholesterol concentrations being decreased (Ginsberg *et al.* 1994), unchanged (Bagdade *et al.* 1992c, Harris, W.S. 1997) or increased (Fumeron *et al.* 1991). The beneficial effects of fish oil on plasma TAG and VLDL concentrations has been well publicised (Harris W.S. 1997; Silva *et al.* 1996, Roche & Gibney, 1996). Reductions of up to 28 % in fasting plasma TAG have been observed as a result of fish oil intake in normolipidaemics (Silva *et al.* 1996). Fish oil intake in hypertriglyceridemics reduces plasma TAG concentrations by 60-80 % (Philipson *et al.* 1985).

The effects of long term-low-dose fish oil supplementation on lipoprotein composition and the function of RCT in normolipidemics has not been examined. The present investigation was designed to determine whether a low-dose of fish oil (0.9g / day) over the long term (16 weeks) had any beneficial effect on plasma lipids and reverse cholesterol transport in a normolipidaemic population. CETP mass and activity, and related lipoproteins were measured to investigate whether low dose fish oil affected RCT, and to elucidate a mechanism which underlined this effect. The study also sought to determine if the expected decrease in plasma TAG concentrations associated with fish oil supplementation had any impact on CETP activity, thereby determining whether TAG commanded a substrate dependent effect on CETP metabolism. Therefore CETP activity was measured using an assay which was dependent upon endogenous apo B lipoprotein concentration (Abbey & Nestel 1994; Lottenberg *et al.* 1996b).

5.2.0: Methods.

5.2.1: Study design

This study was approved by the Ethics Committee of the Federated Dublin Voluntary Hospitals. This study was conducted on an outpatient basis. All patients gave written informed consent to participate in the study. Twenty-eight non-smoking healthy volunteers were recruited (16 males and 12 females). A screening blood sample was taken from each of the subjects to ensure that they adhered to the following inclusion criteria.

Inclusion criteria

Age 18 –60 years

BMI < 25 Kg/m²

Plasma cholesterol < 6.5 mmol/l

Plasma TAG < 2 mmol/l

Gammaglutamyl transferase < 60 IU

Heamoglobin > 11 mg/dl

Not habitual consumers of n-3 PUFA

> 90 minutes strenuous exercise per week

This was a double blind placebo controlled study. Subjects were randomly assigned to receive either 0.9g fish oil per day or a placebo group consuming 0.9g olive oil per day for 16 weeks. Dietary supplements were supplied by Hoffmann La Roche. Study compliance was assessed by a tablet count and EPA/DHA incorporation into platelet phospholipid.

5.2.2: Study subjects

The study was conducted in the nutrition laboratory at the Trinity Centre, St. James Hospital, Dublin 8. Each study day began between 07:30 and 08:00 hrs following a 12 h over night fast. Subjects were asked to abstain from alcohol and exercise 24 hrs before a postprandial day. During postprandial investigations each subject was asked to abstain from food and drink with the exception of water, decaffeinated coffee and diet drinks. The test meal was prepared each day. Each subject received 40g fat (Flora Sun Flower Oil), 150g of skimmed milk and 5 g of orange modjul flavour system (Scientific Hospital Supplies, UK Limited) which was mixed with water.

A 21-gauge 32mm venous catheter (Abbott Ireland Ltd., Dublin, Ireland) was inserted into the antecubital vein of the non-dominant forearm. Blood samples for CETP analysis and platelet phospholipid composition were collected in 5 ml citrated tubes ((0.106M-sodium citrate) (Starstead monovette). Blood samples for lipid analysis were collected into 10 ml heparinised vacutainers. All blood samples were drawn before test meal was administered. The test meal was given and consumed within 15 minutes. A further blood sample was drawn 6 hrs after the test meal. All samples were centrifuged (3000 rpm for 15 min) at room temperature. Plasma was taken off, mixed and aliquoted into 0.5 ml fractions. Samples for CETP analysis were snap frozen under liquid N₂ and stored (-70 °C) until CETP mass and activity was assayed. Plasma for lipid analysis was immediately frozen and stored at -20 °C.

5.2.3: Laboratory methods

Analysis of plasma TAG (Triglyceride PAP, Biomerieux, France), cholesterol (Cholesterol PAP, Biomerieux, France), NEFA (Acyl Co A synthetase-acyl Co A oxidase) Wako Chemicals, Gmbh, Germany), phospholipid (Phospholipid PAP, Biomerieux, France) and apo A1 (Biomerieux, France) was carried out. HDL cholesterol, TAG, phospholipid and apo A1 concentrations were measured using the methods mentioned previously following precipitation of HDL with Immuno Quantolip HDL (Immuno AG, Vienna, Austria) precipitating reagent. Chylomicrons were isolate using ultracentrifugation technique as described in section 2.18.1 and were analysed for cholesterol and TAG concentrations. Plasma lipids were assayed on a Technicon RA-XT Chemistry Analyser (Technicon Inc., Tarrytown, NY., U.S.A.) as detailed in chapter 2

CETP activity was determined using a radiolabelled H³CE in HDL as a donor particle and endogenous apo B lipoproteins as acceptor particles as detailed in section 2.15.0. CETP mass was determined using a alkaline phosphatase based sandwich ELISA devised by Clarke *et al.* (1995). Platelet phospholipid and HDL CE fatty acids were analysed using gas chromatography as detailed in section 2.18.5.

5.2.4: Data Analysis

All statistical analysis was carried out on an Apple Macintosh statistical package Data Desk 4.1 (Data Description Inc., NY., U.S.A). The distribution of the data for each variable was assessed. Some of the variables were transformed to normalise the

distribution of the data sets to give data a normal gaussian distribution. Repeated measures ANOVA was used to investigate statistical changes in biochemical parameters as a result of dietary intervention in control and fish oil groups.

5.3.0: Results

5.3.1: Plasma lipids and CETP analysis

Twenty-eight subjects, 16 males and 12 females participated in this study. The total study group had a mean age of 40 (± 12.74) years, a weight of (± 12.61) kg and a BMI of 24.88 (± 2.407) kg/m². Body weight remained stable in both groups during the study. The mean plasma lipids and CETP concentrations for the study group were total cholesterol, 5.42 (± 1.124) mmol/l, total TAG, 1.32 (± 0.721) mmol/l, NEFA, 0.449 (± 0.244), CETP mass, 1.906 (± 0.391) μ g/ml and CETP activity, 23.70 (± 1.19) % CE transfer/ 3 hr.

CETP mass, activity and plasma lipids at 0 h and 6 h for control and intervention groups are presented in tables 5.3.1.1 and 5.3.1.2. Using repeated measures analysis of variance (ANOVA), CETP activity, plasma TAG and NEFA concentrations increased significantly in both dietary groups ($P \leq 0.0001$) by 6 h, at week 0 and 16 in response to 40g fat test meal. Plasma cholesterol did not change significantly in either group, however LDL cholesterol concentrations did decrease significantly ($P = 0.01$) in both dietary groups 6 h after ingestion of test meal pre and post study.

Repeated measures ANOVA demonstrated no significant effect of dietary intervention with fish oil on fasting or 6 h CETP mass or activity, although decreases in postprandial CETP activity approached significance ($P = 0.07$). Fasting TAG concentrations were significantly reduced ($P = 0.03$) in the fish oil group, while 6 h plasma TAG concentrations were non-significantly ($P = 0.06$) reduced in the fish oil group. Non-significant reductions in plasma TAG concentrations at 0 h and 6 h were observed in the control group. Fasting and 6 h plasma and LDL cholesterol concentrations increased non-significantly by 3.2 % and 1.5 % respectively in the fish oil group. There was a 12.7 % decrease in fasting CETP activity while 6 h CETP activity increased (+ 5.3 %) in the control group pre and post intervention. There was a 13-14 % reduction in LDL cholesterol concentrations at 0 h and 6 h in the control group as a result of dietary intervention.

Table 5.3.1.1: Effect of 16 weeks of dietary intervention with 0.9g/day fish oil on CETP mass, activity and plasma lipids (n 14).

	Pre trial		Post trial	
	0 hr	6 hr	0 hr	6 hr
CETP mass (mg/ml)	1.868 (0.406)	2.156 (0.99)	1.898 (0.362)	2.045 (0.440)
CETP activity % CE transfer/3hr	24.03 (7.35)	28.22* (8.68)	23.38 (4.61)	25.16* (4.54)
Total cholesterol (mmol/l)	5.222 (1.278)	5.199 (1.050)	5.695 (1.270)	5.637 (1.14)
LDL cholesterol (mmol/l)	3.658 (1.134)	3.01† (0.954)	3.77 (1.233)	3.203† (0.954)
NEFA (mmol/l)	0.466 (0.253)	0.662* (0.336)	0.522 (0.462)	0.670* (0.380)
Total triglyceride (mmol/l)	1.38 (0.88)	1.599§ (1.07)	1.14‡ (0.944)	1.465§ (0.692)

Values reported represent mean values and standard deviations

*P < 0.0001, † P < 0.003, §P < 0.016 Significant difference between 0 hr and 6 hr

‡P < 0.01 Significant difference between pre and post intervention

Table 5.3.1.2: Effect of 16 weeks of dietary intervention in the placebo group on CETP mass, activity and plasma lipids (*n* 14).

	Pre trial		Post trial	
	0 hr	6 hr	0 hr	6 hr
CETP mass (mg/ml)	1.940 (0.385)	2.02 (0.638)	1.898 (0.512)	2.03 (0.510)
CETP activity % CE transfer /3 hr	25.49 (6.26)	28.80* (7.62)	22.26 (6.51)	30.35* (9.03)
Total cholesterol (mmol/l)	5.622 (1.278)	5.47 (1.431)	5.03 (1.161)	4.990 (1.15)
LDL cholesterol (mmol/l)	3.833 (1.073)	3.309§ (1.108)	3.263 (1.070)	2.842§ (0.833)
NEFA (mmol/l)	0.452 (0.239)	0.612* (0.227)	0.416 (0.223)	0.579* (0.340)
Total triglyceride (mmol/l)	1.26 (0.56)	1.476† (0.664)	1.05 (0.365)	1.287† (0.518)

Values reported represent mean and standard deviations of the placebo group (*n* 14).

* $P < 0.0001$, † $P < 0.003$, § $P < 0.016$ Significant difference between 0 hr and 6 hr

5.3.2: HDL analysis

The total study group had a mean total HDL cholesterol concentration of 1.46 ($\pm$ 0.399) mmol/l, HDL phospholipid of 1.65 ($\pm$ 0.363) mmol/l, HDL apo A1 of 1.38 ($\pm$ 0.381) g/l and HDL TAG of 0.315 ($\pm$ 0.117) mmol/l. Cholesterol and phospholipid concentrations of the HDL subfractions of fish oil and control groups pre and post trial at 0 h and 6 h are presented in figures 5.3.2.1 to 5.3.2.4. Repeated measures ANOVA revealed that there was significant ($P \leq 0.0001$) increases in total HDL cholesterol, phospholipid, HDL₃ cholesterol and phospholipid concentrations in both dietary intervention groups 6 h after administration of test meal at week 0 and week 16. Intake of 0.9g/day fish oil for 16 weeks resulted in significant ($P \leq 0.03$) increases in fasting and postprandial total HDL cholesterol and HDL₃ cholesterol concentrations. HDL₂ cholesterol concentrations at 6 h increased significantly ($P \leq 0.03$) from week 0 at 6 h HDL₂ cholesterol concentrations as result of fish oil intake. Total HDL phospholipid concentrations increased in this group, however this change only approached significance ($P < 0.06$) (See figure 5.3.2.2). In the control group increases in postprandial HDL₂ cholesterol concentrations at week 16 approached significance ($P = 0.06$) when compared to week 0 concentrations. No other changes in cholesterol or phospholipid concentrations in HDL subfractions were observed in this group as a result of intervention (See figure 5.3.2.3 and 5.3.2.4). There was a significant ($P = 0.01$) increase in 6 h HDL apo A1 concentrations as a result of fish oil intake, while TAG concentrations of the HDL subfractions remained unchanged see tables 5.3.2.1. and 5.3.2.2. No changes in HDL apo A1 and TAG concentrations were found in the control over 16 weeks (See tables 5.3.2.1 to 5.3.2.2)

Figure 5.3.2.1

Changes in cholesterol concentrations of the HDL subfractions pre and post dietary intervention with 0.9 g n-3 PUFA / day (n 14).

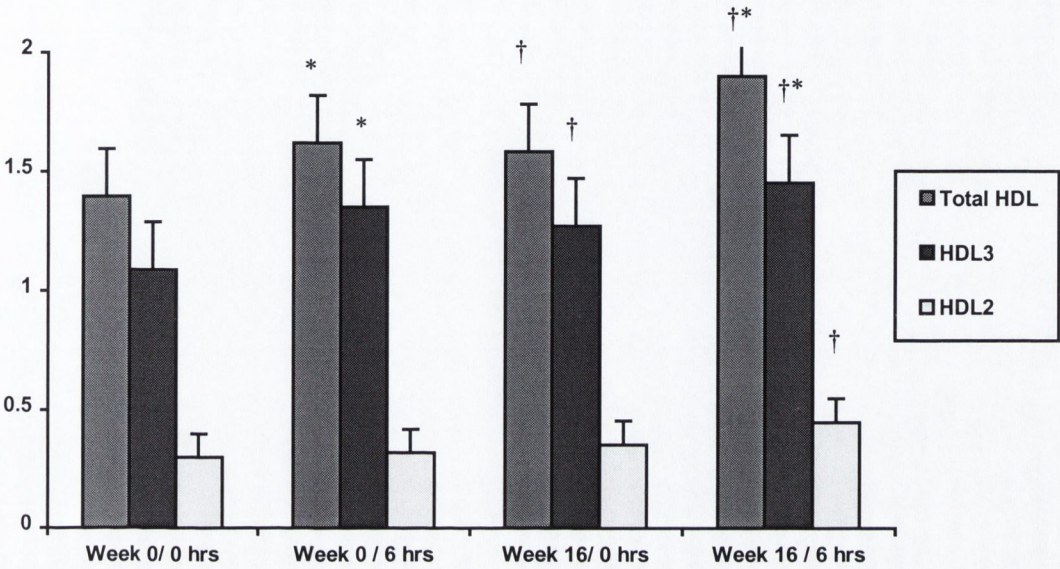
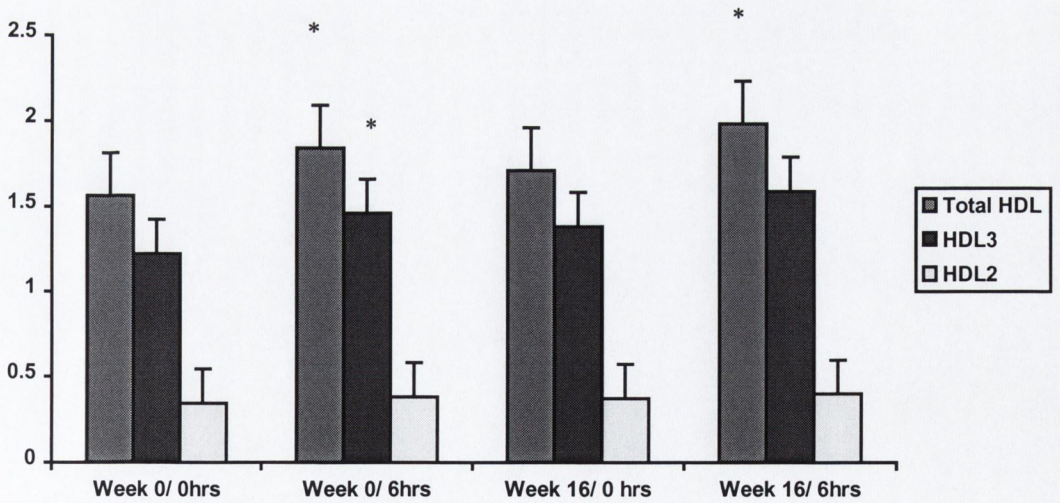


Figure 5.3.2.2.

Changes in phospholipid concentrations of the HDL subfractions pre and post dietary intervention with 0.9 g day n-3 / PUFA (n 14).



Values presented represent mean and standard deviations
* $P \leq 0.0001$ Significant difference between 0 hrs and 6 hrs.
† $P \leq 0.03$ Significant difference between week 0 and week 16.

Figure 5.3.2.3.

Changes in cholesterol concentrations of the HDL subfractions in placebo group pre and post study (*n* 14).

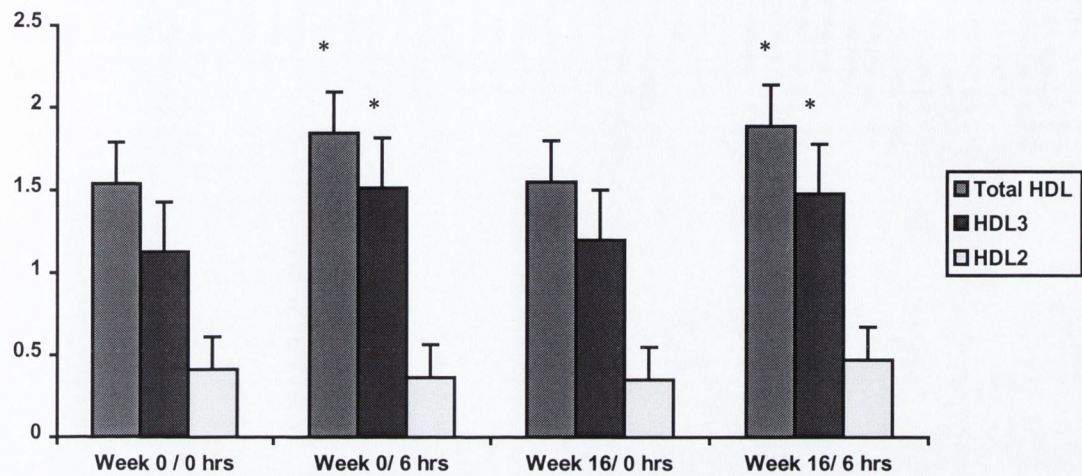
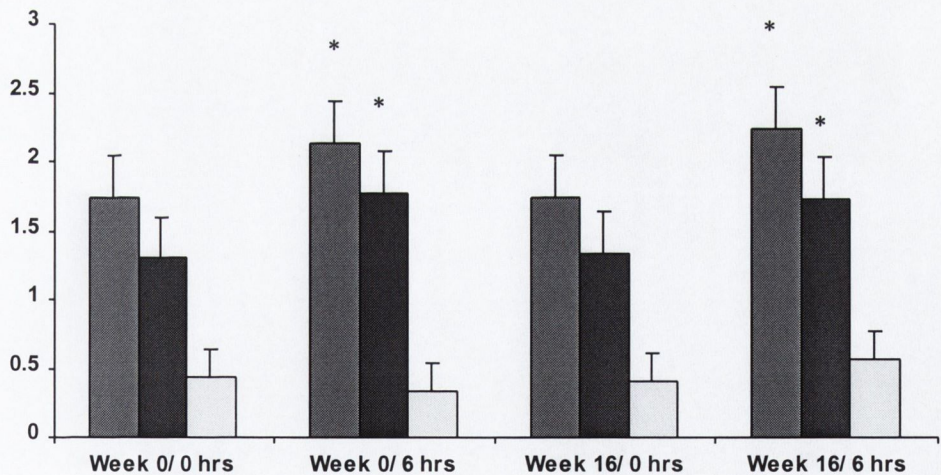


Figure 5.3.2.4.

Changes in phospholipid concentrations of the HDL subfractions in placebo group pre and post study (*n* 14).



Values shown represent means and standard deviations
* $P \leq 0.0001$ Significant difference between 0 hrs and 6 hrs

Table 5.3.2.1: Mean values and standard deviations for apo A1 and TAG concentrations of HDL subfractions in the fasted and postprandial states pre and post intervention with 0.9g/d fish oil for 16 weeks (*n* 14).

	Total HDL				HDL ₃				HDL ₂			
	Week 0		Week 16		Week 0		Week 16		Week 0		Week 16	
	0 hour	6 hour	0 hour	6 hour	0 hour	6 hour	0 hour	6 hour	0 hour	6 hour	0 hour	6 hour
Apo A1 (g/l)	1.31	1.30	0.984	1.43*	1.15	1.08	0.79	1.17	0.16	0.22	0.23	0.25
	(0.32)	(0.21)	(0.39)	(0.47)	(0.38)	(0.19)	(0.11)	(0.39)	(0.12)	(0.19)	(0.27)	(0.21)
TAG (mmol/l)	0.306	0.339	0.40	0.41	0.17	0.26	0.27	0.25	0.213	0.27	0.13	0.153
	(0.17)	(0.207)	(0.31)	(0.31)	(0.11)	(0.36)	(0.31)	(0.11)	(0.11)	(0.33)	(0.09)	(0.12)

Values reported represent mean and standard deviation, of total study group.

*P = 0.01 Significant difference between 0h and 6 h.

Table 5.3.2.2: Mean values and standard deviations for apo A1 and TAG concentrations of HDL subfractions in the fasted and postprandial states pre and post intervention in control group after 16 weeks (*n* 14).

	Total HDL				HDL ₃				HDL ₂			
	Week 0		Week 16		Week 0		Week 16		Week 0		Week 16	
	0 hour	6 hour	0 hour	6 hour	0 hour	6 hour	0 hour	6 hour	0 hour	6 hour	0 hour	6 hour
Apo A1 (g/l)	1.48	1.41	1.27	1.49	1.21	1.23	1.02	1.26	0.27	0.17	0.25	0.24
	(0.57)	(0.28)	(0.48)	(1.07)	(0.42)	(0.27)	(0.40)	(0.27)	(0.18)	(0.18)	(0.21)	(0.16)
TAG (mmol/l)	0.33	0.37	0.54	0.41	0.20	0.19	0.17	0.19	0.12	0.17	0.24	0.21
	(0.09)	(0.15)	(0.42)	(0.40)	(0.10)	(0.10)	(0.09)	(0.15)	(0.06)	(0.18)	(0.17)	(0.25)

Values reported represent mean and standard deviation, of total study group.

5.3.3: Chylomicron lipid composition

Chylomicron and triglyceride poor lipoprotein cholesterol and TAG concentrations are presented in tables 5.3.3.1 and 5.3.3.2. Repeated measures ANOVA demonstrated that there was significant increases ($P \leq 0.002$) in chylomicron TAG concentrations at 6 h as a result of ingestion of test meal in treatment and control groups at week 0 and 16. No significant change in chylomicron TAG concentrations was noted in 0 h and 6 h in either group as a result of dietary intervention using fish oil supplementation for 16 weeks. TPL TAG concentrations did not change 6 h after ingestion of test meal at week 0 or 16, however there was an increase in postprandial TPL TAG concentration over 16 weeks in the control group. No significant changes in TPL TAG concentrations of the fish oil group were observed throughout the study. No significant changes in chylomicron or TPL cholesterol concentrations were observed in either group throughout the duration of the study.

Table 5.3.3.1: Effect of 16 weeks dietary intervention with 0.9 g/d fish oil on chylomicron and triglyceride poor lipoprotein fraction lipid concentrations (n 14)

	Chylomicron				Triglyceride poor fraction			
	Week 0		Week 16		Week 0		Week 16	
	0 hour	6 hour	0 hour	6 hour	0 hour	6 hour	0 hour	6 hour
Cholesterol (mmol/l)	0.34 (0.61)	0.21 (0.27)	0.22 (0.19)	0.32 (0.33)	4.99 (1.15)	4.48 (1.41)	5.31 (0.85)	5.03 (0.84)
Triglyceride (mmol/l)	0.38 (0.27)	0.59* (0.46)	0.40 (0.43)	0.58** (0.46)	0.66 (0.42)	0.64 (0.32)	0.70 (0.29)	0.71 (0.31)

Values reported represent mean and standard deviations

*P = 0.01; **P = 0.03 significant difference between 0 and 6 hr

Table 5.3.3.2: Effect of 16 weeks dietary intervention in control group on chylomicron and triglyceride poor lipoprotein fraction lipid concentrations (n 14)

	Chylomicron				Triglyceride poor fraction			
	Week 0		Week 16		Week 0		Week 16	
	0 hour	6 hour	0 hour	6 hour	0 hour	6 hour	0 hour	6 hour
Cholesterol (mmol/l)	0.14 (0.08)	0.18 (0.16)	0.13 (0.05)	0.13 (0.07)	5.26 (0.77)	5.29 (1.49)	5.29 (1.49)	5.10 (1.71)
Triglyceride (mmol/l)	0.33 (0.22)	0.53* (0.23)	0.27 (0.17)	0.44** (0.25)	0.56 (0.17)	0.50 (0.20)	0.70 (0.28)	0.73† (0.28)

Values reported represent mean and standard deviations

*P = 0.002; **P = 0.005 significant difference between 0 and 6 hr

†P = 0.009 significant difference between week 0 and week 16

5.3.4: Fatty acid composition

Fatty acid composition of platelet phospholipids of fish oil and control groups at week 0 and 16 are presented in table 5.3.4.1. Significant increases ($P \leq 0.0001$) in C20:5(n-3) and C22:6 (n-3) fatty acid composition of platelets phospholipids at week 16 was noted as result of fish oil intake. Significant increases in C18:0 ($P \leq 0.05$) and significant ($P \leq 0.05$) decreases in C16:1 and C18:2 (n-6) compositions were noted as a result of fish oil supplementation. Platelet phospholipid C18:0 increased significantly ($P = 0.02$) in control group at week 16 while C18:2 (n-6) was significantly ($P < 0.03$) reduced post intervention in this group. No significant changes were note in any of the other platelet phospholipid fatty acid composition in control or fish oil group over 16 weeks of study.

Fatty acid composition of HDL cholesterol esters are presented in table 5.3.4.2. A significant ($P = 0.02$) increase in C20:5 (n-3) composition from week 0 to week 16. DHA was not detected in CEs of HDL before or after fish oil supplementation. The percentage composition of all other fatty acid in HDL CE did not change significantly throughout the study in either supplementation group.

Table 5.3.4.1: Platelet fatty acid composition w/w % pre and post dietary intervention for 16 weeks in fish oil and placebo groups.

Fatty acid	Placebo (<i>n</i> 14)		Fish oil (<i>n</i> 14)	
	Pre Trial	Post Trial	Pre Trial	Pre Trial
C16:0	14.24 (2.13)	13.06 (1.66)	14.59 (1.66)	13.04* (1.71)
C16:1	0.732 (0.247)	0.617 (0.14)	0.71 (0.13)	0.60* (0.09)
C18:0	19.13 (1.66)	21.46* (1.98)	19.19 (2.12)	21.80* (3.33)
C18:1	15.67 (2.07)	15.22 (1.82)	15.67 (1.20)	14.75 (1.73)
C18:2 n-6	6.45 (0.74)	5.91* (0.51)	6.51 (0.61)	5.73* (0.59)
C20:4 n-6	29.71 (3.85)	31.45 (4.21)	30.59 (2.64)	31.14 (4.26)
C20:5 n-3	0.98 (0.48)	1.02 (0.29)	0.77 (0.39)	2.19** (0.43)
C22:6 n-3	2.53 (0.57)	2.59 (0.61)	2.25 (0.60)	3.19** (0.67)
Other	10.55 (0.91)	8.67 (0.12)	9.72 (1.21)	7.56 (0.83)

Values reported represent mean and standard deviations of study group

*P ≤ 0.05 **P ≤ 0.0001 Significant difference pre and post intervention

Table 5.3.4.2: Fatty acid composition w/w % of HDL cholesteryl esters in healthy volunteers as a result of dietary intervention with placebo oil and fish oil.

Fatty acid	Placebo group (<i>n</i> 14)		Fish oil group (<i>n</i> 14)	
	Pre Trial	Post Trial	Pre Trial	Pre Trial
C16:0	16.17 (4.36)	13.00 (6.04)	13.87 (6.93)	14.15 (1.71)
C16:1	2.81 (1.29)	5.45 (1.25)	2.81 (0.21)	2.86 (0.30)
C18:0	2.65 (1.28)	5.41 (6.56)	5.39 (1.38)	9.20 (3.68)
C18:2 (n-6)	54.24 (19.75)	54.09 (10.83)	54.80 (9.28)	53.24 (6.35)
C20:4 (n-6)	2.79 (0.33)	2.69 (1.26)	3.16 (2.30)	2.88 (2.06)
C20:5 (n-3)	0.83 (0.81)	0.87 (0.48)	0.81 (0.25)	1.02* (0.72)
Other	19.98 (2.98)	17.10 (4.25)	18.67 (3.19)	15.95 (1.29)

Values reported represent mean and standard deviation of study group

*P = 0.02 Significant difference per and post trial

5.4.0: Discussion

5.4.1: Plasma lipids

In this study plasma TAG concentrations are reduced as a result of dietary supplementation with fish oil over time as has been reported previously (Harris *et al.* 1989; Abbey *et al.* 1990; Roche and Gibney, 1996). In this study there was a significant ($P = 0.01$) decrease of 15 % in plasma TAG as a result of 0.9g/day intake of n-3 PUFA. The decrease in TAG concentration as a result of low dose fish oil intake in this study is comparable with reductions observed in other studies (Argen *et al.* 1996; Fumeron *et al.* 1991) where decreases of between 18 % and 25 % in fasting plasma TAG concentrations were found. Previous studies have used higher dose fish oil over short term with Harris *et al.* (1988) who administered 28g/d for 3 weeks observing a 43% decrease in plasma TAG concentrations, Williams *et al.* (1992) noted a 24.8 % decrease in plasma TAG concentrations in response to 2.7 g/d for 6 weeks. Argen *et al.* (1996) noted a 26.5 % decrease in plasma TAG concentration using 2.3 g/d for 15 weeks. Decreases in plasma TAG noted in our study in response to 0.9 g/day for 16 weeks were comparable to studies using higher doses of fish oil over short term. Low dose fish oil intake over long term is as effective as higher doses at reducing plasma TAG concentrations. Doses of n-3 PUFA used in our study are equivalent to about 2-3 100g portions of fatty fish per week. There were no significant changes in total plasma cholesterol (Harris W.S. 1997) with a slight increase in LDL cholesterol concentrations (Abbey *et al.* 1990) in those individuals receiving fish oil. Previous studies have demonstrated that n-3 PUFA result in the down regulation of hepatic LDL receptor which account for slight increases in LDL cholesterol as a result of fish oil. In vitro and animal studies reveal that EPA down regulates LDL receptor activity (Roach *et al.* 1987; Wong & Nestel 1987). No significant changes in plasma lipids were noted in the placebo group. However non significant decreases of 12-13 % in LDL cholesterol concentration at 0 h and 6 h at week 16 when compared to week 0 was noted in the control group who were administered olive oil. These changes were comparable to changes observed by Groener *et al.* (1991); Jansen *et al.* (2000) in normolipidaemic subjects who were taking MUFA. Animal studies reveal that MUFA reduces LDL cholesterol concentrations by inhibiting LDL receptor suppression (Kurushima *et al.* 1995).

The effects of postprandial lipaemia at 6h were investigated in control and intervention groups at week 0 and week 16. There was a significant ($P \leq 0.0001$)

increase in CETP activity and increases in CETP mass approached significance ($P = 0.06$) at 6 h after ingestion of test meal similar to finding of Tall *et al.* (1986). Plasma TAG and NEFA concentrations increased significantly in response to test meal at 6 hours. Both study groups demonstrated a typical postprandial response to consumption of test meal at week 0 and week 16. Six hour LDL cholesterol concentrations decrease significantly ($P < 0.05$) in both groups at week 0 and 16 in response to test meal in agreement with the findings of Dubois *et al.* (1994).

5.4.2: Chylomicrons

Chylomicron TAG and cholesterol concentrations did not change significantly in either fish oil or control groups at either 0 or 6 h after test meal between week 0 and 16. This confirms findings of Bordin *et al.* (1998) & Illingworth *et al.* (1993), which indicate that fish oil intake, reduces VLDL TAG concentrations. The reduction in plasma TAG concentration observed in this study was not accounted for by reductions in chylomicron TAG concentrations in the late postprandial phase but more likely by reduced VLDL TAG concentrations. Unfortunately we did not measure VLDL TAG concentrations to confirm these findings. At 6 h after ingestion of test meal significant increases in chylomicron TAG concentrations was observed at week 0 and 16, which is expected due to the increase output of chylomicrons during postprandial lipaemia (Levy *et al.* 1991; Goldberg & Schonfield 1985).

5.4.3: Incorporation of EPA and DHA into phospholipid and Cholesterol esters

There was a significant increase in EPA and DHA content of platelet phospholipids as a result of dietary intervention with fish oil similar to the findings of Hjartaker *et al.* (1997), Zuijdgeest van Leeuwen *et al.* (1999), and Bjerve *et al.* (1993). EPA and DHA increased 2.84 and 1.41 times baseline values respectively, similar to increases of 3.5 – 9 times and 1.4 – 1.7 times increase in EPA and DHA composition of platelet phospholipids found by Prisco *et al.* (1996) in response to 4 g/ day fish oil intake for 4 months and is comparable to the findings of Harris *et al.* (1991) who observed a 125 % increase in platelet phospholipid EPA composition in response to 1.25g / day fish oil intake for 6 months. EPA and DHA were incorporated at the expense of C18:1, C18:2 (n-6) and C16:1 in this study. Only the decreases in C16:1 and C18:2 (n-6) were significant. Sarkkienen *et al.* (1994) and Zuijdgeest van Leeuwen *et*

al. (1999) found decreases in C18:1 as a result of fish oil intake, other studies indicate that n-3 PUFA is incorporated into platelet phospholipids at the expense of n-6 fatty acids (C18:2 n-6) (Hunter *et al.* 1990; Argen *et al.* 1996), which is in agreement with the findings of this current study. These increases in n-3 PUFAs in the fish oil group indicated dietary compliance of this group during the study as have been demonstrated previously by Sarkkinen *et al.* (1994). Abbey *et al.* (1990) demonstrated a four fold increase in HDL CE EPA composition as a result of 3.8g fish oil intake per day for 6 weeks. In this study there was a 13 % increase in HDL CE EPA composition, discrepancies between studies may possibly be explained by greater doses of fish oil administered by Abbey *et al.* (1990). There was a non-significant decrease in C20:4 (n-6) and C18:2 (n-6) concentrations in the HDL CE fraction. This data suggest that incorporation of EPA into HDL CE displaced these fatty acids.

5.4.4: HDL lipids

There was a significant increase in total and HDL₃ cholesterol and phospholipid concentrations at 6 h as a result of ingestion of test meal in both dietary supplementation groups at week 0 and 16. Increases in HDL₂ cholesterol and phospholipid concentrations as a result of meal intake only approached significance in both intervention groups at week 0 and 16 (See figures 5.3.2.1-5.3.2.4) as has been reported previously de Bruin *et al.* (1993) and Sutherland *et al.* (1998). HDL TAG and apo A1 concentrations increased during postprandial lipaemia in the intervention in both group in response to test meals but did not reach significance with the exception of week 16 6 h HDL apo A1 concentration in the fish oil group. The significant increase in HDL₃ cholesterol and phospholipids concentrations at 6 h indicated that there was obviously an increase in HDL₂ cholesterol concentrations earlier in postprandial lipaemia, however through the actions of HL these HDL₂ were converted to HDL₃ (Patsch *et al.* 1987). Considering the evidence of Patsch *et al.* (1987) which indicates that HDL TG increases 4-8 hours after meal intake, it appears that in this current study, this phenomena occurred earlier during postprandial lipaemia. 6 h after ingestion of test meal we see the product of HL activity, which is increased HDL₃ cholesterol and phospholipid concentrations. HL activity may also account for the moderate increases in HDL TAG concentrations during postprandial lipaemia at 6 h. It appears that peak HDL TAG concentrations occurred earlier during lipaemia. Others have reported that the

administration of test meals high in MUFA conserves postprandial HDL cholesterol concentrations in contrast to PUFA meals which reduce postprandial HDL cholesterol concentrations. Competition between chylomicron remnants and HDL for catabolism contributes to the conserving effects of MUFA on postprandial HDL cholesterol concentrations (de Bruin *et al.* 1993).

There were significant increases in cholesterol concentrations in total and HDL₃ subfractions in the fasted and postprandial states and 6 h HDL₂ cholesterol concentrations as a result of receiving dietary supplementation with fish oil for 16 weeks. Other studies (Cobiac *et al.* 1991; Harris *et al.* 1988; Fumreon *et al.* 1991) have reported increases in HDL₂ cholesterol concentration as a result of fish oil intake. An increase of 8 % in the ratio of HDL:LDL cholesterol was noted during 16 week intervention with fish oil. There were no significant changes in the lipid concentration of the HDL subfractions in the placebo group. Fasting HDL cholesterol concentrations decreases slightly as a result of taking placebo. There was a 9 % reduction in LDL:HDL ratios as a result of MUFA supplementation (placebo group). However the fish oil group did have a higher HDL cholesterol concentration than did the placebo group post intervention which may be more beneficial in terms of the positive effect increased HDL concentrations have on cardiovascular risk. However ratios of LDL:HDL were not significantly different between the groups at the end of the study.

5.4.5: Effect of fish oil supplementation on CETP mass and activity

There was no significant change in CETP mass or activity in the fasting and postprandial states between week 0 and 16 in this study as a result of fish oil intake. These results concur with the findings of Lottenburg *et al.* (1996a). Postprandial CETP activity was reduced by 8 %, a decrease which approached significance ($P = 0.06$). Other studies have shown significant effects of n-3 PUFA on CETP metabolism. Abbey *et al.* (1990) demonstrated that CETP activity was significantly reduced by 23 % in response to 3.8g n-3 PUFA for 6 weeks and Bagdade *et al.* (1992c) showed that CETP activity and mass was reduced by 97 % and 13 % respectively as a result of 6 g/d fish oil for 12 weeks in hypercholesterolemics. There are important differences between the current study and previously mentioned studies, where the subject types (hypercholesterolemics) and the higher daily dose of n-3 PUFA was used. Bagdade *et al.* (1992c) concluded that dietary intake of fish oil in hypercholesterolemic subjects

resulted in the normalisation of CETP. It is possible that cholesterol concentrations may regulate CETP activity in these patients' therefore significant decreases in plasma cholesterol noted by Bagdade *et al.* (1992c) may have accounted for a proportion of decrease in CETP activity as a result of marine oil intake. An explanation for the modest results regarding CETP mass and activity in this study maybe that our study group already had normalised CETP mass, activity and plasma cholesterol concentrations. A strong correlation between plasma cholesterol and CETP activity ($r = 0.356$; $P = 0.01$) was noted in this study. Fish oil supplementation didn't exhibit any significant effect on CETP or plasma cholesterol concentrations during this study. In light of the strong relationship between plasma cholesterol and CETP activity, we conclude that if this population had been more at risk of the development of CAD, we may have seen greater reductions in CETP activity similar to the findings of Bagdade *et al.* (1992c) and Abbey *et al.* (1990). The control group which was administered a monounsaturated oil exhibited a 10 % reduction in fasting CETP activity which is in agreement with other studies where effect of MUFA intake on CETP activity was measured (Groener *et al.* 1991; Jansen *et al.* 2000). However postprandial CETP activity was increased post trial in this group.

Although intake of fish oil did not alter CETP mass or activity, n-3 PUFA did seem to have a profound effect on HDL lipid composition. Possible mechanisms for these increases may be attributed to increased EPA content of HDL cholesterol esters in the fish oil group. Increasing chain length and unsaturation of CEs results in a lower rate of transfer of cholesterol esters by CETP, as demonstrated by Morton *et al.* (1986) thereby resulting in CE being retained in the HDL fraction. Incidentally we did not detect DHA in CE of the HDL cholesterol before or after supplementation similar to findings of Abbey *et al.* (1990). Reductions in plasma TAG concentrations and the non-significant reduction in postprandial CETP activity in those receiving fish oil may account for increases in HDL cholesterol, phospholipid and HDL₂ cholesterol concentrations in the fish oil group. A possible mechanism as to how this occurs may be that with lower plasma TAG concentrations and moderately reduced 6h CETP activity, transfer of TAG to the HDL₂ subfraction is reduced. Because of the reduced TAG concentration in HDL₂, HDL₂ high in CE will not be catabolised to HDL₃ as rapidly by HL (Patsch *et al.* 1987; Patsch *et al.* 1984) this would account for increased fasting and postprandial HDL cholesterol and postprandial HDL₂ cholesterol

concentrations. This appears to be confirmed by the inverse correlation ($r = -0.235$; $P = 0.02$) between HDL₂ cholesterol concentration and plasma TAG in this study and as have been reported by others (Sprecher *et al.* 1994).

Abbey *et al.* (1994) and Abbey *et al.* (1990) have shown that there is an increase in A1/A11 ratio in HDL a known inhibitor of CETP activity as a result of n-3 PUFA intake. Unfortunately this ratio was not measured in this study. A study by Fumeron *et al.* (1991) suggests that there is an increase in apo A1 and inhibitory apo A1/A11 concentrations. Studies carried out by Nestel *et al.* (1986) and Phillipson *et al.* (1985) on the effect of fish oil on apo A1 have found decreasing and increasing effects of fish oils on this protein. In this study fasting HDL apo A1 concentrations decreased non-significantly while postprandial apo A1 concentrations increased non-significantly as a result of intervention. It is hard to ascertain from this study and others studies if fish oil exhibit any effect on apo A1 concentrations.

5.4.6: Conclusions

The findings of this study demonstrate that administration of low dose fish oil over 16 week's results in significant reduction of plasma TAG concentrations while MUFA decreased plasma TAG non-significantly. Low dose fish oil intake result in significantly increased HDL cholesterol concentrations and non-significant increases in HDL phospholipid concentrations. In many previous studies the administration of higher doses of fish oil than used in this current study resulted in reduction (Flaten *et al.* 1990) or no change (Harris, W.S. 1997) in HDL cholesterol concentrations. Intake of placebo (MUFA) had no effect on HDL lipids. Non-significant decreases in CETP mass or activity were noted as a result of fish oil and MUFA intake. Intake of fish oil increased LDL cholesterol while MUFA (placebo) decreased LDL cholesterol concentrations. It may be concluded that intake of a low dose of fish oil has a beneficial effect on CAD risk factors by reducing plasma TAG and postprandial CETP activity and increasing HDL cholesterol concentrations.

Chapter 6

Effects of isomeric blends of Conjugated Linoleic Acid on plasma lipids, cholesteryl ester transfer protein mass, activity and lipoprotein metabolism

6.1: Introduction

Conjugated linoleic acid (CLA) is the name given to a mixture of positional and geometric isomers of linoleic acid with conjugated double bonds at positions 9 and 11 or 10 and 12 (Ha *et al.* 1987). These conjugated double bonds may be of *cis* or *trans* configuration. CLA is produced in ruminants by intestinal flora (Kepler *et al.* 1970). Dairy products are the primary source of dietary CLA, with an average estimated daily intake of CLA in humans being about 160 mg (Chin *et al.* 1992).

In recent years a number of publications have demonstrated that CLA exhibits anticarcinogenic effects (Ip *et al.* 1995, Ip *et al.* 1996). CLA exhibits an ability to modulate immune function. Hayak *et al.* (1999) demonstrated that mice whose diets were supplemented with CLA increased IL-2 production, while Chew *et al.* (1997) demonstrated that CLA enhanced T cell mitogen proliferation and inhibited IL-2, with the suppression of phagocytic activity of macrophage using porcine lymphocytes *in vitro*. Evidence to date on the effects of CLA on immune function is not fully understood, therefore more research may reveal the true effect of CLA on immune function.

CLA has been shown to affect body composition by increasing lean to fat ratios in animals. Stangl *et al.* (2000) reported CLA intake resulted in an increase lean body mass in rats when compared to controls. Humans supplemented with CLA at 2.7 g/day induced a 20 % reduction in body fat in non-obese subjects (Atkinson *et al.* 1999). Gudmundsson *et al.* (1999) demonstrated that supplementation of CLA at 7.2 g/day for 12 weeks significantly reduced body fat, and increased lean body mass in moderately obese humans. Gavino *et al.* (2000) suggests that the *trans*-10, *cis*-12 isomer of CLA is active in reducing body fat accumulation, which confirmed the findings of Park *et al.* (1999a) where *trans*-10, *cis*-12 isomer was associated with reducing body fat in mice.

Several studies have demonstrated that dietary supplementation with CLA prevents diet induced atherosclerotic plaque development in rabbits and hamsters (Lee *et al.* 1994; Nicolosi *et al.* 1997). This may be mediated by a combination of the apparent hypolipidaemic and reduced phagocytosis ability of macrophage (Chew *et al.* 1992) attributed to CLA. Lee *et al.* (1994) demonstrated a 30 % reduction in cholesterol deposition in aorta of rabbits, while Nicolosi *et al.* (1997) noted a reduction of 26 % aortic streak formation in hamsters. CLA supplementation has a beneficial effect on plasma lipids in mouse and hamster models as is evident from the findings of Munday *et al.* (1999) and Nicolosi *et al.* (1997) where significant reductions in plasma TAG concentrations were noted. VLDL TAG concentrations were significantly reduced in

rats as a result of dietary supplementation with CLA (Stangl *et al.* 2000). These findings have important implications in terms of CAD as plasma TAG is a known risk factor of CAD (Stampfer *et al.* 1996). Dietary supplementation with CLA significantly reduces total plasma cholesterol concentrations in hamsters (Gavino *et al.* 2000; Nicolosi *et al.* 1997) and non-HDL (VLDL and LDL) cholesterol concentrations in hamsters (Nicolosi *et al.* 1997).

Possible mechanisms whereby CLA exerts its hypolipidaemic effects are evident from the findings of Yotsumoto *et al.* (1999) suggests that the *trans*-10, *cis*-12 CLA isomer of CLA suppresses hepatic TAG and cholesterol synthesis and apo B secretion in vitro, suggesting a mechanism whereby CLA reduces plasma TAG and cholesterol concentration. CLA is a ligand for PPARs, which are involved in the regulation and expression of genes involved in lipid metabolism. PPAR α is expressed in the liver and regulates genes that control lipid metabolism. Moya-Carmena *et al.* (1999a) demonstrated that *cis*-9, *trans*-11 isomer of CLA is a more potent activator of PPAR α than the *trans*-10, *cis*-12 isomer of CLA. Therefore CLA may modulate lipid metabolism in the liver. PPAR γ regulates lipid metabolism in the adipocyte and is also activated by CLA. Houseknecht *et al.* (1998) demonstrated that CLA increased PPAR γ responsive genes that include LPL, acyl-Co-A synthase, and the fatty acid binding protein. Increase LPL in the adipocyte will result in increased removal of TAG rich lipoproteins, thereby reducing plasma TAG concentrations

The *cis*-9, *trans*-11-isomer of CLA is the most predominant naturally occurring isomer and is most readily incorporated into tissue phospholipids where an isomeric blend of CLA is administered (Ha *et al.* 1990). The *cis*-9, *trans*-11 isomer of CLA makes up between 0.12-0.50 % total fatty acids in serum (Fogerty *et al.* 1988). The *trans*-10, *cis*-12 isomer of CLA is not easily detected in tissue and plasma possibly due to preferential metabolism of this isomer as suggested by Sebedios *et al.* (1997).

A number of studies have demonstrated that dietary supplementation with CLA reduces plasma lipid concentrations in animal models (Nicolosi *et al.* 1997; Lee *et al.* 1994). Based on these findings we investigated whether CLA supplementation in humans had any effect on lipid metabolism. To date there has been no investigation into the effects of CLA on plasma lipid metabolism in humans. This study investigated the effects of two isomeric blends of CLA on lipid metabolism, CETP mass and activity in a large normolipidaemic human population. The two isomeric blends of CLA were made up as follows. 1) a 50:50 isomeric blend of the *cis*-9, *trans*-11 and *trans*-10, *cis*-

12 isomers of CLA. This blend of CLA will be referred to as the 50:50 *cis*-9, *trans*-10 isomeric blend of CLA. 2) a 80:20 isomeric mix of the *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers of CLA. This will be referred to as the 80:20 *cis*-9, *trans*-10 isomeric blend of CLA. The two isomer blends allowed us to determine whether the effects of CLA on lipid metabolism are specific to isomeric blends of CLA. Incorporation of isomers of CLA into total plasma fatty acid composition was also accessed. A dose of 3g/d CLA intake, estimated to be about 1-1.5 % total dietary fat intake was administered over 8 weeks. We hypothesise that daily intake of CLA at this concentration in humans should elicit an effect on lipid metabolism in humans based on the findings of Nicolosi *et al.* (1997) where CLA administered at 0.6-1.1 % energy had an effect on lipid metabolism and development of atherosclerosis.

6.2.0: Methods

6.2.1: Study Design

This study was approved by the Ethics Committee of the Federated Dublin Voluntary Hospitals. The study was conducted on an outpatient basis. 51 healthy non-smoking healthy volunteers aged between 18-60 years were recruited from the personnel of Trinity College and St. James Hospital, Dublin. All volunteers gave a written informed consent to participate in the study. A fasting screening blood sample was taken from each volunteer to ensure that they adhered to the following biochemical parameters.

Inclusion criteria

Age 18 –60 years

BMI < 25 Kg/m²

Plasma Cholesterol < 6.5 mmol/l

Plasma TAG < 2 mmol/l

Gammaglutamyl transferase < 60 IU

Haemoglobin > 11mg/dl

Not habitual consumers of n-3 PUFA

< 90 minutes strenuous exercise per week

A dietary questionnaire was given to each subject to make an assessment of their dietary intake of dairy products. Any subjects who had an estimated intake of CLA greater than 300mg / day as calculated from the quantity of dairy products consumed per day were excluded. Any volunteers who had a high intake of oily fish, consumed fish oil capsules or other dietary supplements were excluded from the study. This was a double blind placebo controlled study. Volunteers were randomly assigned into three groups receiving 3.0 g/day of either 1) 50:50 *cis*-9, *trans*-10 isomeric blend of CLA (*n* 16), or 2) 80:20 *cis*-9, *trans*-10 isomeric blend of CLA (*n* 17) or 3) linoleic acid (*n* 18). CLA supplements were supplied by Loders Crooklann, B.V., The Netherlands. Study compliance was measured using a pill count and by incorporation of the *cis*-9, *trans*-11 isomer of CLA into total plasma lipids.

6.2.2: Study subjects

This study was conducted at the Nutrition Laboratory, in the Trinity Centre for Health Sciences at St. James Hospital, Dublin 8. Subjects attended the Nutrition Laboratory for blood sampling between 07:30 –08:00 h following a 12 h over night fast at weeks 0, 4 and 8. Subjects were also asked to abstain from alcohol 24 h prior to blood sampling. Subjects were given 4 weeks supply of capsules following blood sampling at week 0 and 4.

Blood was taken in 10 ml EDTA vacutainer (Beckton & Dickinson, Plymouth, UK). Blood samples were centrifuged at 3000 rpm for 10 minutes. Plasma for CETP and plasma lipid analysis was stored at -70°C . Plasma for HDL LDL and VLDL isolation was stored at 4°C and these subfractions were isolated as detailed in chapter 2.

6.2.3: Laboratory methods

Analysis of plasma TAG (TAG PAP Biomerieux, France), cholesterol (PAP, Biomerieux, France), NEFA (Acyl Co A synthetase-acyl Co A oxidase) Wako Chemicals, Gmbh, Germany), phospholipid (PAP, Biomerieux, France), apo A1 (Biomerieux, France) and apo B (Biomerieux, France) concentrations were carried out. HDL cholesterol, TAG, phospholipid and apo A1 concentrations were measured using the methods mentioned previously following precipitation of HDL with Immuno Quantolip HDL (Immuno AG, Vienna, Austria) precipitating reagent. VLDL were isolated using ultracentrifugation as described in section 2.16.2 and were analysed for cholesterol and TAG concentrations. Plasma lipids were assayed on a Technicon RA-XT Chemistry Analyser (Technicon Inc., Tarrytown, NY., U.S.A.) as detailed in chapter 2

CETP activity was determined using a radiolabelled H^3CE in HDL as a donor particle and endogenous apo B lipoproteins as acceptor particles as detailed in chapter 2. CETP mass was determined using an alkaline phosphatase based sandwich ELISA assay devised by Clarke *et al.* (1995). Total plasma lipids were analysed using gas chromatography using chromatography as outlined in section 2.18.6.

6.2.4: Data Analysis

All statistical analysis was carried out on an Apple Macintosh statistical package Data Desk 4.1 (Data Description Inc., NY., U.S.A). The distribution of the data for each variable was assessed. Some of the variables were transformed to normalise the distribution of the data sets to give data a normal gaussian distribution. Repeated measures ANOVA was used to investigate statistical changes in biochemical parameters as a result of dietary intervention in control and those receiving isomeric blends of CLA.

6.3.0: Results:

6.3.1: Total study group

Fifty-one subjects 18 males and 33 females participated in this study. The total study group had a mean age of 31.6 (± 10.03) years, a mean weight of 68.53 (± 11.23) kg and a mean BMI of 23.33 kg/m². There was no significant change in body weight in the 50:50 *cis*-9, *trans*-10 or 80:20 *cis*-9, *trans*-10 CLA groups or the linoleic acid group as a result of dietary supplementation. The mean plasma lipid and CETP concentrations for the total group were as follows, plasma cholesterol 5.04 (± 1.00) mmol/l, plasma TAG 1.10 (± 0.38) mmol/l, plasma glucose 4.96 (± 0.39) mmol/l, plasma NEFA 0.34 (± 0.25) mmol/l, CETP mass 2.33 (± 0.53) mg/ml and CETP activity 21.66 (± 7.09) CE % transfer / 3 hr. There was no significant difference in these variables between the three dietary supplementation groups in this study

6.3.2: Effects of supplementation on plasma lipids and CETP

Plasma lipid and CETP concentrations of the three dietary groups at week 0 and week 16 are presented in table 6.3.2. Using repeated measures analysis of variance plasma TAG concentrations were significantly ($P \leq 0.0001$) reduced in the 50:50 *cis*-9, *trans*-10 CLA group at week 8 when compared to week 0 values in this group. Non significant reductions of 7.4 % and 8.7 % in plasma TAG concentrations were observed in the 80:20 *cis*-9, *trans*-10 isomer blend of CLA and linoleic acid groups as a result of dietary intervention over 8 weeks. A non-significant decrease of 8 % was observed in LDL cholesterol concentrations in the 50:50 *cis*-9, *trans*-10 and 80:20 *cis*-9, *trans*-10 CLA groups by week 8. A 3 % decrease in LDL cholesterol concentrations was observed as a result of supplementation with linoleic acid for 8 weeks. No significant changes in CETP mass or activity were observed as a result of dietary supplementation with the isomeric blends of CLA used in this study. Plasma NEFA and glucose concentrations remained unchanged in all of the dietary intervention groups throughout the study.

Table 6.3.2: Effect of dietary supplementation using isomeric blends of CLA and linoleic acid at 3 g/d for 8 weeks on plasma lipids and CETP mass and activity

	50:50 <i>cis</i> -9, <i>trans</i> -10 isomer blend (n 16)				80:20 <i>cis</i> -9, <i>trans</i> -10 isomer blend (n 17)				Control: linoleic acid (n 18)			
	Week 0		Week 8		Week 0		Week 8		Week 0		Week 8	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Cholesterol (mmol/l)	4.93	1.36	4.84	0.95	5.01	0.49	4.92	0.70	5.18	1.17	5.28	1.02
Triglyceride (mmol/l)	1.20	0.39	0.95*	0.31	1.08	0.32	1.00	0.40	1.03	0.43	0.94	0.33
Glucose (mmol/l)	4.94	0.31	4.85	0.40	4.97	0.49	4.88	0.41	4.97	0.37	5.21	0.51
NEFA (mmol/l)	0.36	0.26	0.37	0.19	0.33	0.14	0.38	0.19	0.33	0.35	0.33	0.22
LDL cholesterol (mmol/l)	1.65	0.83	1.53	0.64	1.69	0.70	1.54	0.59	1.77	1.04	1.71	0.71
CETP activity (CE Transfer %)	19.26	6.81	20.53	8.01	23.13	6.01	23.51	7.49	22.6	8.46	22.40	9.98
CE mass (mg/ml)	2.12	0.77	2.45	0.86	2.62	0.58	2.34	0.77	2.27	0.26	1.98	0.70

Mean values were significantly different between week 0 and week 8: *P = 0.005

6.3.3: VLDL lipid composition

VLDL and TPL cholesterol and TAG concentrations for the 50:50 *cis*-9, *trans*-10 and the 80:20 *cis*-9, *trans*-10 isomer blends of CLA and linoleic acid groups at week 0 and 8 are presented in tables 6.3.3.1, 6.3.3.2. and 6.3.3.3. VLDL cholesterol concentrations were significantly ($P = 0.05$) reduced in the 80:20 *cis*-9, *trans*-10 CLA group at week 8. There was a non-significant decrease of 24.5 % in VLDL TAG concentrations in this group as a result of CLA supplementation over 8 weeks. In the 50:50 *cis*-9, *trans*-10 CLA group a non-significant decrease of 27.5 % in VLDL cholesterol and 29.4 % in VLDL TAG concentrations was observed between week 0 and week 8. In the linoleic acid group a 12 % increase in VLDL TAG concentrations was noted with VLDL cholesterol concentrations remaining unchanged between week 0 and week 8.

When the data for VLDL cholesterol and TAG concentrations in both CLA groups were pooled, repeated measures ANOVA revealed that VLDL cholesterol and TAG concentrations were significantly ($P \leq 0.05$) reduced as a result of 8 weeks of dietary supplementation with CLA.

No significant change in TPL TAG and cholesterol concentrations was observed in any of the supplementation groups during the trial. TPL apo B concentrations remained unchanged in all study groups as a result of dietary supplementation.

Table 6.3.3.1: Effect of dietary supplementation of a 50:50 *cis*-9, *trans*-10 isomer blend of CLA at 3 g/ d for 8 weeks on very low density lipoprotein and triglyceride poor lipoprotein fraction lipid concentrations (*n* 16)

	Week 0				Week 8			
	VLDL		TPL		VLDL		TPL	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Cholesterol mmol/l	0.29	0.14	3.81	0.97	0.21	0.08	3.92	1.08
Triglyceride mmol/l	0.68	0.38	0.42	0.16	0.48	0.22	0.38	0.15

Values reported represent mean and standard deviations

Table 6.3.3.2: Effect of dietary supplementation using linoleic acid at 3 g/ d for 8 week on very low density lipoprotein and triglyceride poor lipoprotein lipid concentrations (n 18)

	Week 0				Week 8			
	VLDL		TPL		VLDL		TPL	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Cholesterol mmol/l	0.31	0.22	3.95	0.94	0.26	0.13	4.02	0.95
Triglyceride mmol/l	0.52	0.36	0.33	0.09	0.58	0.29	0.34	0.12

Values reported represent mean and standard deviations

Table 6.3.5.3: Effect of dietary supplementation of a 80:20 *cis*-9, *trans*-10 isomer blend of CLA at 3 g/ d for 8 weeks on very low density lipoprotein and triglyceride poor lipoprotein lipid concentrations (n 17)

	Week 0				Week 8			
	VLDL		TPL		VLDL		TPL	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Cholesterol mmol/l	0.31	0.16	3.60	0.56	0.21*	0.13	4.15	0.94
Triglyceride mmol/l	0.61	0.30	0.36	0.08	0.46	0.27	0.34	0.08

Values reported represent mean and standard deviations

Significant difference between week 0 and week 8 *P < 0.05

Table 6.3.3.4: The effect of dietary supplementation using isomeric blends of CLA and linoleic acid at 3g /d for 8 weeks on triglyceride poor lipoprotein apo B concentrations (g/l).

	<u>Week 0</u>		<u>Week 8</u>	
	Mean	SD	Mean	SD
50:50 <i>cis</i> -9, <i>trans</i> -10 isomeric blend	0.66	0.19	0.79	0.77
80:20 <i>cis</i> -9, <i>trans</i> -10 isomeric blend	0.65	0.16	0.72	0.18
Linoleic acid	0.64	0.20	0.73	0.20

Values reported represent mean and standard deviation in parenthesis

6.3.4: HDL Analysis

The mean total HDL lipid concentrations of the total study group were as follows, HDL cholesterol 1.43 (± 0.44) mmol/l, HDL phospholipid 1.57 (± 0.39) mmol/l, total HDL TAG 0.19 (± 0.09) mmol/l and HDL apo A1 1.26 (± 0.26) g/l. The lipid and apo A1 concentrations of the HDL subfractions of each dietary supplementation group at week 0 and week 8 are presented in tables 6.3.4.1 to 6.3.4.3. No significant changes in HDL subfraction composition were noted as a result of dietary supplementation with isomeric blends of CLA or linoleic acid. A non-significant increase of 11 % in total HDL cholesterol and 8.6 % in total HDL phospholipid was observed in the linoleic acid group pre and post intervention. There was a 29 % increase in HDL₂ cholesterol composition in this group over 8 week dietary supplementation period (See table 6.3.4.1).

LDL/HDL cholesterol ratios were reduced by 2.4 % in the 50:50 *cis*-9, *trans*-10 CLA group, 11.18 % in the linoleic acid group and 7.2 % in the 80:20 *cis*-9, *trans*-10 CLA group.

Table 6.3.4.1: Effect of dietary supplementation using linoleic acid at 3g/d for 8 weeks on lipid and apo A1 concentrations of the HDL subfractions (*n* 18)

	HDL		Week 0 HDL ₃		HDL ₂		HDL		Week 8 HDL ₃		HDL ₂	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Cholesterol (mmol/l)	1.27	0.31	1.04	0.24	0.24	0.17	1.42	0.36	1.11	0.18	0.31	0.21
Phospholipid (mmol/l)	1.39	0.24	1.17	0.19	0.20	0.13	1.51	0.31	1.21	0.15	0.30	0.20
Triglyceride (mmol/l)	0.18	0.03	0.26	0.33	0.06	0.36	0.17	0.06	0.24	0.36	0.06	0.39
Apo A1 (g/l)	1.19	0.24	1.06	0.15	0.18	0.39	1.26	0.24	1.11	0.09	0.13	0.18

Values represent mean and standard deviations
NSD

Table 6.3.4.2: Effect of dietary supplementation using a 80:20 *cis*-9, *trans*-10 isomeric blend of CLA at 3g /d for 8 weeks on lipid and apo A1 concentrations of the HDL subfractions (*n* 16)

	Week 0				Week 8							
	HDL		HDL ₃		HDL ₂		HDL		HDL ₃		HDL ₂	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Cholesterol (mmol/l)	1.44	0.44	1.06	0.20	0.39	0.31	1.47	0.37	1.05	0.19	0.41	0.28
Phospholipid (mmol/l)	1.58	0.41	1.21	0.19	0.40	0.27	1.57	0.34	1.19	0.19	0.37	0.26
Triglyceride (mmol/l)	0.18	0.06	0.17	0.06	0.15	0.18	0.18	0.06	0.18	0.06	0.09	0.06
Apo A1 (g/l)	1.32	0.22	1.08	0.15	0.21	0.21	1.21	0.21	1.14	0.33	0.06	0.39

Values represent mean and standard deviations
NSD

Table 6.3.4.3: Effect of dietary supplementation using a 50:50 *cis*-9, *trans*-10 isomeric blend of CLA at 3g/d for 8 weeks on lipid and apo A1 concentrations of the HDL subfractions (*n* 16)

	Week 0				Week 8							
	HDL		HDL ₃		HDL ₂		HDL		HDL ₃		HDL ₂	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Cholesterol (mmol/l)	1.59	0.57	1.14	0.23	0.47	0.26	1.54	0.42	1.07	0.21	0.41	0.27
Phospholipid (mmol/l)	1.75	0.56	1.32	0.31	0.43	0.22	1.66	0.38	1.18	0.19	0.45	0.24
Triglyceride (mmol/l)	0.21	0.09	0.19	0.06	0.30	0.03	0.21	0.09	0.48	0.84	0.30	0.81
Apo A1 (g/l)	1.27	0.33	1.11	1.50	0.19	0.27	1.33	0.27	1.04	0.15	0.28	0.15

Values represent mean and standard deviations
NSD

6.3.5: Fatty acid composition of total plasma lipids

Fatty acid composition of total plasma lipids are presented in table 6.3.5. Significant increases of 87 % ($P = 0.001$) and 90 % ($P \leq 0.0001$) in C18:2 *cis*-9, *trans*-11 isomer were observed in the 50:50 *cis*-9, *trans*-10 and 80:20 *cis*-9, *trans*-10 CLA groups respectively. There was a significant ($P = 0.01$) reduction in C18:3 n-3 in the 50:50 *cis*-9, *trans*-10 CLA group, while C20:5 n-3 decreased significantly ($P = 0.02$) in the 80:20 *cis*-9, *trans*-10 CLA group as a result dietary supplementation with this isomeric blend of CLA over 8 weeks. No other significant changes in the fatty acid composition in either of the CLA groups where observed. No significant changes in the fatty acid composition of total plasma lipids were noted as a result of supplementation with linoleic acid.

Table 6.3.5: Fatty acid composition of total plasma lipids as a result of dietary supplementation using isomeric blends of CLA and linoleic acid at 3g/d for 8 weeks

Fatty acid	50:50 <i>cis</i> -9, <i>trans</i> -10 isomer blend (n 16)		80:20 <i>cis</i> -9, <i>trans</i> -10 isomer blend (n 17)		Linoleic acid (n 18)	
	Week 0	Week 8	Week 0	Week 8	Week 0	Week 8
C16:0	25.38 (2.15)	24.80 (2.13)	25.10 (1.98)	24.61 (1.63)	24.63 (2.15)	24.98 (1.48)
C16:1	2.25 (0.72)	2.14 (0.64)	2.76 (0.86)	3.16 (2.14)	2.39 (2.24)	1.84 (0.72)
C18:0	9.15 (2.03)	8.62 (1.07)	9.01 (1.18)	9.33 (1.22)	9.35 (0.87)	9.23 (2.57)
C18:1	21.40 (3.82)	22.84 (2.55)	21.14 (2.28)	20.96 (1.90)	21.51 (4.05)	20.16 (3.77)
C18:2 n-6	28.49 (4.21)	27.25 (3.39)	28.83 (3.42)	29.44 (3.32)	29.31 (4.54)	29.44 (2.83)
C18:3 n-6	0.27 (0.12)	0.19 (0.10)	0.25 (0.08)	0.298 (0.12)	0.40 (0.28)	0.29 (0.08)
C18:3 n-3	0.74 (0.29)	0.50* (0.35)	0.68 (0.29)	0.58 (0.27)	0.61 (0.26)	0.62 (0.23)
c-9, t-11	0.33 (0.20)	0.62† (0.26)	0.42 (0.23)	0.80‡ (0.29)	0.40 (0.16)	0.28 (0.12)
C20:2	0.28 (0.08)	0.19 (0.07)	0.29 (0.12)	0.27 (0.07)	0.26 (0.06)	0.27 (0.06)
C20:3 n-6	1.54 (0.67)	1.24 (0.53)	1.77 (0.36)	1.79 (0.29)	1.66 (0.54)	1.88 (0.32)
C20:4 n-6	7.32 (1.17)	6.53 (1.78)	7.13 (1.61)	6.94 (1.74)	7.17 (1.35)	7.42 (1.31)
C20:5 n-3	1.04 (0.48)	1.02 (0.59)	1.19 (0.52)	0.90** (0.47)	0.94 (0.33)	1.05 (0.55)
C22:4 n-6	0.31 (0.09)	0.27 (0.13)	0.41 (0.33)	0.32 (0.17)	0.41 (0.33)	0.37 (0.12)
C22:6 n-3	2.32 (0.86)	2.27 (0.73)	2.17 (0.65)	2.13 (0.61)	2.11 (0.66)	2.03 (0.84)

Values represent mean and standard deviation in parenthesis

Significance difference between week 0 and week 8 *P = 0.01; **P = 0.02; †P = 0.001;

‡P ≤ 0.0001

6.4.0: Discussion

6.4.1: Plasma lipids

In this study a significant ($P = 0.005$) reduction in plasma TAG concentration of 20 % was observed in those receiving the 50:50 *cis*-9, *trans*-10 isomeric blend of CLA. This is comparable with the findings of Munday *et al.* (1999), Stangl *et al.* (2000) and Nicolosi *et al.* (1997) who observed decreases in plasma TAG concentration as a result of dietary supplementation with CLA in mice and rat and hamster models. Nicolosi *et al.* (1997) noted reductions in plasma TAG concentrations of 28 % and 36 % as a result of CLA supplementation for 11 weeks at 0.06 % and 0.11 % of energy intake in hamsters. Non-significant decreases of 7.4 % and 8.7 % in plasma TAG concentrations were noted in both the 80:20 *cis*-9, *trans*-10 isomeric blend and linoleic acid group. Nicolosi *et al.* (1997) noted decreases in plasma TAG concentrations in hamsters where diets were supplemented with linoleic acid over 11 weeks were comparable with reductions noted in CLA diets.

From the findings of this study the *trans*-10, *cis*-12 isomer of CLA appears to be potent at reducing plasma TAG concentrations. Non-significant reductions in plasma TAG concentrations were noted as a result of supplementation with the 80:20 *cis*-9, *trans*-10 CLA isomer blend. The *cis*-9, *trans*-11 isomer of CLA does not appear to have the same hypotriglyceridaemic effects as the *trans*-10, *cis*-12 isomer. The substantially lower intake of *trans*-10, *cis*-12 isomer in the 80:20 *cis*-9, *trans*-10 group may explain why reductions in plasma TAG concentrations did not reach significance. Our findings on the different effects of specific isomeric blends of CLA, with the *trans*-10, *cis*-12 isomer appearing to be effective in lowering plasma TAG concentrations are confirmed by Gavino *et al.* (2000). Gavino *et al.* (2000) demonstrated that a CLA isomer mix reduced plasma TAG concentrations, while pure *cis*-9, *trans*-11 isomer of CLA did not exhibit any effect on plasma TAG concentrations in Golden Syrian hamsters. Evidence from in vitro experiments by Yotsumoto *et al.* (1999) demonstrated that the *trans*-10, *cis*-12 isomer of CLA inhibits hepatic TAG synthesis. Therefore a reduction in hepatic TAG synthesis may contribute to decrease in plasma TAG as a result of CLA intake noted in this study.

6.4.2: Very Low Density Lipoproteins

Significant decreases in plasma TAG and moderate reductions in LDL cholesterol concentrations attributed with CLA intake in this study may be the product of the reductions in VLDL lipids observed in both CLA supplementation groups. A

significant decrease ($P \leq 0.05$) in VLDL cholesterol concentrations was observed in the 80:20 *cis*-9, *trans*-10 CLA group. Non-significant decreases in VLDL TAG concentrations were observed in both CLA groups in this study. However when VLDL TAG and VLDL cholesterol concentrations of both CLA grouping are pooled and compared pre and post intervention, VLDL TAG and cholesterol concentrations were significantly ($P \leq 0.05$) reduced by dietary supplementation with CLA by week 8. These findings are in agreement with the findings of Stangl (2000) were significant reductions in VLDL TAG concentrations were noted in rats and Nicolosi *et al.* (1997) who observed reductions in non HDL cholesterol concentrations in hamsters as a result of dietary supplementation with CLA. An in-vitro study by Moya-Camarena (1999a) has demonstrated that the *cis*-9, *trans*-11 isomer of CLA is a PPAR alpha agonist. PPAR alpha is important in hepatic lipid metabolism. The fibrate class of drugs mediate their action on lipid metabolism through PPAR alpha in a similar manner to CLA (Moya-Camarena *et al.* 1999a,b). The reductions in VLDL cholesterol concentrations in this current study suggest that *cis*-9, *trans*-11 isomer of CLA may be active in the down regulation of hepatic cholesterol synthesis. The *cis*-9, *trans*-11 comprised 80 % of total CLA supplemented in this group with *trans*-10, *cis*-12 comprising 20 % of total CLA. The PPAR mechanism of action of the *cis*-9, *trans*-11 isomer on cholesterol metabolism may possibly account for reductions in VLDL cholesterol concentrations associated with the 80:20 *cis*-9, *trans*-10 isomeric blend of CLA. A non-significant reduction of 24.5 % in VLDL TAG concentration was observed with the 80:20 *cis*-9, *trans*-10 isomeric blend of CLA, with similar non-significant reductions in VLDL TAG concentrations in the 50:50 *cis*-9, *trans*-10 CLA group. Houseknecht *et al.* (1998) demonstrated that CLA increased the activation of PPAR responsive genes which include LPL of the adipocyte, acyl Co A synthase and the fatty acid binding protein. These decreases in VLDL TAG concentrations in this current study may be accounted for by CLA in particular by the *trans*-10, *cis*-12 isomer of CLA increasing adipocyte LPL activity resulting in reductions in TAG concentrations. Yotsumoto *et al.* (1999) demonstrated in vitro, that the *trans*-10, *cis*-12 isomer suppresses hepatic lipid synthesis and apo B secretion. One or a combination of these in vitro findings may explain a mechanism whereby the isomeric blends of CLA reduce VLDL lipid concentrations in the current study.

6.4.3: Low Density Lipoprotein

LDL cholesterol concentrations were reduced non significantly by 8% in both CLA groups in this current study similar to the findings of Nicolosi *et al.* (1997) where non HDL cholesterol concentrations were reduced by between 9 % and 13 % in response to CLA supplementation administered at doses of between 0.06 % and 1.1 % of energy intake. These decreases in LDL cholesterol concentration may be attributed to the reductions observed in VLDL cholesterol concentrations as a result of CLA supplementation. VLDL is metabolised to LDL, because VLDL is a precursor for LDL, reductions in VLDL cholesterol concentration thought the action of PPAR may be ultimately responsible for the non-significant reductions in LDL cholesterol concentrations as a result of CLA supplementation.

6.4.4: HDL subfractions, CETP mass and activity

No significant changes in the lipid composition of the HDL subfractions was observed as a result dietary supplementation with isomeric blends of CLA or linoleic acid. Other investigators accessing the effects of CLA supplementation on coronary artery disease have also found that CLA supplementation had no effect on total HDL cholesterol concentrations (Lee *et al.* 1994; Gavino *et al.* 2000; Stangl *et al.* 2000; Nicolosi *et al.* 1997). Dietary supplementation with linoleic acid appeared to have the greatest effect on total HDL cholesterol and phospholipid composition in this current study, with non significant increases of 11 % and 8.6 % respectively in these lipid concentrations similar to the finding of Singer *et al.* (1990) who demonstrated that dietary supplementation using sunflower oil (high in linoleic acid) resulted in moderate increases in HDL cholesterol concentrations. CETP mass and activity was measured in this trial. No changes in CETP mass and activity were noted in either CLA or linoleic acid dietary supplementation groups over the course of the study, which is surprising considering that VLDL lipids, an important source of substrate for CETP activity was reduced by CLA supplementation. Postprandial CETP mass and activity was not measured in this study. It is possible that reductions VLDL TAG and cholesterol concentrations may effect postprandial CETP mass and activity.

6.4.5: LDL/HDL cholesterol ratios

LDL/HDL cholesterol ratios were reduced non-significantly by 2.4 % in the 50:50 *cis*-9, *trans*-10 CLA group and by 7.2 % in the 80:20 *cis*-9, *trans*-10 CLA group over 8 weeks of dietary supplementation. This is in agreement with the findings of Lee

et al. (1994) where a significant decrease in LDL/HDL cholesterol ratio was observed in rabbits fed an atherogenic diet supplemented with 0.5g/day CLA for 12 weeks. Dietary supplementation with linoleic acid for eight weeks in this current investigation resulted in an 11 % reduction in LDL/HDL cholesterol ratios. This is in agreement with the findings of Singer *et al.* (1990) where a calculated decrease of 17 % in LDL/HDL cholesterol ratios was noted in hyperlipidproteemia subjects as a result of dietary supplementation with sunflower oil which is high in linoleic acid. Nydahl *et al.* (1994), Dupont *et al.* (1990) demonstrated that linoleic acids didn't alter HDL cholesterol concentrations significantly but reduced LDL cholesterol concentrations which is in agreement with the findings of this study. The decreases in LDL/HDL cholesterol ratios of the 80:20 *cis*-9, *trans*-10 CLA and linoleic acid groups in the current study have important implication in CAD as decreases in LDL/HDL cholesterol ratios decreases CAD risk

6.4.6: Body weight

No change in body weight was observed in this study as a result of dietary supplementation with isomeric blends of CLA over 8 weeks. This is in contrast to the findings of Gumundsen *et al.* (1999) where reductions in the body weight of moderately obese and obese humans was observed in response to 3.6g/day CLA over 12 weeks. Nicolosi *et al.* (1997) found no changes in body weight in hamsters supplemented with CLA as part of a controlled diet up to 8 weeks. However by week 11, body weights were significantly decreased in hamsters consuming CLA by between 12-16 % when compared to control animals. Based on these results it is possible that longer dietary supplementation period with CLA than that which was used in this current study or a higher daily intake of CLA may affect body weight in humans. Stangl *et al.* (2000) demonstrated an increase in lean tissue to fat tissue in rats as a result of dietary supplementation with CLA. Gavino *et al.* (2000) demonstrated that the *trans*-10, *cis*-12 isomer of CLA appeared to be responsible for the body compositional change in rats. Body composition measurements were not carried out in this study, therefore it would be impossible to determine whether changes in body composition occurred in this study group.

6.4.7: Fatty acid composition of total plasma lipids

The mean concentration of *cis*-9, *trans*-11 isomer was 0.38 % of total plasma lipid fatty acid composition at week 0 was comparable with concentrations of this

isomer of CLA found in total plasma lipid in humans by Jiang *et al.* (1999). A significant increase ($P \leq 0.0001$) of 90 % and ($P < 0.001$) 87 % in the *cis*-9, *trans*-11 CLA isomer was observed in total plasma lipids of both the 50:50 *cis*-9, *trans*-10 and 80:20 *cis*-9, *trans*-10 CLA groups respectively as a result of dietary supplementation with these isomeric blends of CLA for 8 weeks. Similar increases in the incorporation of *cis*-9, *trans*-11 isomer of CLA into plasma phospholipids in humans was observed by Britton *et al.* (1992) as a result of increased intake of food products high in *cis*-9, *trans*-11 isomer of CLA for 3 weeks. Huang *et al.* (1994) demonstrated that CLA increased by 19-27 % as a result of increased intake of dairy products in humans. Stangl *et al.* (2000) demonstrated a 20 fold increased in serum as a result of intake of CLA at 1 % of total dietary intake for 6 weeks. The *trans*-10, *cis*-12 isomer was not detected in the week 0 total plasma lipids in this study, while it was only detected in some of the week 8 samples. It appears that the *trans*-10, *cis*-12 isomer of CLA is not incorporated efficiently into total plasma lipids. An explanation for this finding may be that the *trans*-10, *cis*-12 isomer is catabolised into C 20:4 5,8,12,14 and C 20:3 8,12,14 via desaturation and elongation pathways as has been demonstrated by Sebedios *et al.* (1997), and therefore cannot be detected by gas chromatography. We did not investigate whether there was an increase in the metabolites of the *trans*-10, *cis*-12 isomer in this study. Martin *et al.* (2000) hypothesise that the *trans*-10, *cis*-12 isomer of CLA is more easily oxidised due to its structure allowing it to bypass a number of rate limiting steps in the peroxisomal β oxidation pathway suggesting why there is difficulty in the detection of this isomer of CLA in plasma.

It was difficult to ascertain from the data on total plasma lipid composition which fatty acids were displaced by the incorporation of the *cis*-9, *trans*-11 isomer of CLA as this fatty acid composed less than 1 % of total fatty acids in total plasma lipid fatty acid composition. Significant reductions ($P < 0.02$) were noted in C18: 2 (n-3) composition of the 50/50 *cis*-9, *trans*-10 CLA group and C20:5 n-3 of the 80/20 *cis*-9, *trans*-10 CLA group respectively. However non-significant reductions were observed in other fatty acids of total plasma lipids in these supplementation groups. Stangl *et al.* (2000) noted slight decreases in C18:3 n-6, C18:2 n-6, C20:3 n-6 and C20:4 n-6 in a swine model consuming CLA suggesting these decreases were attributed to incorporation of CLA fatty acids. No significant changes in the fatty acid composition of total plasma lipids was observed in the linoleic acid group as a result of 8 weeks dietary supplementation.

The incorporation of *cis*-9, *trans*-11 isomer of CLA into plasma phospholipids was also measured (data not presented). There was almost a doubling of the presence of this isomer in plasma phospholipids in the CLA groupings. However this increase was not significant due to the very low concentrations of this isomer of CLA present in the phospholipid fraction and high variability in concentrations of this isomer between the subjects.

6.4.8: Conclusions

The findings of this study indicate that CLA supplementation in humans has beneficial effect in terms of coronary artery disease risk factors with the reduction in plasma TAG concentrations being accounted for by reduction in VLDL TAG concentrations. The decrease in plasma TAG associated with intake of the 50:50 *cis*-9, *trans*-10 isomer blend of CLA equates to a reduction of 12.5 % in CAD risk according to the findings of Stampfer *et al.* (1996). Significant reductions in VLDL cholesterol concentrations were observed as a result of supplementation with the 80:20 *cis*-9, *trans*-10 isomeric blend of CLA. HDL cholesterol concentrations are unaffected by CLA supplementation, while LDL cholesterol concentrations are reduced. The findings of this study indicate a clinically positive effect of CLA on CAD risk by conserving the cardioprotective HDL cholesterol concentrations and reducing the potentially atherogenic VLDL and LDL cholesterol concentrations. Modifications in lipid metabolism are not accounted for by alterations in CETP mass or activity, suggesting that effects of CLA on lipid metabolism may be mediated through a molecular mechanism.

Chapter 7

General Discussion

Coronary artery disease is characterised by atherogenesis, with high LDL cholesterol or so called “bad cholesterol” and low HDL cholesterol or the “good cholesterol” concentrations being significant risk factors for the development of CAD. The distribution of cholesterol within lipoprotein fractions is determined by CETP activity. Therefore an understanding of the mechanisms involved in neutral lipid transfer is important to understand how and whether dietary and drug therapies are effective in reducing CAD disease risk factors.

The studies outlined in this thesis were designed to establish whether CETP contributed to either a pro or anti-atherogenic lipoprotein profile in a normolipidaemic population. Strong associations between genotype of CETP mass and activity indicate that in a normolipidaemic population there is a specific genotype for CETP which are at reduced risk of CAD when compared to the majority of the population. The reduction in risk of CAD is attributed to reduced CETP mass and activity in this group. Plasma cholesterol concentrations also impact on CETP metabolism with elevated cholesterol resulting in elevated CETP concentrations and increased risk factors for the development of CAD. CETP mass and activity increases during postprandial lipaemia. Data from chapter 4 of this thesis suggests that individuals with increased plasma cholesterol concentrations have increased postprandial CETP activity when compared to individuals with lower plasma cholesterol grouping considering both groups consumed the same test meal. These findings also revealed that subjects with moderately elevated plasma cholesterol have a more atherogenic lipoprotein profile than subjects with normal plasma cholesterol concentrations. This difference in lipoprotein profile between these groups may be attributed to the increased redistribution of CE into apo B lipoproteins as a result of exaggerated increase in CETP activity in subjects with moderately elevated plasma cholesterol particularly during postprandial lipaemia. Therefore postprandial CETP activity in particular may be a good indicator for the development of CAD risk factors.

Dietary supplementation using low dose fish oil and a novel fatty acid known as conjugated linoleic acid over long term exhibit beneficial effects in terms of CAD risk in humans. Chapter 5 and 6 demonstrate that long term low dose fish oil intake and dietary supplementation using isomeric blends of CLA was beneficial in terms of reducing CAD risk factors in a normolipidaemic population. Results from the low dose fish oil study indicated that small amounts of fish oil may be incorporated into foods, allowing greater intake of n-3 PUFA in the general population in the form of functional

foods. Similarly, dairy products could be fortified with CLA if it is demonstrated that low dose CLA supplementation is effective in reducing CAD risk factors in humans. Now that it has been established that CLA has beneficial effects on lipoprotein metabolism, it is imperative that a dose response study be carried out to determine the minimum and most effective dose of CLA which is beneficial in terms of CAD prevention

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