A Study of Omega-3 Fatty Acid Therapy in Patients with Nephrotic Syndrome

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Abstract

Patients with nephrotic range proteinuria have a higher risk of cardiovascular disease through qualitative and quantative changes in lipids and lipoproteins. The aim of this study was to examine the effect of omega-3 fatty acids derived from fish oil in this population.

Treatment with omega-3 fatty acids in these patients was well tolerated and had a number of beneficial effects. They reduced small dense LDL concentration, remnant lipoproteins, VLDL and triglyceride levels. Postprandial lipaemia was improved with an improvement in chylomicron clearance. However, we found that treatment increased LDL-C and although there was a redistribution to protective HDL2 rather than HDL3, HDL-C was not significantly increased. Furthermore, there was no improvement in endothelial function or inflammatory markers.

Thus, we do not recommend treatment with omega-3 fatty acids alone for dyslipidaemia in this population of patients. The combination of omega-3 fatty acids with a statin merits further work.

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Abbreviations

ABCA1	adenosine triphosphate- binding cassette protein A1
ACE	Angiotensin Converting Enzyme
ACh	Acetylcholine
AFCAPS/ TEXCAPS	Air Force/ Texas Coronary Atherosclerosis Prevention Study
ALP	Atherogenic Lipoprotein Phenotype
Apo	Apolipoprotein
BP	Blood Pressure
CAD	Coronary Artery Disease
CARE	Cholesterol and Recurrent Events Study
CETP	Cholesteryl Ester Transfer Protein
CHD	Coronary Heart Disease
CrCl	Creatinine Clearance
CV	Cardiovascular
d	Density
Da	Daltons
DART	Diet and Reinfarction Trial
DBP	Diastolic Blood Pressure
DHA	Docosahexenoic acid
eGFR	Estimated Glomerular Filtration Rate
EPA	Eicosapentenoic acid

FCHL	Familial Combined Hyperlipidaemia
HDL	High Density Lipoprotein
HL	Hepatic Lipase
HOPE	Heart Outcomes Evaluation Trial
HMG CoA	3-hydroxy, 3-methyl glutaryl coenzyme A
hs-CRP	Highly sensitive C- Reactive Protein
I-CAM	Intercellular Adhesion Molecule-1
IDL	Intermediate Density Lipoprotein
IQR	Inter- quartile range
IMT	Intima- media Thickness
LCAT	Lecithin Cholesterol Acyltransferase
LDI	Laser Doppler Imaging
LDL	Low density Lipoprotein
LNA	Alpha Linolenic Acid
LPL	Lipoprotein Lipase
MDRD	Modification of Diet in Renal Disease
MI	Myocardial Infarction
MRFIT	Multiple Risk Factor Intervention Trial
NaBr	Sodium Bromide
NaCl	Sodium Chloride
NO	Nitric Oxide
NMR	Nuclear Magnetic Resonance
PLAC-1	Pravastatin Limitation of Atherosclerosis in Coronary Arteries Trial

RLP-C	Remnant-like lipoprotein - Cholesterol
RLP-TG	Remnant-like Lipoprotein - Triglyceride
SCIMO	Study on Prevention of Coronary Atherosclerosis by Intervention with Marine Omega-3 fatty acids
SD	Standard Deviation
Sf	Svedberg Flotation Units
SNP	Sodium Nitroprusside
SRB1	Scavenger Receptor Class B1
TNT	Treating to New Targets
VA-HIT	Veterans Affairs High Density Lipoprotein Cholesterol Intervention Trial
VLDL	Very Low Density Lipoprotein
vWF	Von Willebrand Factor
WOSCOPS	West of Scotland Coronary Prevention Study

Summary

Nephrotic range proteinuria is associated with increased risk from CHD. This is thought to be in part through qualitative and quantative change in lipids and lipoproteins. Among the lipid and lipoprotein abnormalities possessed by these patients, increased concentration of triglyceride rich lipoproteins, lipoprotein remnants and excess small dense LDL contribute significantly to this risk. Furthermore, postprandial lipaemia plays an important role in the development of atherosclerosis as triglyceride rich lipoproteins are mainly produced in the postprandial state. This suggests that abnormalities of postprandial lipoprotein metabolism may contribute to the increase in triglyceride rich lipoproteins observed in this population. Low levels of HDL are an important risk factor for CVD independent of other factors and increasing these levels have been shown to reduce this risk. Experimental and clinical data has shown that endothelial dysfunction and inflammation is important early in the development of atherosclerosis. Both epidemiological data and randomised controlled trials have shown that increased intake of omega-3 fatty acids is associated with reduced CV disease. Omega-3 fatty acids act through a number of mechanisms. Their main effect on lipoprotein metabolism results in a reduction in triglyceride levels. They also have anti-thrombotic, anti-inflammatory and anti-arrhythmic properties contributing to their beneficial effect on CV disease.

The main hypothesis of this research was that omega-3 fatty acids have the ability to correct the dyslipidaemia of proteinuric renal disease. First, we aimed to examine whether omega-3 fatty acids provided adequate triglyceride

lowering effects and consequent benefits on LDL phenotype and HDL concentration. Next, we aimed to examine whether omega-3 fatty acids improved postprandial lipaemia in this population and finally we aimed to assess their effect on endothelial function.

We recruited 19 patients with primary glomerular disease and nephrotic range proteinuria and compared them with normal controls. Patients and controls were treated with 4g daily of omega-3 fatty acids for a period of 8 weeks. Treatment was generally well tolerated with only one patient withdrawing due to adverse gastrointestinal side effects. An additional patient withdrew prior to commencing the study, therefore we analysed 17 patients.

At baseline, patients with nephrotic range proteinuria had increased concentration of total cholesterol, plasma triglyceride, VLDL, remnant lipoproteins and small dense LDL (LDL III). Omega-3 fatty acids reduced triglyceride rich lipoproteins, remnant lipoproteins and LDL III in the patients with nephrotic range proteinuria but also increased LDL-C. However, the increase in LDL-C was also associated with a redistribution to larger, lighter and potentially less atherogenic LDL.

Postprandial lipaemic response was assessed following a fat load both pre and post treatment with omega-3 fatty acids. Postprandial chylomicron concentration was greater in the patients with nephrotic range proteinuria compared with the controls at baseline but postprandial $VLDL_1$ concentration did not differ between the two groups. Treatment with omega-3 fatty acids reduced postprandial chylomicron in the patient group but not the controls. Postprandial $VLDL_1$ was not affected by treatment in patients or controls,

however peak postprandial $VLDL_1$ concentration was significantly lowered in all subjects.

On examining the structure and composition of these chylomicrons, we found that patients with nephrotic range proteinuria had significantly higher levels of apolipoprotein CII, CIII and E per particle with greater quantities of triglyceride, total cholesterol, free cholesterol and phospholipid indicating that these particles are bigger. We did not demonstrate any difference in the structure or composition of $VLDL_1$ particles between patients and controls. However, despite the reduction in postprandial chylomicron concentration observed post treatment; omega-3 fatty acids did not influence the structure or composition of either chylomicrons or $VLDL_1$ in patients or controls. This suggests that any improvement in postprandial dyslipidaemia results from reduced chylomicron synthesis rather than any change in their structure.

HDL subfractions were measured in both patients and controls at baseline and following treatment with omega-3 fatty acids. There was no difference in the concentration of these subfractions in patients compared with controls. Treatment had a beneficial effect on the HDL subfractions with an increase in protective HDL_2 and a reduction in HDL_3 concentration in spite of any change in HDL-C.

Finally, we measured microvascular endothelial function using the technique of laser doppler imaging and also by analysing markers of endothelial function and inflammation in the patients compared with the controls. We were unable to demonstrate a difference in endothelial function but plasma PLA_2 were increased in the patients at baseline. Omega-3 fatty acids did not affect endothelial function or markers of inflammation in patients or controls.

In conclusion, in this research we have shown that omega-3 fatty acids have a number of beneficial effects on lipids and lipoproteins in patients with nephrotic range proteinuria. However, any potential benefit that may result has to be balanced against the increase in LDL-C and so monotherapy with omega-3 fatty acids in this population may not be advisable. We suggest that the combination of omega-3 fatty acids and HMG Co A reductase inhibitor merits further investigation to ascertain whether this is the optimum therapeutic option to achieve the dual goal of reducing triglyceride rich atherogenic lipoproteins as well as LDL-C in this high-risk patient population.

Chapter 1: Introduction

1.1 Cardiovascular Risk and Proteinuria

The presence of excess urinary protein is associated with increases in both cardiovascular disease (CVD) and coronary heart disease (CHD)(1). An increasing number of studies have suggested that microalbuminuria is a risk factor for CVD. This risk has been shown to increase as the level of proteinuria increases. In the 'HOPE' (Heart Outcomes Evaluation Trial),which examined 3577 diabetics aged 55 or over with either one previous cardiovascular event or risk factor, microalbuminuria was associated with increased risk of the combined end-point of myocardial infarction, stroke or cardiovascular (CV) death in patients with and without diabetes(2). This risk increased as the level of proteinuria increased. In studies examining the general population, this risk is evident even at low levels of albuminuria, independent of other CV risk factors(3;4). In a population based study examining over 40,000 subjects, increased urinary albumin excretion was associated with increased CV mortality independent of other risk factors(3). Klausen et al found a 2 fold increase in the relative risk of CHD and a 1.9 fold increase in relative risk of death in subjects with low levels of microalbuminuria independent of age, sex, creatinine clearance (CrCl), diabetes mellitus, hypertension and plasma lipids(4). This risk is higher in patients with persistently positive dipstick proteinuria as found in the MRFIT study(5). This showed that patients with 1+ of persistently positive dipstick proteinuria was associated with a 2 fold relative risk of death from CHD, increasing to 2.5 fold with 2+ proteinuria(5). Furthermore, Miettinen et al demonstrated that clinical proteinuria (>300mg/l) was associated with a 5 fold

increase in the incidence of stroke in non-diabetics(6). This risk increases further in patients with nephrotic range proteinuria. Ordonez et al showed that patients with nephrotic range proteinuria (>3g/24hrs) possessed a 3 fold increase in the risk of CHD death and 5.5 fold increase of myocardial infarction independent of hypertension and smoking (7). There are, however, no prospective intervention trials examining risk reduction in patients with nephrotic range proteinuria.

The precise mechanism behind the association of proteinuria with CV risk is unknown. Glomerular capillary leaking of albumin may be a manifestation of a widespread vascular process caused by atherosclerosis (8;9). A link between microalbuminuria and endothelial dysfunction has been observed (10). Non-diabetic hypertensive patients with microalbuminuria have been shown to have higher levels of von Willebrand factor (vWF) antigen than those without microalbuminuria(11). vWF is a glycoprotein which is secreted in larger amounts when there is damage to the vascular endothelium, therefore microalbuminuria may reflect damage to the systemic vascular endothelium.

It is not clear whether proteinuria leads to increased CV risk or whether proteinuria is a marker of increased risk. However, nephrotic range proteinuria is associated with a number of qualitative and quantative changes in lipids and lipoproteins (described later), which contribute to the increased CV risk.

1.2 Normal Lipoprotein Metabolism

1.2.1 Lipids

Lipids play an important role in metabolism as they are both a source of energy, are essential for the structure of cell membranes and precursors to essential vitamins and hormones. Cholesterol and phospholipid are required for the formation of the cell membrane. Cholesterol also acts as a precursor for vitamin D, bile acids and steroid hormones. Triglycerides provide a high energy source. These lipids are hydrophobic neutral particles and cannot be transported in the circulation in their free form. As a result, they are bound to proteins and other lipids to form amphipathic lipoprotein particles.

1.2.2 Lipoproteins

Lipoproteins are globular particles with differing lipid and protein content. They can be classified by a number of different methods; size, density, flotation constant and electrophoretic mobility. The most widely used method of classification is when lipoproteins are classified by density ultracentrifugation (12).This divides lipoproteins into 5 main classes: chylomicrons, very-low density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL) and high density lipoprotein (HDL). The properties of these are outlined in table 1.1.

Lipoproteins may also be classified according to their protein component (apolipoprotein). Each lipoprotein contains one or more apolipoproteins. Individual apolipoproteins control lipoprotein metabolism in a variety of ways such as by binding to specific cell membrane receptors or by acting as co-

factors for enzymes involved in lipoprotein metabolism. They are also essential for the structural integrity of lipoprotein particles. These properties are outlined in table 1.2.

Chylomicrons are the largest lipoprotein and are also the lightest. They are synthesised in the intestine from dietary fat. The main apolipoprotein is apo B-48 but acquires apo CII and E. Chylomicrons have a high triglyceride content. VLDL are the next step down in terms of density and size. They contain 5 – 12% protein, 50-55% triglyceride, 18 –20% phospholipid, 12 –15% cholesteryl ester and 8 –10% free cholesterol. They differ from chylomicrons in that they are synthesised in hepatocytes and their main function is the transport of endogenously synthesised triglyceride. Furthermore, in contrast to chylomicrons, the main apolipoprotein is B-100. They also contain apo CII, apo CIII and apo E. VLDL can be separated into 2 subfractions by cumulative- gradient ultracentrifugation according to their flotation coefficients. $VLDL_1$ (Sf 60-400) is larger, lighter and rich in triglyceride while $VLDL_2$ (Sf 20-60) is smaller, denser and more enriched in cholesteryl ester. $VLDL_1$ and $VLDL_2$ production is regulated independently. $VLDL_1$ is associated with raised triglycerides (13). It is metabolised slowly and is integral to the formation of small dense LDL, whereas $VLDL_2$ is metabolised more rapidly to IDL and LDL.

IDL is smaller than VLDL and more dense. It is composed of 10-12% protein, 24- 30% triglyceride, 25-27% phospholipid, 32 –35% cholesteryl ester and 8-10% free cholesterol. IDL is primarily derived from triglyceride hydrolysis of VLDL. IDL is taken up by the liver or undergoes further triglyceride hydrolysis

to become LDL. IDL contains both apo-B100 and apo E. Increased plasma IDL concentration has been associated with atherogenesis(14).

LDL is smaller than IDL and more dense. LDL contains 20-22% protein, 10-15% triglyceride, 20-28% phospholipid, 37-48% cholesteryl ester and 8-10% free cholesterol. LDL is the main transporter of cholesterol and cholesteryl esters. The main apolipoprotein of LDL is B-100 accounting for approximately 25% of the particle mass. Excess LDL is associated with atherogenesis. LDL, itself, can be further subdivided by a number of different methods including size, density and electrophoretic mobility. This will be discussed further later. HDL is the smallest of the lipoproteins and the most dense. HDL contains approximately 55% protein, 3-15% triglyceride, 15-30% cholesteryl ester, 24-46% phospholipid, 15-30% cholesteryl ester and 2-10% free cholesterol. The main function of HDL is in reverse cholesterol transport as it takes cholesterol from peripheral tissues to the liver. The main apolipoprotein constituents of HDL are AI, and II and also contain apolipoproteins CII, CIII and Apo E. HDL may be separated into 2 subclasses, HDL_2 and HDL_3. HDL_2 is larger and richer in lipids and HDL_3 is smaller and denser. There is an important inverse relationship between HDL and coronary artery disease risk, which will also be discussed later.

Table 1.1: Properties of Plasma Lipoproteins

Lipoprotein	Density (kg/l)	Particle Size (nm)	Flotation Coefficient (Sf)	Molecular Weight
Chylomicron	<0.94	75-1200	>400	50-1000 x 10^6
VLDL	0.94-1.006	30-80	20-400	10-80 x 10^6
IDL	1.006 –1.019	25-35	12-20	5-10 x 10^6
LDL	1.019-1.063	18-25	0-12	2-3 x 10^6
HDL	1.063-1.21	5-12	0-9*	65-386 x 10^3

*at solvent density 1.20kg/l

Adapted with permission (15)

Table 1.2: Plasma Apolipoproteins

Apolipoprotein	Particle	Main Function	Molecular Weight
A-I	HDL	Accepts cholesterol Ligand for HDL binding LCAT activator	28,000
AII	HDL	Ligand for HDL LCAT cofactor	17,500
A-IV	HDL	Ligand for HDL LCAT cofactor	46,000
B-48	Chylomicrons	Forms structure	264,000
B-100	VLDL, IDL, LDL	Forms structure Ligand for LDL receptor	512,000
CII	Chylomicrons, VLDL	Activates LPL	9000
CIII	Chylomicrons, VLDL	LPL inhibitor	9000
E	Chylomicrons, VLDL, IDL, Lipoprotein Remnants	Ligand for IDL receptor, LDL-RP & VLDL Receptor	34,000

LCAT=Lecithin cholesterol acyltransferase, LPL= Lipoprotein lipase, LDL-RP= LDL receptor- related protein

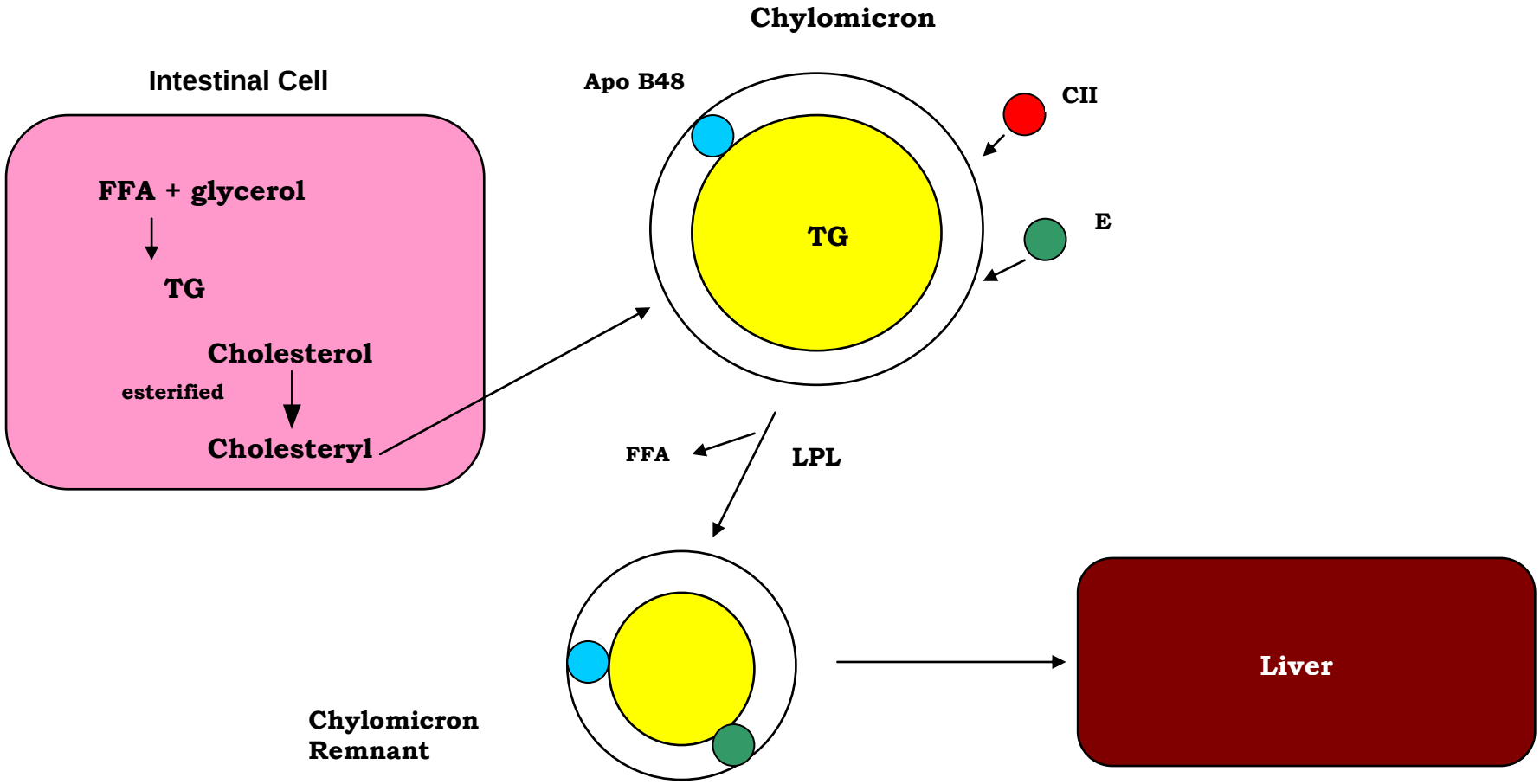

Figure 1.1 Exogenous Lipoprotein Metabolism

Dietary cholesterol and fatty acids are absorbed in the intestine where triglyceride is synthesised from free fatty acids with glycerol and cholesterol is esterified to form cholesteryl esters. These are assembled into chylomicrons. Apo CII and E are acquired as the chylomicrons enter the circulation. LPL hydrolyses the core triglyceride making the chylomicrons progressively smaller and releasing free fatty acids which are used as an energy source, converted to triglyceride or stored in adipose tissue. The end products are chylomicron remnants, which are cleared from the circulation by hepatic chylomicron remnant receptor.
(FFA = Free fatty acids, TG = Triglyceride, LPL= Lipoprotein Lipase)

1.2.3 Exogenous Lipid Metabolism

Lipoprotein metabolism can be considered as either exogenous or endogenous pathways. The exogenous pathway involves transport of dietary fat from the intestine (mainly in the form of triglyceride) as chylomicrons to the liver (fig 1.1). This pathway begins with the intestinal absorption of dietary cholesterol and fatty acids. Triglyceride is synthesised from the combination of free fatty acids with glycerol and absorbed cholesterol is esterified to form cholesteryl esters within the small intestine. These are assembled to form chylomicrons. The main apolipoprotein is B-48 but CII and E are acquired as the chylomicrons enter the circulation. Apo-B48 permits lipid binding to the chylomicrons but does not bind to the LDL receptor thereby preventing premature clearance of chylomicrons from the circulation. Apo C II is a cofactor for lipoprotein lipase (LPL), which hydrolyses the core triglyceride thus making the chylomicrons progressively smaller and at the same time releases free fatty acids. These free fatty acids are used as an energy source, converted to triglyceride or stored in adipose tissue. The end product of chylomicron metabolism is chylomicron remnants, which are cleared from the circulation by hepatic chylomicron remnant receptor, for which apo E is a ligand. Surface components are transferred from the chylomicron remnants to HDL.

1.2.4 Endogenous Lipid Metabolism

The endogenous pathway involves transport of lipids, which are synthesised in hepatocytes to peripheral tissues. This pathway begins with the synthesis of VLDL particles in the liver, which are released as nascent VLDL particles

containing apo A-I, and B-100. Apolipoproteins CII, CIII and apoE are picked up from circulating HDL. As VLDL passes through the circulation, its structure is altered. Triglyceride is removed through the action of LPL (activated by apo C-II) generating either VLDL remnants or IDL. Surface components including the C and E apolipoproteins (originally donated from HDL) are transferred to HDL. In addition to this hydrolysis, a large amount of exchange takes place between VLDL and HDL. Cholesteryl ester is transferred from HDL to VLDL in an exchange reaction that concomitantly transfers triglyceride from VLDL to HDL. This is mediated through cholesteryl ester transfer protein (CETP). Therefore, VLDL particles lose triglyceride via lipolysis and become cholesteryl ester enriched via the action of CETP thereby forming IDL. Further hydrolysis of IDL is mediated via hepatic lipase allowing further depletion of triglyceride and loss of apoE, which in turn leads to the formation of a smaller particle LDL.

1.2.5 HDL Metabolism

The protective effect of HDL is largely through reverse cholesterol transport, which removes excess cholesterol from cells and atherosclerotic plaques. Small nascent HDL particles are synthesised in the liver and intestinal cells. These particles acquire free cholesterol from peripheral tissue via the adenosine triphosphate- binding cassette protein A1 receptor (ABCA1). Free cholesterol is esterified by lecithin cholesterol acyltransferase (LCAT) forming cholesteryl ester, which moves to the particle core and results in larger, mature HDL particles. These particles transport cholesterol to the liver by interaction with the scavenger receptor class B1 receptor (SRB1) which frees

them to recirculate and participate in another cycle of reverse transport. The major apolipoprotein on HDL is A-1. Apo A-1 is involved at many points throughout this pathway namely interaction with the ABCA1 receptor, activation of LCAT and recognition of the SRB1 receptor. This pathway allows the removal of cholesterol from peripheral sites such as arterial vessel walls. Both the HDL mediated reverse cholesterol transport pathway in combination with the endogenous lipoprotein pathway involving the apo B containing lipoproteins (VLDL, IDL and HDL) allow exchange of cholesterol and triglyceride mediated by CETP. Thus, cholesterol within HDL is exchanged with triglyceride within Apo B containing particles enabling transport to the liver and removal from the circulation by the LDL receptor(16).

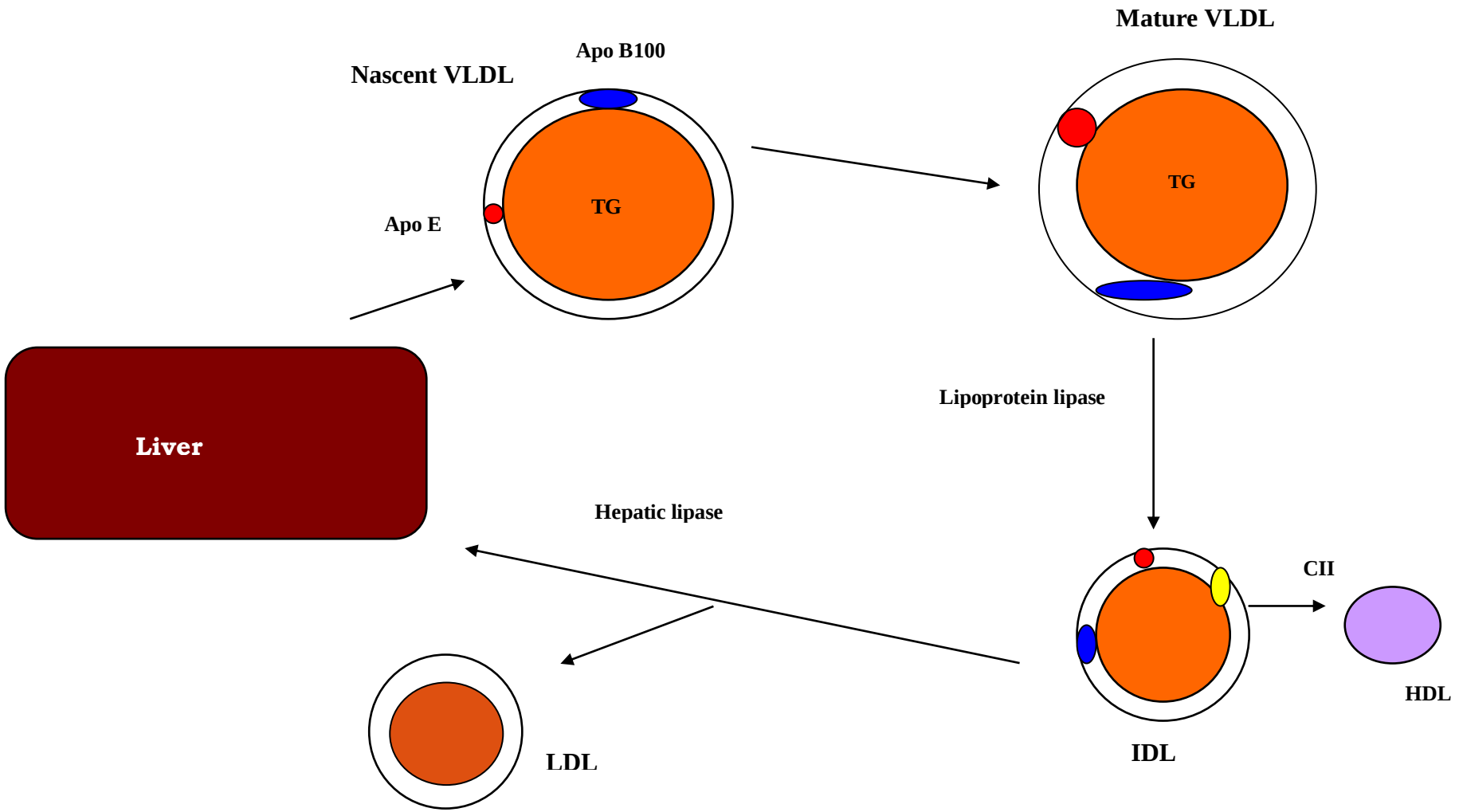

Figure 1.2 Endogenous Lipoprotein Metabolism

VLDL is synthesised in the liver and released as nascent VLDL particles containing apo A-I, and B-100. Apolipoproteins CII, CIII and apoE are picked up from circulating HDL. As VLDL passes through the circulation, triglyceride is removed through the action of LPL (activated by apo C-II) generating either VLDL remnants or IDL. Further hydrolysis of IDL is mediated via hepatic lipase allowing further depletion of triglyceride and loss of apoE, which in turn leads to the formation of a smaller particle LDL.
(TG= Triglyceride)

1.3 Dyslipidaemia and Atherosclerosis

1.3.1 Cholesterol and LDL-C

The association between hypercholesterolaemia and CVD was initially based on epidemiological data. Studies have demonstrated a continuous graded correlation between cholesterol and risk of CHD (17;18). The impact of cholesterol lowering in both primary prevention and secondary prevention of CVD is now well established.

The West of Scotland Coronary Prevention Study (WOSCOPS) is one of the most important studies examining the use of statins in primary prevention. Treatment with pravastatin significantly reduced cholesterol and LDL-C levels as well as CV end-points(19). A subsequent meta-analysis of primary prevention trials using statins also shows reductions in cholesterol and CVD mortality(20).

There have been many trials examining the effect of cholesterol lowering in secondary prevention. A number of studies have looked at the effect of cholesterol lowering on the regression of atherosclerosis. These studies evaluated regression either through coronary angiography (21-23) or ultrasonic techniques (24;25). Furthermore, there have been several large studies assessing clinical outcome with lipid lowering. The Scandinavian Simvastatin Survival Study (4S) found a 25% reduction in cholesterol and 35% reduction in LDL-C with a significant reduction in mortality in over 4000 patients treated with simvastatin(26). More recently, in the 'Treating to New Targets' (TNT) study, LDL-C levels were significantly lower (2.0 mmol/l with 80mg of atorvastatin vs 2.6 mmol/l with 10mg). Fewer patients reached the

primary end-point which was a major CV event in the high dose arm (27). The benefit of lipid lowering in patients in the absence of substantially elevated lipid levels has been observed. The Cholesterol and Recurrent Events (CARE) study included patients with a mean total cholesterol of 5.4mmol/l, and LDL-C of 3.6mmol/l. Treatment with pravastatin significantly reduced the combined end-point of coronary death and non fatal myocardial infarction (28).

1.3.2 HDL

Low levels of plasma HDL-C are a significant independent predictor of increased risk of CHD. This has been demonstrated in several observational studies. The Framingham Heart study showed that, in the normal population, the risk for myocardial infarction increases by about 25% for every 0.13mmol/l reduction in HDL-C (29). This is also evident in patients already diagnosed with CHD. An analysis of the LIPID and CARE studies encompassing over 13,000 patients, revealed that low HDL-C was a significantly stronger predictor of CHD in patients with LDL-C less than 125mg/dl (3.2mmol/l) compared with those with LDL-C greater than or equal to 125mg/dl (30). This cardioprotective effect is due to, in part, its role in reverse cholesterol transport. Furthermore, HDL is thought have a number of other protective effects. These include anti-inflammatory, anti-oxidant and pro-fibrinolytic effects (31).

There are several studies examining the effect of raising HDL. The Veterans Affairs High Density Lipoprotein Cholesterol Intervention Trial (VA-HIT) compared treatment with gemfibrozil to placebo in over 2000 patients with

CHD. There was 6% increase in mean HDL concentration and significant reductions in nonfatal MI and CHD death in the gemfibrozil arm. This reduction in nonfatal MI and CHD deaths correlated with HDL and was independent of changes in LDL-C or triglyceride concentrations (32). The HDL Atherosclerosis Treatment Study examined a combination of simvastatin and niacin therapy. This combination reduced LDL-C by 42%, increased HDL-C by 26%, induced regression of angiographic atherosclerosis and reduced the likelihood of sustaining a CV event. The magnitude of reduction in CV events and increase in HDL in this study was much greater than in studies which used statins alone, suggesting that the additional effect of raising HDL provides further benefits (33).

1.3.3 Remnant Lipoproteins

Remnant lipoproteins are formed from chylomicrons or VLDL particles, which have been partially metabolised by LPL. These particles are heterogeneous in size and composition and determined by plasma triglyceride concentration. Elevated remnant lipoproteins are associated with atherosclerosis. Patients with established coronary artery disease have been found to have increased levels of remnant lipoproteins(34;35). Karpe et al found that carotid artery intima-media thickness was positively related to baseline plasma remnant-like protein cholesterol (RLP-C) independently of plasma triglyceride and LDL levels (36). Two further Japanese studies have shown that elevated RLP-C levels were predictive of future coronary events in patients with CHD independent of other risk factors (37;38).

1.3.4 Oxidized LDL

It is has been suggested that there is a link between lipids and inflammation in the development of atheroma. LDL, which is present in the lipid fraction of atheroma within the intima, undergoes oxidative modification. These modified lipids have a number of pro-atherogenic properties. They induce expression of adhesion molecules, chemokines, proinflammatory cytokines and other inflammatory mediators. They are also immunogenic and so capable of inciting T cell responses, which in turn can incite local responses within the plaque.

The association of oxidized LDL with CHD has been observed in a number of studies. Elevated plasma levels of oxidized LDL has been observed in patients with stable CHD compared with healthy controls (39). Ehara et al demonstrated that oxidized LDL levels correlated with the severity of acute coronary syndromes (40). In addition, Toshima et al found concentrations of oxidized LDL in patients with angiographically proven CHD to be 1.9 times higher than controls (41).

1.4 Postprandial Lipaemia

Postprandial lipaemia was first suggested to be linked with atherosclerosis in 1979 by Zilversmit (42). Triglyceride rich lipoproteins (TRLs) are mainly produced postprandially (43;44). The triglyceride rich particles (chylomicrons and VLDL) are metabolised by LPL into glycerol and free fatty acids as described earlier. LPL activity is the rate-limiting step in the postprandial phase; therefore competition for this enzyme leads to accumulation of VLDL, as chylomicrons are the preferred substrate for LPL. This competition is more

likely to occur in the presence of fasting hypertriglyceridaemia such as in type II diabetes. Strong positive correlations between fasting triglyceride and postprandial triglyceride concentrations have been observed (45). However, subjects with normal fasting levels of triglyceride have also been found to have impaired lipoprotein clearance (46).

Clinical studies have shown that peak and late postprandial triglyceride concentrations as well as retinyl palmitate (a marker of intestinally derived TRLs) are associated with CHD (47-52). Simpson et al observed a prolonged and exaggerated period of hypertriglyceridaemia following a fat load and increased levels of retinyl palmitate in patients with severe CHD (51). Patients with CAD have also displayed a delay in the normalisation of triglyceride concentration and delayed clearance of retinyl esters (48). Furthermore, in a study by Patsch et al, both the maximal triglyceride increase and the magnitude of the postprandial triglyceride response following a fat load were higher in patients with CHD (50). Increased levels of chylomicron remnants have been shown to be related to the rate of progression of coronary lesion in post myocardial infarction males (53). Carotid intima-media thickness (IMT) is used as a surrogate marker for atherosclerosis. In one study of healthy males, following a fat load, the subsequent triglyceride response was related to IMT independent of other clinical risk factors (54). In summary, it is clear that there is a well-established relationship between postprandial lipaemia and atherosclerosis. Currently, the assessment of postprandial lipaemia is through the administration of a standard fat load followed by regular venesection for eight hours. However, this means of assessment cannot be applied to large numbers of patients in a clinical setting and so a more practical method for the

assessment of postprandial lipaemia would need to be developed before this can be applied as a useful clinical tool.

1.5 The Atherogenic Lipoprotein Phenotype

The Atherogenic lipoprotein phenotype (ALP) is a term used to describe a collection of proatherogenic lipoprotein abnormalities. It was defined by Austin as hypertriglyceridaemia, low HDL-C levels and a predominance of small dense LDL (55).

LDL was first subdivided using gradient gel electrophoresis (56) into a discrete number of subfraction with differing size and density. Subjects with a preponderance of large LDL were labelled as having 'Pattern A' and those with small LDL 'Pattern B'(57) and ALP. 'Pattern B' has been shown to be associated with a three-fold increased risk of myocardial infarction (58). An alternative method of analysis that allows quantification of each LDL subfraction is through density gradient ultracentrifugation. This divides LDL into three main subfractions (LDL I d=1.025-1.034g/ml, LDL II d=1.034-1.044 and LDL III d=1.045-1.060)(59). LDL I is large and predominates in premenopausal women with LDL II being the most common subfraction in males. LDL III is the smallest and most dense subfraction and has been linked with CHD in number of studies(58;60-64) (Fig 1.3).It is thought that the generation of small dense LDL is as a result of defective clearance or overproduction of VLDL (65). Chylomicron metabolism is also affected with increased production of chylomicron and chylomicron remnants and HDL falls with a shift towards smaller, denser HDL_3. ALP has been observed to be

common in a number of conditions: diabetes (66), metabolic syndrome (67), familial combined hyperlipidaemia (68) and patients with nephrotic range proteinuria (69).

Small dense LDL particles have been associated with CHD in a number of studies including cross-sectional, prospective, epidemiological and intervention studies (70). Moreover, the National Cholesterol Education Program Adult Panel III has accepted small dense LDL as a cardiovascular risk factor (71). Whilst, most of the studies have shown that small LDL size is a predictor of CHD on univariate analysis only, several studies found LDL size to be an independent predictor on multivariate analysis. The Standard Five-City project found that patients with myocardial infarctions had smaller LDL in a nested case control study independent of other factors(60). Griffin et al observed that LDL III concentration of > 100 mg/dl conferred a 4.5 fold increase in risk of CHD and 6.9 fold increase risk of myocardial infarction (61). The Quebec Cardiovascular Study examined 2072 men over a period of 13 years. They observed a strong association between small dense LDL and risk of ischaemic heart disease, (72) again independent of other factors. The Pravastatin Limitation of Atherosclerosis in Coronary Arteries Trial (PLAC-1) used the technique of Nuclear Magnetic Resonance (NMR) to examine LDL particle size in patients with cardiovascular disease. They revealed that small LDL particles and not large LDL particles predicted angiographically measured disease progression independent of other lipid levels (73). Liu et al investigated the association between LDL particle size and carotid artery intima thickness (IMT) as a measure of atherosclerosis in asymptomatic family members with familial combined hyperlipidaemia (FCHL). They found that on

multivariate analysis, smaller particle size was associated with increased IMT(74). A further study looking at patients with type II diabetes showed that LDL size was the strongest marker of CHD in a multivariate analysis(75). Thus, the association between small dense LDL and CHD is well established.

Many theories have been suggested as to why small dense LDL is so atherogenic. It has been suggested that small dense LDL is more easily taken up by arterial tissue than larger LDL thereby leading to greater accumulation in atherosclerotic plaques (76). Another study suggested that the increased atherogenicity of small dense LDL was due to decreased hepatic clearance by the LDL receptor and enhanced binding to LDL receptor independent sites in extra-hepatic tissues such as the arterial wall (77). There is an increase in oxidative susceptibility and reduced antioxidant properties in small dense LDL further contributing to atherogenicity (78).

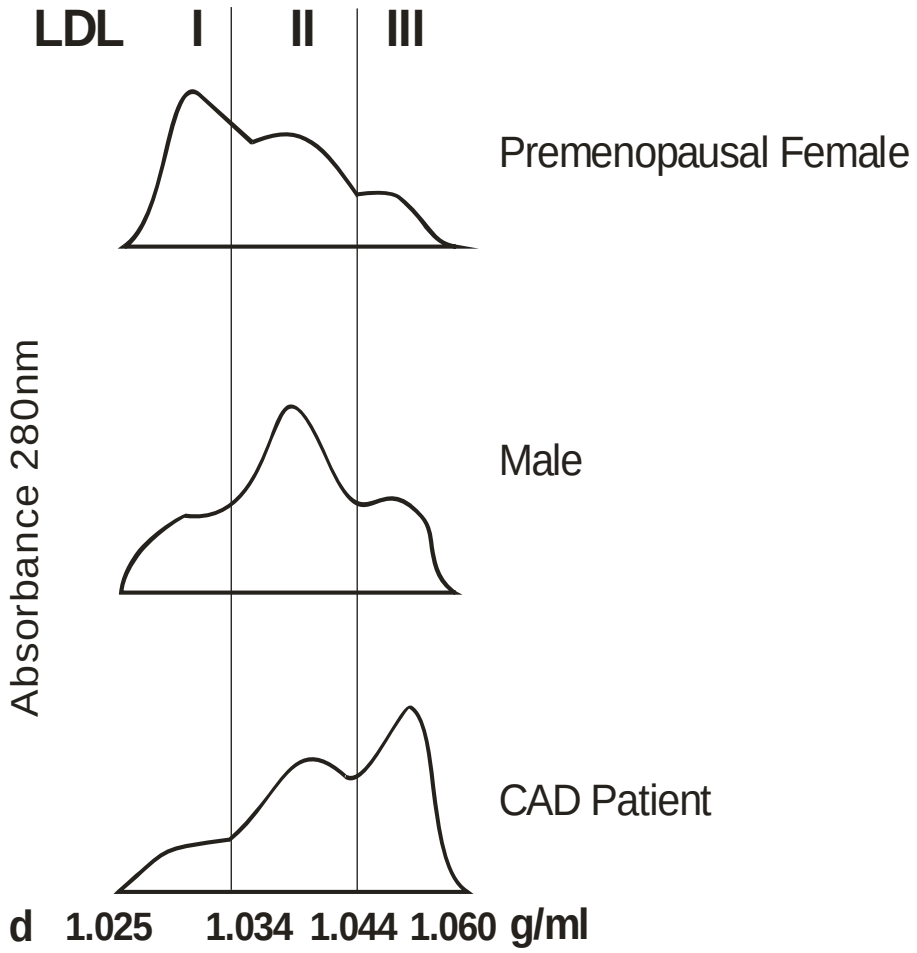

Figure 1.3 Typical LDL Subfractions in Different Populations

1.6 Normal Endothelial Function

Endothelial dysfunction is thought to play an important role in the initiation and progression of atherosclerosis. It is defined as alterations in the normal properties of the endothelium, which leads to impairment of organ function. Normal endothelium reduces vascular tone, regulates vascular permeability, limits platelet adhesion and aggregation, prevents activation of the coagulation cascade and restricts leucocyte adhesion. Endothelial activation is the loss of endothelial anti-inflammatory properties through the increased expression of adhesive molecules such as e-selectin, ICAM and VCAM. Nitric oxide (NO) is one of the most important mediators released by the endothelium. It is a potent vasodilator that inhibits inflammation, growth of vascular smooth muscle and aggregation of platelets.

Many different means of assessing endothelial dysfunction have been employed most of which usually measures an endothelial cell response to a stimulus. Endothelium dependant vasodilatation can be assessed in the coronary and peripheral circulation. Coronary angiography has been used to assess changes in vascular diameter in response to an infusion of an endothelium dependant vasodilator such as acetylcholine (Ach). In a healthy vessel, ACh causes a NO mediated vasodilatatory response, which is not observed in patients with endothelial dysfunction. Also, intracoronary Doppler can be used to measure coronary blood flow in response to stimuli (79). The degree of endothelial dysfunction in the peripheral circulation is proportionate to the degree of endothelial dysfunction occurring in the coronary arteries (80). Assessment of endothelial vasomotion in these peripheral arteries has

been shown to correlate with coronary dysfunction (81). Peripheral circulation endothelial function can be assessed in the forearm by intra-arterial infusion of endothelium dependent vasodilators such as acetylcholine (82). Flow mediated dilatation of the brachial artery using high resolution ultrasound is a less invasive approach(83). Further non-invasive methods of assessing endothelial function are currently emerging such as laser doppler imaging. This technique is described later.

1.7 Cardiovascular Disease, Endothelial Function and Inflammation

1.7.1 Endothelial Function

It is now accepted that abnormalities of endothelial function and inflammation play a major role in the development of atherosclerosis. This is supported by both experimental and clinical data. Furthermore, there is significant evidence for the use of inflammatory markers both as predictors of coronary heart disease and prognostic factors for CHD(84-88).

Normal endothelium does not usually support binding of leucocytes but in the presence of early atheromatous changes, patches of arterial endothelial cells express selective adhesion molecules on their surface, which bind to various classes of leucocytes. Vascular Cell Adhesion Molecule- 1 (VCAM-1) expression has found to be increased on endothelial cells overlying atheroma (89). This increase in adhesion molecules is found at sites prone to developing atheroma, such as branch points in the arterial tree, where there is disturbed flow and reduced shear stress, which reduces local production of NO. NO has anti-inflammatory properties and can reduce V-CAM

expression(90). This disturbed flow can also increase expression of intercellular adhesion molecule-1 (ICAM-1) (91). These leucocytes then penetrate the intima where they perpetuate a local inflammatory response with macrophages ingesting lipid and becoming foam cells. The activated leucocytes and arterial cells can release fibrogenic mediators that promote replication of smooth muscle cells leading to the development of an atherosclerotic plaque. Inflammatory processes also contribute to precipitating acute thrombotic complications of atheroma. The activated macrophages abundant in the atheroma can produce proteolytic enzymes capable of degrading the collagen within the fibrous plaque of the cap allowing it to rupture (Figure 1.4).

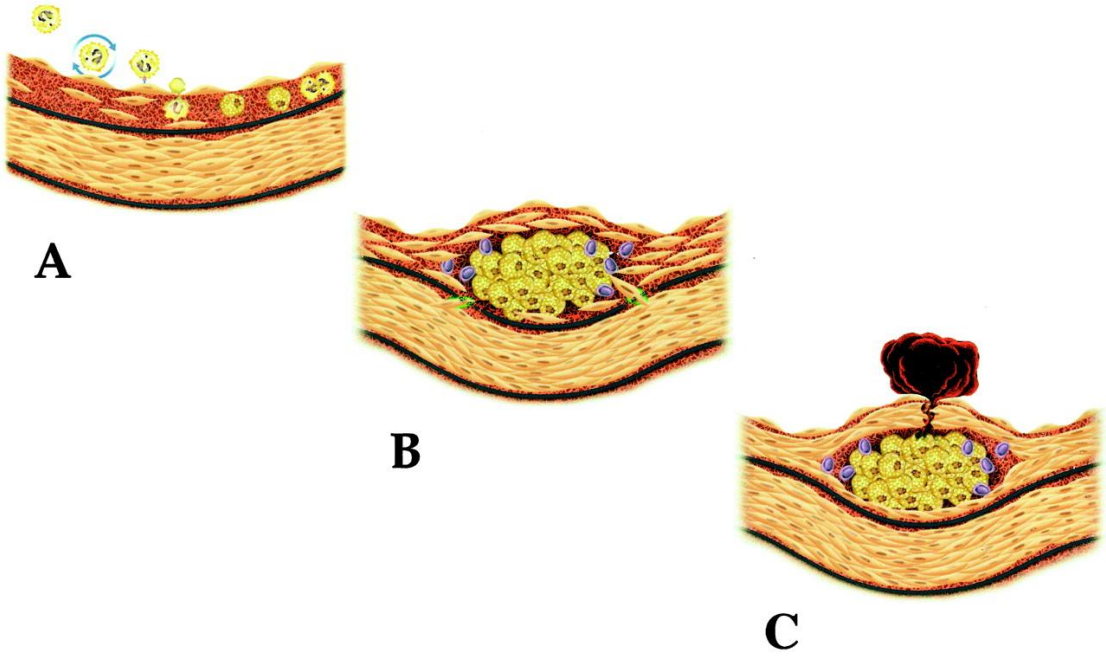

Fig 1.4 Participation of Inflammation in Atherosclerosis

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- **A.** Leucocyte recruitment to the atherosclerotic plaque.
- **B.** T-lymphocytes join macrophages in the intima during lesion evolution
- **C.** Thinning of the fibrous cap caused by alteration of the extracellular matrix induced by collagenases produced by foam cells leading to plaque rupture

1.7.2 Markers of Inflammation

Elevated levels of several inflammatory markers have shown to be predictive of vascular events in several different populations. Prospective epidemiological studies have shown associations with increased levels of cytokines Interleukin-6 (IL-6) and Tumour Necrosis Factor-α (TNF-α) and increased vascular risk(85-87;93). This has also been observed with increased levels of acute phase reactants such as C-reactive protein (CRP), fibrinogen and serum amyloid A(84;85;94;95).

The most relevant clinically of these markers is CRP. It is an acute phase protein, which is a member of the pentraxin family of innate immune response proteins (96). Measurement of highly sensitive CRP (hs-CRP) as a biomarker of inflammation and therefore atherosclerosis confers several advantages over the measurement of other novel biomarkers. CRP has a long half life and is stable without being affected by circadian variation (97). Furthermore, it is easily measured with a standardized high-sensitivity assay available.

Several studies have indicated that increased levels of highly sensitive (hs) CRP are strong predictors of cardiovascular disease in healthy subjects(88) (Fig 1.5). These studies included men, women, middle-aged and elderly subjects. A large British study by Danesh et al showed that higher levels of baseline CRP predicted coronary heart disease after adjustment for other vascular risk factors (84). Thus, CRP is an important biomarker useful for the prediction of vascular risk in most populations of patients independent of other factors.

A number of drugs used in the treatment of CHD have been observed to reduce CRP. In the Physicians' Health Study, aspirin has been shown to

reduce cardiovascular risk to a greater extent in subject with higher levels of CRP (95). A number of studies have shown that statins reduce CRP in patients with hyperlipidaemia. In the Air Force/ Texas Coronary Atherosclerosis Prevention Study (AFCAPS/ TexCAPS), lovastatin reduced CRP by almost 15%. In addition, not only was there a significant reduction in coronary events apparent in patients with increased total cholesterol to HDL ratio but a reduction was also present in patients with an increased CRP but no increase in cholesterol:HDL ratio(98).

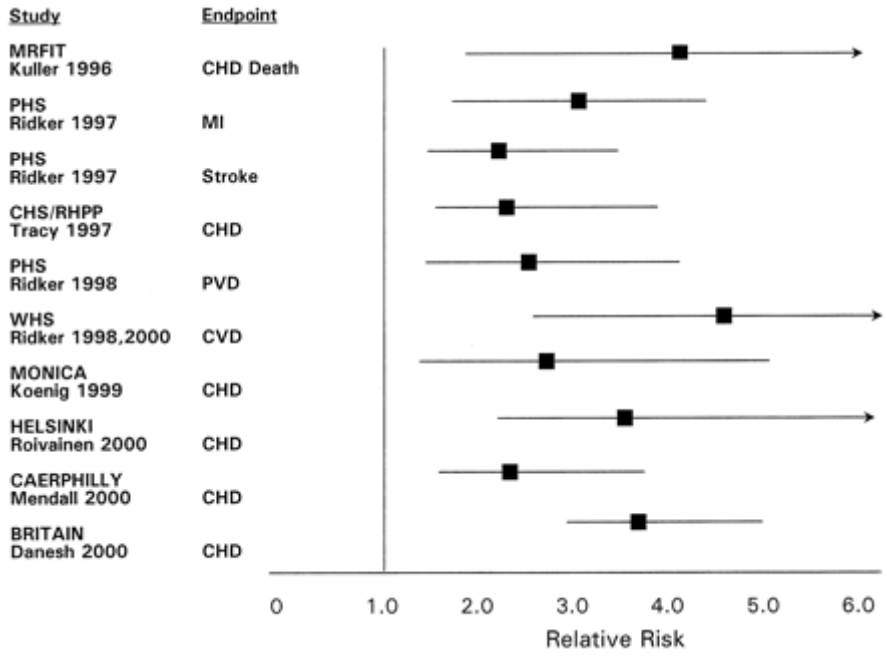

Fig 1.5 Prospective studies of hsCRP as a predictor for future vascular events

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1.8 Omega –3 Fatty acids

The cardioprotective effects of omega-3 fatty acids were first noted following the observation that the Greenland Inuit had a low mortality from CHD despite a high incidence of smoking and obesity. In 1975, Dyerberg and Bang suggested that this was due to the high content of omega-3 fatty acids in the Inuit diet which comprised mainly of fish, seal and whale (99).

Omega-3 fatty acids are polyunsaturated fatty acids. These omega-3 fatty acids are alpha linolenic acid (LNA), eicosapentenoic acid (EPA) and docosahexenoic acid (DHA) (Fig 1.6). LNA is a plant-based source of omega-3 fatty acid while EPA and DHA are obtained from marine sources. They are essential fatty acids and so must be consumed in the diet. Omega- 3 fatty acids have a variety of potential cardioprotective properties including effects on lipoproteins, inflammation, thrombosis and endothelial function.

1.8.1 Omega-3 Fatty Acids and CHD: Observational Studies

The observation that the Greenland eskimos had lower mortality due to their high fish consumption has lead to several observational studies. A study examining middle aged men in the town of Zupthen in the Netherlands found that men who rarely or never ate fish had a higher rate of CHD than those who ate fish more than once a week(100). Similarly, the Chicago Western Electric Study also showed an inverse relationship between fish consumption and death from CHD (101) as did the Multiple Risk Factor Intervention Trial (MRFIT)(102). Zhang et al found that fish consumption was associated with reduced risk from all cause mortality, IHD and stroke across 36 countries (103). In women, the Nurses' Health Study found that CHD deaths were 50%

lower in those women who consumed fish five times a week (104;105). A subgroup of these women with diabetes showed a stronger relationship with a 60% reduction in CHD death in those who ate fish five times a week(106). However, not all observational studies have yielded similar results, with some studies showing a lack of association. The Health Professional Follow-up study showed no association between dietary fish consumption and the risk of coronary disease (107). Also, the US Physicians Health study found no association between the risk of MI, non-sudden cardiac death or total CV mortality but fish consumption was associated with a reduction in total mortality (108). There was an inverse association between fish consumption and 25 year mortality in the Seven Countries Study but this was no longer significant once the effects of saturated fats, flavinoids and smoking were taken into account (109).

Various theories behind the conflicting data have been postulated. End-point definitions, experimental design, study populations and type of fish consumed have all thought to influence the varying results. However, a recent meta-analysis of 13 published cohort studies found a consistent inverse association between fish consumption and CHD mortality rates(110).

1.8.2 Randomised Controlled Trials of Omega-3 Fatty Acids

Several trials have suggested favourable effects of omega-3 fatty acids in reducing the risk of CHD. The diet and reinfarction trial (DART) included 2033 Welsh men(111). It found that patients who received dietary advice on fish had a relative reduction in mortality of 29% during the 2 year follow-up mainly because of reductions in deaths from CHD. The Physician's Health Study

(112) found a reduced risk of sudden cardiac death in subjects with increased blood levels of omega- 3 fatty acids. The largest randomised controlled trial examining the efficacy of omega-3 fatty acids in secondary prevention of CHD was the Italian GISSI-Prevenzione trial, which examined 11, 324 patients with a recent myocardial infarction. Patients were randomised to either 300mg of vitamin E or 850mg of omega-3 fatty acids, both or neither. After 3.5 years, the omega 3 fatty acid group had a reduction in relative risk of 15% in the composite end-point of total mortality, non-fatal myocardial infarction and stroke with a 20% reduction in all cause mortality and 45% reduction in sudden death(113). The Study on Prevention of Coronary Atherosclerosis by Intervention with Marine Omega-3 fatty acids (SCIMO) Study demonstrated the positive effects of omega-3 fatty acid supplementation on coronary artery disease which was assessed angiographically (114). In spite of these large studies showing a beneficial effect from fish oil, a recent meta-analysis examining 48 randomised controlled trials found that omega-3 fatty acids had no effect on total mortality or combined CV events(115).

1.8.3 Effect of Omega-3 Fatty Acids on Lipoproteins

In a review of human studies, Harris et al reported that 4g daily of omega-3 fatty acids reduced triglyceride levels by 25 to 30% with a decrease in HDL of 1 to 3%. However, LDL cholesterol was found to increase by 5 to 10%. The triglyceride lowering effect was dose dependant (116).

Human and animal studies have shown that omega-3 fatty acids inhibit hepatic triglyceride synthesis and secretion of VLDL from the liver (117-119). The primary mechanism of triglyceride lowering is thought to be via reduced

production rather than enhanced clearance of triglyceride rich lipoproteins as activity of either postheparin LPL or hepatic lipase has been shown not to be stimulated by omega-3 fatty acids (120-122).

The LDL raising effect of omega-3 fatty acids is well recognised and has been observed in both healthy (123) and hyperlipidaemic subjects (124). This effect is rare at doses of 1g per day(125). At higher doses (3-5g), the LDL raising effect may be offset by a redistribution of LDL to larger sizes(126), thus potentially reducing its atherogenicity. The mechanisms behind the LDL raising effect of omega-3 fatty acids are unclear. It has been suggested that the fish oil down regulates the LDL receptor (127;128). Changes in LDL particle size occur as omega-3 fatty acids suppresses CETP activity thus reducing the transfer of cholesteryl ester from HDL to VLDL and LDL thereby favouring large cholesterol rich LDL(129).

Most studies show a beneficial effect of omega-3 fatty acids on HDL. The reduction in activity of CETP also affects HDL particles favouring large cholesterol rich HDL (HDL_2) again through reducing transfer of cholesteryl esters from HDL to VLDL(129).

Omega-3 fatty acids have also been shown to reduce triglyceride levels postprandially (130). This effect has been observed in healthy men (131) and patients with hypertriglyceridaemia (120). Moreover, they lower postprandial chylomicrons and chylomicron remnants (122;131).

1.8.4 Effect of Omega-3 Fatty Acids on Thrombosis

Omega-3 fatty acids are thought to reduce the risk of thrombosis by affecting platelet function and haemostasis. The observation that the Greenland

Eskimos had reduced platelet aggregation and prolonged bleeding times suggested an important mechanism relating to the reduction of CHD(132). They act by displacing arachidonic acid from platelet phospholipid stores, decreasing the available substrate for thromboxane A2 synthesis and so reducing the ability of thromboxane A2 to induce platelet aggregation, thereby resulting in prolongation of bleeding time(133). The results from studies examining the effect of omega-3 fatty acids on haemostatic and fibrinolytic factors are conflicting(134-137). Thus, omega-3 fatty acids have beneficial effect on platelet aggregation but their effect on thrombosis is yet to be established.

1.8.5 Effect of Omega-3 fatty Acids on Arrhythmias

The effect of omega-3 fatty acids on reducing CHD mortality may, in part, be attributable to their anti-dysrhythmic effects. Several studies have suggested that omega-3 fatty acids are useful in the prevention of atrial and ventricular dysrhythmias. These observations have arisen as a result of animal and cell culture studies and more recently clinical randomised controlled trials. Studies examining rats have revealed that pre-treatment with omega-3 fatty acids prevented the initiation and reduced the severity of arrhythmias in response to various stimuli (138). In cultured rat cardiac myocytes, omega-3 fatty acids inhibited induced tachyarrhythmias (139). These properties may be as a result of incorporation of omega-3 fatty acids into myocardial membrane phospholipids affecting electrophysiological properties. In patients with implantable cardiac defibrillators, the effect of omega-3 fatty acids on arrhythmic events is uncertain but there is no reduction in mortality (140-142).

1.8.6 Omega-3 Fatty Acids and Inflammation

Once again, it was first suggested that omega-3 fatty acids had anti-inflammatory properties from the epidemiological observations of the low incidence of autoimmune and inflammatory disorders in the Greenland Eskimos (143). As discussed previously, inflammation plays a key role in the development of atherosclerosis and so the anti-inflammatory properties of omega-3 fatty acids are thought to contribute further to their cardioprotective effects.

Consumption of omega-3 fatty acids increases EPA in the cell membrane, which competes with arachidonic acid as a substrate for cycloxygenase and lipoxygenase enzymes. Those derived from arachidonic acid are pro-inflammatory whereas those derived from omega-3 fatty acids are anti-inflammatory(144). Furthermore, they suppress the production of pro-inflammatory cytokines and reduce expression of cell adhesion molecules such as I-CAM, V-CAM and e-selectin (145).

The beneficial anti-inflammatory effects of omega-3 fatty acids have been seen in a number of conditions other than CHD. Studies have suggested benefits in patients with inflammatory bowel disease (146), rheumatoid arthritis (147) and asthma (148).

1.8.7 Omega-3 Fatty Acids and Endothelial Function

Evidence from in vitro and in vivo studies have shown that omega-3 fatty acids are thought to have a beneficial effect on vascular endothelium. Chin et al showed an improvement in endothelial function with omega-3 fatty acids using forearm venous occlusion plethysmography (149;150). Goodfellow

examined endothelial function using non-invasive ultrasonic vessel wall tracking of brachial artery flow mediated dilatation (151) and found an improvement in the group treated with fish oil. In vitro, Goode et al demonstrated an improvement in endothelial function with an in vitro study of small arteries from subcutaneous gluteal fat biopsies in hypercholesterolaemic patients treated with omega-3 fatty acids(152).

The mechanism behind these improvements is unclear. It has been suggested that omega-3 fatty acids cause changes in the composition of the membrane bilipid layer. The in vitro study by Goode et al found that the greatest improvement in endothelial function occurred in patients with greater EPA and DHA levels in their red cell membrane. Therefore, changes in the membrane fluidity may promote increased release of endothelium derived NO in response to ACh(152).

1.8.8 Omega-3 Fatty Acids and Hypertension

As a result of clinical and animal studies, it has been postulated that omega-3 fatty acids have hypotensive properties. They are thought to stimulate prostaglandins that control sodium and water excretion, inhibit thromboxane, which is a vasoconstrictor, and regulate renin release(153). A meta-analysis of 31 studies showed a significant reduction of 3.4 mmHg systolic and 2.0 mmHg diastolic pressure with a 5.6g consumption of omega-3 fatty acids(154). Similarly, a further meta-analysis of 17 trials found a 5.5 mmHg systolic and 3.5mmHg reduction in diastolic blood pressure with daily doses of greater than 3g(155).

1.8.9 Omega-3 Fatty Acids in the Treatment of IgA Nephropathy

Trials of omega-3 fatty acids in the treatment of IgA nephropathy have shown conflicting results. A trial from the Mayo clinic treated patients with 12g daily of omega-3 fatty acids. They found at 4 years that patients receiving the fish oil had a significantly lower incidence of doubling of serum creatinine and a lower incidence of death or end stage renal failure(156). Other studies have failed to confirm these results and a meta-analysis of five randomised controlled trials concluded that the variation in results were due to differences in the duration of follow-up and once this was adjusted for statistically, there was no benefit from fish oil treatment (157). A more recent trial by the Southwest Paediatric Nephrology Study elucidated no significant difference in renal progression observed at 3 years in the patients treated with 4g daily of fish oil(158). In summary, there is currently no clear evidence supporting the use of omega-3 fatty acids in IgA nephropathy.

1.8.10 Tolerability of Omega-3 Fatty Acids

The Food and Drug Administration has ruled that intakes of up to 3g daily of marine omega-3 fatty acids are generally recognized as safe for inclusion into the diet. This includes particular consideration of bleeding tendencies and their effect on LDL-C.

Omega-3 fatty acids are generally well tolerated, with a fishy aftertaste as the most commonly reported side effect in most studies. In the GISSI Prevention study, when 0.85g of omega-3 fatty acids were given daily to subjects for 3.5 years, gastrointestinal upset and nausea were the most commonly reported side effect (4.9% and 1.4% respectively)(113). In a study examining 275

patients given 6.9g daily of omega-3 fatty acids for 6 months, gastrointestinal upset was reported in 8% of patients (159).

In summary, both observational and randomised controlled trials have suggested that omega-3 fatty acids have cardioprotective properties. In addition to their effect on lipids and lipoproteins, they have been shown to have anti-inflammatory, anti-thrombotic, anti-hypertensive, anti-arrhythmic properties and improve endothelial function as well as being well tolerated. However, it is difficult to compare the results of these studies due to differing doses and treatment duration.

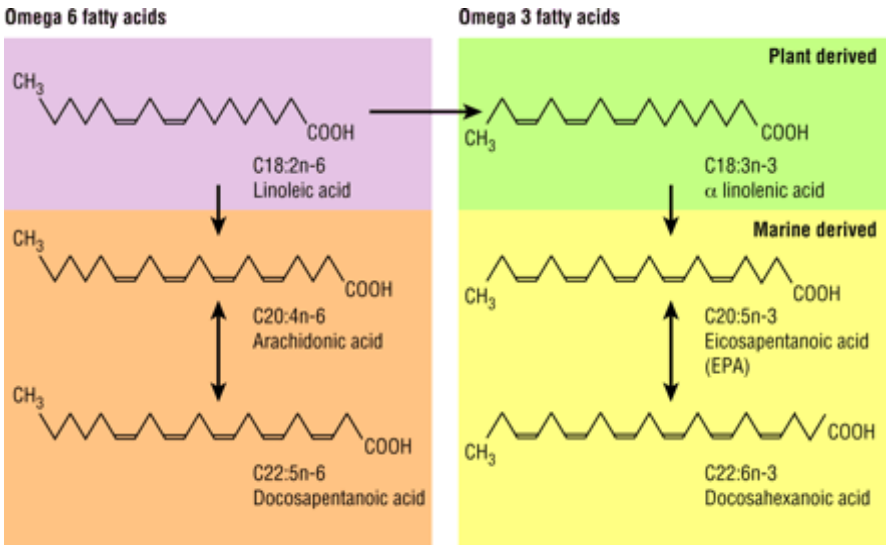

Figure 1.6 Structures of Omega-3 Fatty Acids

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Structures of the two classes of polyunsaturated fatty acids. The omega 3 fatty acids have their first double bond at the third carbon molecule from the methyl (CH_3) end of the fatty acid, whereas the omega 6 fatty acids have their first double bond at the sixth carbon molecule. The chemical names for each fatty acid are also given: the number of carbon atoms is given first, followed by the number of double bonds and the position of the first double bond. Omega-6 linoleic acid can be desaturated in certain plants to form omega-3 α linolenic acid. Whereas linoleic acid is mainly converted into arachidonic acid, αlinolenic is elongated and desaturated into eicosapentenoic acid and then docosahexenoic acid

1.9 Proteinuric Renal Disease

1.9.1 Dyslipidaemia of Proteinuric Renal Disease

The prevalence and extent of dyslipidaemia in proteinuric renal disease is variable depending on the aetiology of the renal dysfunction, the degree of the renal impairment and level of proteinuria. The association between nephrotic syndrome and hyperlipidaemia was first reported in 1917(161). However, despite extensive clinical and animal work, the mechanism behind this has not yet been fully established.

The development of nephrotic range proteinuria is associated with abnormal lipoprotein metabolism and both qualitative and quantitative changes in lipids and lipoproteins. Total cholesterol and LDL are invariably raised (162;163). Triglyceride levels may also be elevated (164) and VLDL is raised (162;163). Furthermore, it has been shown that patients with nephrotic range proteinuria have excess LDL III (165).

This pattern of abnormalities is probably a result of a number of different mechanisms. Firstly, the increase in plasma LDL is thought to be due to increased LDL synthesis (166) and reduced catabolism of LDL. Indeed a previous study in our laboratory by Warwick et al showed that there was a significant reduction in the rate of catabolism of Apo B, which is the main constituent of LDL (167;168). Furthermore, Vaziri et al have suggested that not only does proteinuria lead to the up-regulation of 3-hydroxy-3-methylglutaryl (HMG) CoA reductase (the rate-limiting enzyme required for cholesterol biosynthesis) but also leads to a deficiency in receptor mediated LDL clearance. This results in a limitation of hepatic cholesterol uptake and leads to an increase in cholesterol and LDL levels (169). Furthermore, it has

been shown that, in patients with nephrotic range proteinuria, there is an increase in HDL_3 with a reduction in protective cholesterol rich HDL_2 (170). Proteinuria is thought to lead to urinary losses of lecithin: cholesterol acyltransferase (LCAT) which has a similar molecular weight to albumin(171). This limits HDL mediated uptake of surplus cholesterol from extra hepatic tissues leading to HDL abnormalities and subsequent effect on VLDL and chylomicrons (172). The increase in triglyceride rich lipoproteins has been shown to be due to impaired chylomicron (173;174) and VLDL clearance (173). Deighan et al reported a deficiency of VLDL apolipoproteins in proteinuria and hypothesised that this deficiency resulted in its defective clearance (175). Impaired lipoprotein lipase activity has been observed in patients with proteinuria. LPL is the rate-limiting enzyme involved in lipolysis of chylomicrons and VLDL. LPL binds to heparan sulphate proteoglycans on the cell surface of the endothelium. Many studies have shown a reduction in LPL activity in both nephrotic humans and animals (169).

Data from the Framingham heart study has shown that patients with proteinuria have RLP-C and RLP-TG concentrations 2-3 fold higher than the reference range for males(176). In patients with nephrotic range proteinuria, it has been demonstrated that there is an excess of remnant lipoproteins as well as LDL III thereby significantly increasing cardiovascular risk(177). In this study, plasma triglyceride level was the most important factor determining LDL III and remnant lipoprotein levels.

The formation of LDL III requires excess triglycerides and hepatic lipase. Triglyceride transfers via CETP from $VLDL_1$ to LDL in exchange for

cholesteryl ester. This produces a triglyceride enriched LDL particle, which is hydrolysed by hepatic lipase resulting in the formation of small dense LDLIII. This is also the suggested mechanism for small dense LDL formation in proteinurics due to higher levels of triglyceride and the strong correlation between triglyceride concentration and LDL III concentrations.

1.9.2 Endothelial Function in Proteinuric Renal Disease

It has been suggested that proteinuria itself, or pathophysiological changes associated with it, may cause endothelial dysfunction. There are a number of theories to explain the possible link between proteinuria and endothelial dysfunction. These include elevated blood pressure, dyslipidaemia, hypoalbuminaemia, disordered coagulation pathways, inflammation and oxidative stress that all may occur in proteinuric patients, particularly those with the nephrotic syndrome. Endothelial function has been shown to be abnormal by both venous occlusion plethysmography(178) and brachial artery ultrasound(179), in patients with proteinuria.

3.5 Hypotheses and Aims

1.10.1 Hypotheses

The purpose of this project is to examine the ability of omega-3 fatty acids derived from fish oils to correct the dyslipidaemia observed in patients with nephrotic range proteinuria, thus reducing the risk of coronary heart disease (CHD), and to clarify the mechanism underlying this dyslipidaemia.

It is important to establish a lipid lowering therapy that is effective, tailored to the specific problem in proteinuric patients but is also safe and well tolerated.

Fish oil derived fatty acids lower plasma triglyceride by reducing VLDL production and at the start of the study, Omacor, a therapeutic agent containing omega-3 fatty acids in a concentrated form had recently become available and seemed an ideal therapy for this population.

Our main hypotheses are (i) Omacor will be well tolerated in subjects with nephrotic range proteinuria and will give adequate triglyceride lowering with consequent benefits on levels of small dense LDL and HDL. (ii) VLDL and not chylomicrons (intestinally derived lipoproteins) possess structural abnormalities that delay clearance and result in hypertriglyceridaemia. Fish oils will correct this. (iii) Omacor will improve endothelial function in proteinuric patients.

1.10.2 Aims

The following questions summarise the rationale behind this research:

In patients with nephrotic range proteinuria:

1. Does treatment with omega-3 fatty acids give adequate triglyceride control with consequent benefits for levels of small dense LDL and remnant lipoproteins?
2. Is postprandial lipaemia improved with omega-3 fatty acids?
3. Do omega-3 fatty acids have an effect on chylomicron and $VLDL_1$ structure and composition?
4. Are HDL subfractions affected by treatment?
5. What is the effect of treatment on endothelial function and inflammation?

Chapter 2: Methods

2.1 Study Population

19 Subjects with primary glomerular disease, nephrotic range proteinuria (defined as either >3g of proteinuria or >2g of albuminuria per 24 hours) and serum creatinine <250µmol/l were recruited. Patients with diabetes mellitus or who were on steroids, other immunosuppressive agents or warfarin treatment were excluded. Comparisons were made with age and sex matched controls. If used, lipid-lowering treatment was stopped 4 weeks prior to the study and restarted following the study. All patients and controls gave written consent prior to participating and Glasgow Royal Infirmary Research Ethics Committee approved the study. Patients and controls attended after an overnight fast. The laser Doppler scan was carried out as outlined in chapter 8. An intravenous cannula was inserted and fasting samples were taken. A fat load amounting to 90g of fat in the form of “Calogen” drink was given to the subject. Subsequent samples were taken at 2, 4, 6 and 8 hours following the fat load. This procedure was repeated after 8 weeks treatment with Omega-3 fatty acids (Omacor) 2g twice daily.

2.2 Statistical Power

Sample size calculations are to give 80% power at $\alpha = 0.05$. Omacor reduces triglyceride levels by 37% in subjects with hypertriglyceridaemia and 27% in familial combined hyperlipidaemia(180). To detect a 30% change in triglyceride concentration, 17 subjects will be required.

Based on results from a previous study comparing VLDL apolipoprotein content in subjects with proteinuria and controls, each VLDL particle in controls contained 10 moles of apoCII, 30 moles of apoCIII and 0.5 moles of

apoE(175). A meaningful difference in apoCII between proteinurics and controls would be 4 moles per lipoprotein particle. Using a standard deviation (SD) of 2.5 for this measurement, 8 subjects will be required in each group. For apoCIII, 17 subjects would be required for a meaningful change of 15 moles/particle(SD 15). For apoE, 17 subjects would be required for a difference between proteinurics and controls of 0.3 moles/particle (SD of 0.3).

The apolipoprotein content of patients and controls post treatment with Omacor will be compared by ANOVA. For apoCII to detect a difference of 4 moles/particle (SD 2.5), 8 patients will be required. For apoCIII, (difference of 15 moles/particle, SD 15), sample size =17. For apoE (difference of 0.3 moles/particle (SD 0.3), sample size =17.

The sample size being studied is small and so if the standard deviation exceeded those predicted or if there is marked heterogeneity within the population, there will be a significant chance of a Type II error. Given that a number of variables studied are inter-related, formal statistical advice was that correction for multiple comparisons was not necessary. However, we acknowledge that multiple analyses could result in statistically significant results by chance and so therefore we have endeavoured to avoid over interpreting the data.

2.3 β Quantification of Lipoproteins

VLDL cholesterol, LDL cholesterol and HDL cholesterol were measured according to the Lipid Research Clinics Program Manual of Laboratory Operations (1975)(12). VLDL (d 1.006g/ml) was isolated by ultracentrifugation at 35 000 rpm at 4°C for 18 hours in a Beckman Ti 50.2 rotor. LDL was

precipitated by adding an equal volume of 92mmol/l sodium heparin ($5x10^5$) / Mn Cl_2(181). This was followed by centrifugation at 10,000 rpm at 4°C for 30 minutes. HDL remained in solution. Cholesterol measurements were carried out on total plasma, VLDL and HDL. The HDL level was subtracted from the cholesterol content of the supernatant giving LDL cholesterol. All coefficients of variations (CV) were less than 2%.

2.4 Preparation of Chylomicrons

Chylomicrons were isolated by a short ultracentrifugation at a density of 1.006g/ml. 2ml of plasma was overlayered with 4ml d=1.006g/ml solution and centrifuged in a Beckman Ti 40.3 rotor at 4°C for 30 minutes at 10,000rpm. The chylomicrons were removed carefully using a finely drawn glass Pasteur pipette in 2ml. The 2 ml removed were then overlayered with 4ml d=1.006g/ml solution and centrifuged in a Beckman Ti 40.3 rotor for 16 hours at 15°C at 35,000rpm for 16 hours. The purified chylomicrons were removed using a finely drawn Pasteur glass pipette.

2.5 Isolation of $VLDL_1$ (S_f 60-400), $VLDL_2$ (S_f 20-60), IDL (S_f 12-20) and LDL (S_f 0-12)

$VLDL_1$ (S_f 60-400), $VLDL_2$ (S_f 20-60), IDL (S_f 12-20), LDL (S_f 0-12) were isolated from fresh plasma by modification of the cumulative gradient ultracentrifugation method (15). Stock solutions of density 1.006g/ml and 1.182g/ml were prepared using NaCl, NaBr and Na_2EDTA. These were used to make 6 density solutions, which ranged from 1.0588 to 1.0988g/ml. The density of 2mls of plasma (after removal of chylomicrons described above)

was adjusted to 1.118g/ml using 0.341g NaCl. The adjusted plasma was carefully overlayered by the density solutions as described in Table 2.1. Ultracentrifugation of the sample was performed as outlined in Table 2.2. Samples were carefully removed after each run using a finely drawn Pasteur pipette.

$VLDL_1$ was removed in 1ml, $VLDL_2$ and IDL in 0.5ml, and LDL in 1ml. After removal of $VLDL_1$, The sample was overlayered with 1ml of 1.0988g/ml density solution.

Table 2.1 Density Gradients for Separation of $VLDL_1$, $VLDL_2$, IDL and LDL

Solution	Density (mg/l)	Volume (mls)	Position
6	1.0588	2	Top
5	1.0641	2	
4	1.0722	2	
3	1.0790	2	
2	1.0860	1	
1	1.0988	1	
Plasma	1.118 (adjusted)	2	
	1.182	0.5	Bottom

(Adapted with permission(15))

Table 2.2 Conditions for Preparation of $VLDL_1$, $VLDL_2$, IDL and LDL

	S_f	Speed (rpm)	Time (h/min)
$VLDL_1$	60-400	39,000	1.38
$VLDL_2$	20-60	18,500	15.41
IDL	12-20	39,000	2.35
LDL	0-12	30,000	21.10

(Adapted with permission(15))

2.6 LDL Subfraction Analysis

Three LDL subfractions were separated directly from fresh plasma by nonequilibrium density gradient ultracentrifugation(59). Again, stock solutions of density 1.006g/ml and 1.182g/ml were prepared using NaCl, NaBr and Na_2EDTA. These were used to make 6 density solutions, which ranged from 1.060g/ml to 1.019g/ml. The density of plasma was adjusted to 1.09g/ml by using KBr. The adjusted plasma was carefully overlayered as described in Table 2.3 in an ultraclear Beckman SW 40 tube, which had been coated with polyvinyl alcohol. This was centrifuged for 24h at 40,000rpm at 23°C in a swinging bucket rotor with slow acceleration and no deceleration. The gradient containing separated LDL subfractions was displaced upwards by a

dense hydrophobic material (Maxidens 1.9g/ml), which was introduced using a constant infusion pump at a flow rate of 0.5ml/min. The presence of 3 fractions (LDL I d 1.025-1.034 g/ml, LDL II d 1.034-1.044 g/ml and LDL III d 1.044-1.063 g/ml) was detected by continuous monitoring at 280nm. The individual subfraction areas were quantified, corrected for previously calculated extinction coefficients and expressed as a percentage of total LDL. The lipoprotein mass of LDL (d 1.019 –1.063 g/ml) was determined and used to generate individual subfraction concentrations in mg lipoprotein per 100ml plasma.

Table 2.3 Density Gradient for LDL Subfractions

Solution	Density (mg/l)	Volume (ml)	Position
6	1.019	1	Top
5	1.024	2	
4	1.034	2	
3	1.045	1	
2	1.056	1	
1	1.060	1	
Plasma	1.09 (adjusted)	3	
	1.182	0.5	Bottom

(Adapted with permission (15)).

2.7 Sequential preparation of HDL_2 and HDL_3

HDL_2 (d 1.063 – 1.125 g/ml) and HDL_3 (d 1.125 – 1.215 g/ml) were separated using sequential density ultracentrifugation. The remaining 4ml of infranatant following LDL separation with a density of 1.019- 1.063 g/ml was adjusted to d 1.125 g/ml by the addition of NaBr solution at density 1.3104 g/ml. After a 24 hour spin at 15°C at 35, 0000 rpm in a Beckman 50.4 fixed angle rotor in a Beckman L8-60M ultracentrifuge, during which the lighter particles floated to the top and heavier particles sank, the top 2 ml containing HDL_2 at d 1.063-1.125g/ml were removed carefully from the top. The infranatant was adjusted to d 1.215 g/ml by addition of NaBr solution at d 1.3104 g/ml. After a further 24-hour spin again at 15°C and 35,000 rpm, the top 2 ml containing HDL_3 at d 1.125- 1.215 g/ml were aspirated.

2.8 Compositional Analysis: Cholesterol, Free Cholesterol, Triglyceride, Phospholipid and Protein

Total lipoprotein composition of $VLDL_1$, $VLDL_2$, IDL, LDL d 1.019 – 1.063, HDL_2 and HDL_3 were quantified as follows. Total cholesterol was measured by enzymatic hydrolysis of cholesteryl esters to form free cholesterol followed by oxidation to give hydrogen peroxide. This is quantified by formation of a coloured product at 505nm (Roche Diagnostics, UK, Kit 11491458216). Free cholesterol was assayed by the same method omitting the enzyme cholesteryl esterase. (Wako Chemicals: Alpha Laboratories, UK Kit 279-47106). Cholesteryl ester was calculated as the total cholesterol minus the free cholesterol times 1.68 to correct for mass of esters. Triglyceride was assayed by enzymatic hydrolysis followed by enzymatic determination of liberated

glycerol by colorimetry at 505nm (Roche Diagnostics, UK, Kit 12016648122). Phospholipid was determined by an enzymatic colorimetric assay method using absorbance at 500nm (Wako Chemicals through Alpha Laboratories, UK). Lipid analyses were performed on IL 600 clinical chemistry analyser (Instrument Laboratories, Cheshire, UK). Protein was analysed by modification of the method by Lowry et al(182). The free cholesterol, cholesteryl ester, triglyceride, phospholipid and protein content of the $VLDL_1$, $VLDL_2$, IDL and LDL collected were assayed and their lipoprotein concentration were calculated as the sum of these products. CVs for assays were: cholesterol 2.86%, triglyceride 3.83%, free cholesterol 2.15%, phospholipid 3.82% and protein 5.06%.

2.9 Remnant Lipoprotein

RLP-C was separated from plasma by an immunoaffinity mixed gel containing monoclonal antibodies to human apo B-100 and human apo A-1. (Japan Immunoresearch lab, Takasaki, Japan). The unbound fraction was measured after 2 hours incubation on automatic shaker cholesterol by an ultrasensitive enzymatic spectrophotometric assay using an I lab 600 clinical chemistry analyser. Interassay CV was 5.8%.

2.10 Highly Sensitive CRP

C reactive protein (CRP) was measured by an in house double antibody sandwich enzyme linked immunosorbent assay with rabbit anti-human CRP and peroxide conjugated rabbit anti-human CRP(183). Interassay CV was 2.72%.

2.11 I-CAM and V-CAM

ICAM was measured using a quantative sandwich enzyme immunoassay from R&D Systems (Abingdon, Oxfordshire) Quantikline Human Soluble ICAM-1 Immunoassay Kit BBE 1B. VCAM was analysed using a quantative sandwich enzyme immunoassay commercially available kit from R&D Systems (Abington, Oxfordshire, UK). Interassay CV was less than 5%.

2.12 Insulin and Glucose

Insulin was measured using a solid phase two-site immunoassay purchased from Mercodia (Uppsala, Sweden). Glucose was analysed using a commercially available kit from Randox Laboratories, Crumlin, UK. The principle of this assay is glucose-6-phosphate is oxidised in the presence of NADP and glucose-6-phosphate dehydrogenase. The rate of NADPH formation during the reaction is directly proportional to the glucose concentration. Insulin CV was 4% and glucose 2%.

2.13 Oxidised LDL

Oxidised LDL was measured using a solid phase two-site antibody immunoassay in which two monoclonal antibodies are directed against separate antigenic determinants on the same oxidised LDL apoB molecule. This is commercially available from Mercodia (Uppsala, Sweden).Interassay CV 16.4%.

2.14 Apolipoprotein Measurements

Apolipoprotein were analysed A1, B, CII, CIII and E were analysed on an IL600 clinical chemistry analyser (Instrument Laboratories, Warrington, Cheshire, UK) using Immunoturbidimetric commercially available kits from WAKO Chemicals through Alpha Laboratories, UK. CVs were as follows: AI 5.23%, B 1.54%, CII 2.46%, CIII 7.31% and E 5.78%.

2.15 HDL Gel Electrophoresis

HDL sizes were determined using non-denaturing polyacrylamide gel electrophoresis using 2-30% linear gradient pre-poured slab gels from Alamo Gels, San Antonio, Texas. The gels were scanned by laser densitometry and particle size was determined using high molecular weight standards(184) [HDL_{2b} 9.7- 12 nm; HDL_{2a} 8.8- 9.7 nm; HDL_{3a} 8.2- 8.8 nm, HDL_{3b} 7.8- 8.2 nm; HDL_{3c} 7.2- 7.8 nm]. HDL CVs less than 2%.

Chapter 3: Baseline Patient & Control Characteristics

3.1 Baseline Characteristics

19 patients who fulfilled the inclusion criteria outlined in the methods section (Chapter 2) were recruited from the renal out-patient clinic of Glasgow Royal Infirmary. One patient withdrew prior to the first visit and one patient withdrew during the treatment period as he became intolerant of Omacor due to gastrointestinal upset (diarrhoea). Therefore data for 18 patients at baseline and 17 patients post treatment were analysed. In the patient group, median serum creatinine was 143μmol/l (IQR 118-163). This represented a median estimated glomerular filtration rate (eGFR) of 46ml/min (IQR 38-58) using 4 variable MDRD formula(185). Mean 24 hour urinary protein excretion was 4.3g (IQR 3.4-6.0) and mean serum albumin was 36g/dl (IQR 31- 40). Primary renal diagnoses were as follows: 8 idiopathic membranous nephropathy, 3 focal segmental glomerulosclerosis, 2 mesangiocapillary glomerulonephritis, 2 IgA nephropathy with a further 3 patients with chronic glomerulonephritis that was unable to be classified further on renal biopsy. 16 out of the 18 patients (84%) were on statin therapy prior to the study and all of the patients were on either an ACE inhibitor or Angiotensin receptor antagonist with 2 on dual therapy.

Baseline data was compared with that obtained from 17 control patients who were age and sex matched (table 3.1). We attempted to match patients and controls for triglyceride. However, in view of the significant hypertriglyceridaemia observed in the patient population, it proved difficult to screen and recruit controls with higher triglyceride levels. This explains why the triglyceride levels in the control population were higher than expected in a normal population but still lower than the patient group.

3.2 Lipids and Lipoproteins

The results for baseline lipids, lipoproteins, insulin and glucose are outlined in table 3.1. Plasma cholesterol was higher in the patient population (p=0.003). Plasma triglyceride was also increased in the patient population (p=0.01). However, with the initial attempts to match for triglyceride, the mean triglyceride observed for the control patients was higher than would be expected in a normal population. Total VLDL-C was higher in the patients (p=0.004) however, LDL-C and HDL-C did not differ significantly despite the mean LDL-C in the patient group being 0.5mmol/l higher than controls. Notably, fasting insulin and glucose levels were similar despite the observed difference in plasma triglyceride and there was no relationship between baseline triglyceride concentration and either insulin or fasting glucose in both patients and controls.

3.3 Plasma Lipoprotein Concentrations

Total VLDL concentration in the patients was increased due to an increase in both larger, lighter $VLDL_1$ and smaller, denser $VLDL_2$ subfractions although only the increase $VLDL_2$ reached statistical significance (p=0.02). Despite similar LDL-C levels, total LDL lipoprotein concentration was higher in the patient group (p=0.03), however no difference in IDL concentration was observed (table 3.2).

3.4 LDL Subfractions and Remnant Lipoproteins

The plasma concentration of small dense LDL (LDLIII) was increased threefold (p=0.03) in the patient population. LDL II was the major subfraction in controls whereas LDL III was the major subfraction in the patient population with its mean percentage being twice that in controls. The concentrations of, and relative percentages of LDL I and II did not differ significantly between the groups.

Remnant-like lipoproteins were increased in patients with nephrotic range proteinuria. There was an almost three fold increase in the concentration of RLP-Cholesterol (RLP-C) in the patient group (p=0.03) and although the median RLP-Triglyceride (RLP-TG) concentration was doubled, this did not reach statistical significance (table 3.2).

3.5 Factors associated with Baseline LDL III and Remnant Lipoproteins in Patients and Controls

Tables 3.3 and 3.4 outline these relationships. In the patient group, LDL III concentration and LDLIII percentage correlated with plasma triglyceride concentrations, $VLDL_1$, $VLDL_2$ and remnant lipoproteins. There is a greater correlation between % LDL III and RLP than LDL III concentration as shown in table 3.3. No relationship was observed between LDL III and RLP baseline renal function, proteinuria, HDL-C, insulin or fasting glucose.

When examining factors associated with LDL III and RLP across the two groups studied, similarities and differences were identified. In the control group, LDL III concentration and %LDLIII also correlated closely with plasma

triglyceride concentrations, $VLDL_1$, $VLDL_2$ and remnant lipoproteins. Also in the control group, there was a much closer correlation between LDL III concentration and remnant lipoproteins. In the control group, an inverse relationship between both LDL III concentration and RLP-TG with HDL was observed. This was not observed in the patient group however in multivariate analysis, subject group was not an independent predictor for LDL III concentration, %LDL III or RLP.

3.6 Discussion

Analysis of β quantification of lipids at baseline demonstrated that patients had higher levels of total cholesterol, triglyceride and VLDL-C and no difference in LDL-C or HDL-C. Detailed lipoprotein analysis demonstrated an increase in both VLDL subfractions with a 51% increase in $VLDL_2$ but a non-significant 39% increase in $VLDL_1$ in patients with nephrotic range proteinuria. Higher levels of small, dense atherogenic LDL III were observed in the patient group in sufficient quantities that is likely to increase cardiovascular risk. Furthermore, a three-fold increase in remnant lipoprotein cholesterol was found in the patients with proteinuria. No direct relationship was observed between either RLP or LDL III and urinary protein, plasma albumin or renal function.

The results observed in our study are in keeping with previously published studies examining patients with nephrotic range proteinuria both in our laboratory and by other authors. The most commonly observed abnormality observed in patients with nephrotic range proteinuria is a raised plasma

cholesterol (162). Triglyceride levels are often raised but their range is more variable(164). These changes have been found to correlate inversely with serum albumin(186). Furthermore, previous studies have shown that LDL-C levels are invariably raised in these patients(163) and VLDL levels are frequently increased. A previous study from our laboratory has demonstrated that patients with nephrotic range proteinuria possessed excess remnant lipoproteins as well as raised LDL III in quantities significantly increasing CV risk. Plasma triglyceride levels have been shown to be the most important factor determining both LDLIII and remnant lipoprotein levels(187) . Data from the Framingham heart study has shown that patients with urinary dipstick analysis positive for protein have RLP-C and RLP-TG concentrations 2-3 fold higher than the reference range for males(176). In contrast to previous work from our laboratory, the 63% rise in median $VLDL_1$ concentration we observed in patients compared with controls was not significant. It is recognized that the $VLDL_1$ subfraction predominates in patients with hypertriglyceridaemia. In keeping with this, plasma triglycerides were elevated in the patient group. However marked heterogeneity was seen in the control group. We suspect that this results from the higher than normal triglyceride concentration obtained in our control population and accounts for the lack of difference observed in the $VLDL_1$ results. We did not observe any difference in HDL-C concentration. This is a frequent finding with a number of authors not identifying any difference in HDL levels in patients with nephrotic range proteinuria without renal failure(170;186;188). Thus, our baseline results are in keeping with previously published work in patients with nephrotic range proteinuria.

We did not find subject group to be an independent factor predicting LDL III concentration, % LDL III or remnant lipoproteins suggesting that the differences observed between the two populations are due to differing baseline parameters, and almost certainly, the increased plasma triglyceride levels seen in the patients. Correlations between LDL III concentration, remnant lipoproteins and plasma triglyceride concentration were tighter in the control population. Plasma triglyceride is the most important determinant of LDL III formation followed by hepatic lipase activity. Increased triglyceride levels lead to triglyceride enrichment of LDL II. Hepatic lipase hydrolyses the triglyceride thereby shrinking it to LDL III(65). This may explain why we observed a weaker correlation between LDL III and triglyceride in the patient group as it is likely that due to the hypertriglyceridaemia, hepatic lipase was not rate-limiting.

Increasing number of studies has shown that higher LDL III concentrations are associated with increased cardiovascular risk. LDL III concentration >100mg/dl confers a seven-fold increase in risk of myocardial infarction (61). Moreover, there is evidence to suggest that remnant lipoproteins are atherogenic and an independent risk factor for coronary heart disease (34;35). In summary, this combination of increases in total cholesterol, triglyceride, VLDL, LDL III and remnant lipoproteins are all likely to contribute to the significantly increased risk of cardiovascular disease observed in patients with proteinuria.

	Patients	**Controls**
	n= 18	**n=17**
	(All median & IQR)	
Age (years)	63.0 (55.8- 71.0)	61 (47.0-70.5)
Sex (M:F)	15:3	14: 3
Cholesterol (mmol/l)	6.2 (5.5- 7.5)	5.5 (4.7 – 5.9)*
Triglyceride (mmol/l)	2.1 (1.8- 2.6)	1.4(1.1 – 2.0) **
VLDL-C (mmol/l)	0.7 (0.6 -1.2)	0.5(0.3- 0.7) †
LDL-C (mmol/l)	4.2 (3.3- 5.4)	3.7 (3.0- 4.3)
HDL-C (mmol/l)	1.0 (0.90 -1.1)	1.1 (0.9- 1.4)
Insulin (mmo/l)	10.4 (7.9 -16.8)	8.4 (3.8- 15.8)
Glucose (mmol/l)	5.3 (4.8 -6.2)	5.3 (4.4- 5.9)

Table 3.1 Baseline Patient and Control Characteristics

Baseline patient characteristics compared with controls. Prior to statistical analysis, any parameter which was not normally distributed was normalised by log transformation. All data were compared using unpaired t-tests. Statistical differences are indicated (* p=0.003, **p=0.01, †p=0.004).

	Patient (n=18) (All median & IQR)	**Control** (n=17)	**p**
Total VLDL	182 (122- 240)	107 (78- 175)	ns
$VLDL_1$ (mg/dl)	106 (62-138)	65(35- 115)	ns
$VLDL_2$ (mg/dl)	88 (44-106)	43 (35- 61)	0.02
IDL (mg/dl)	59 (44- 82)	53 (43- 64)	ns
LDL I conc(mg/dl)	43 (21- 62)	29 (15-57)	ns
LDL II conc(mg/dl)	198 (112- 246)	167 (73-261)	ns
LDL III conc(mg/dl)	195 (64-237)	53 (30-182)	0.03
T-LDL conc(mg/dl)	411 (278-492)	315 (254-374)	0.03
% LDL I	8.9(5.5-15.8)	8.1 (3.8-16.1)	ns
%LDL II	41.1 (34.3-61.6)	65.2 (27.3-68.3)	ns
%LDL III	48.4 (17.8-60.3)	24.7 (12.0-63.9)	0.07
RLP- C (mg/dl)	14.8 (6.0- 20.5)	5.6 (4.1- 12.7)	0.03
RLP-TG (mg/dl)	46.5 (22.2- 51.3)	23.8 (20.8 – 49.2)	ns

Table 3.2 Patient and Control Lipoproteins at Baseline

	LDL III conc (r^2%)	LDL III% (r^2%)	RLP-C (r^2%)	RLP-TG (r^2%)
Triglyceride	32.7 [c]	53.5 [a]	64.1 [a]	71.5 [a]
$VLDL_1$ conc	30.5 [c]	50.4 [a]	72.4 [a]	79.7 [a]
$VLDL_2$ conc	52.7 [a]	56.0 [a]	36.8 [b]	30.7 [c]
RLP-C	19.8 [d]	40.5 [d]	_	_
RLP-TG	20.4 [d]	45.7 [b]	_	_
HDL-C	0.0	0.0	0.0	0.0

Table 3.3 Factors associated with LDL III and Remnant Lipoprotein Concentration in Patients at Baseline

Linear regression analysis of baseline LDL III and remnant lipoproteins with baseline triglyceride, $VLDL_1$, $VLDL_2$, remnant lipoproteins and HDL in patient group. r^2% = coefficient of determination and level of statistical significance is indicated: [a] $p<0.001$, [b] $p<0.005$, [c] $p<0.01$, [d] $p<0.05$.

	LDL III conc (r^2%)	LDL III% (r^2%)	RLP-C (r^2%)	RLP-TG (r^2%)
Triglyceride	70.0 [a]	49.4 [a]	80.1 [a]	77.6 [a]
$VLDL_1$ conc	64.5 [a]	37.8 [b]	68.5 [a]	60.7 [a]
$VLDL_2$ conc	59.4 [a]	32.4 [d]	44.3 [b]	19.4 [d]
RLP-C	67.2 [a]	59.0 [a]	_	_
RLP-TG	49.1 [a]	39.5 [b]	_	_
HDL-C	30.8 [d]	6.5	16.8	27.7 [d]

Table 3.4 Factors associated with LDL III and Remnant Lipoprotein Concentration in Controls at Baseline

Linear regression analysis of baseline LDL III and remnant lipoproteins with baseline triglyceride, $VLDL_1$, $VLDL_2$, remnant lipoproteins and HDL in controls group. r^2% = coefficient of determination and level of statistical significance is indicated: [a] $p<0.001$, [b] $p<0.005$, [c] $p<0.01$, [d] $p<0.05$.

Chapter 4: The Effect of Omega-3 Fatty Acids on Atherogenic Lipoproteins in Proteinuric Patients

4.1 Introduction

It is known that patients with nephrotic range proteinuria posses the atherogenic lipoprotein phenotype characterised by hypertriglyceridaemia, small dense LDL and low HDL-C(187). These abnormalities are proatherogenic conferring an increase in cardiovascular risk with an LDL III concentration of > 100 mg/dl which has been associated with a seven-fold increase in the risk of myocardial infarction (61). Remnant lipoproteins and remnant lipoprotein cholesterol (RLP-C) are also known to be proatherogenic(189) and an independent risk factor for coronary artery disease (190). Their presence is characteristically observed in patients with hypertriglyceridaemia. Deighan et al have previously shown that excess levels of both LDLIII and RLP-C were present in patients with nephrotic range proteinuria and these levels were closely correlated with triglyceride concentration (187).

Omega-3 fatty acids have cardioprotective effects through a number of mechanisms. These have been discussed in chapter 1 and include triglyceride reduction. Theoretically therefore, they would appear to be an ideal treatment to reduce the hypertriglyceridaemia present in patients with proteinuria and as a result, have beneficial effects on both small dense LDL and remnant lipoproteins.

Our aim, therefore, was to examine the effect of high dose omega-3 fatty acids on atherogenic triglyceride rich lipoproteins in patients with nephrotic range proteinuria and also in age and sex matched controls. In particular we aimed to assess changes in small dense LDL and remnant lipoproteins.

4.2 Subjects and Methods

18 patients with nephrotic range proteinuria and 17 controls were recruited as described in chapter 3. One patient did not tolerate omega-3 fatty acids and so 17 patients and 17 controls were analysed. Samples following a ten hour fast were taken at baseline and after an eight-week treatment period with 4g of omega-3 fatty acids daily (Omacor –Solvay). Patients receiving lipid-lowering therapy had their treatment stopped for a period of four weeks prior to inclusion into the study.

4.3 Statistics

Statistical analyses were carried out using MINITAB 13.1 for Windows (Minitab inc). Results are shown as median and interquartile ranges (IQR). Prior to statistical analysis, any factor that was not normally distributed was normalised by log transformation. Patient and control results were compared using unpaired t-tests. Results pre and post treatment were compared using paired t-tests. Linear regression analysis was performed to identify significant correlations. Multivariate analysis was performed using stepwise regression and a general linear model.

4.4 Results

4.4.1 Lipids and Lipoproteins

Changes in lipids and lipoproteins in patients following treatment with omega-3 fatty acids are outlined in table 4.1. In proteinuric patients we found that 4g daily of omega-3 fatty acids reduced plasma triglyceride (TG) concentration by

a mean of 0.45mmol/l (95%CI: 0.16 to 0.74, p=0.005). Plasma VLDL-C also fell following treatment (mean decrease 0.38mmol/l 95% CI: 0.01 to 0.75, p=0.04). This was mainly due to a decrease in larger TG rich $VLDL_1$ rather than $VLDL_2$ with a mean reduction of $VLDL_1$ 33.2mg/dl (95% CI 4.2 to 62.2, p=0.03). It is noteworthy that total plasma cholesterol increased by a mean of 0.25mmol/l (95% CI: 0.0 to 0.5, p=0.05) with an increase in LDL-C in proteinuric patients following treatment with omega-3 fatty acids with a mean increase of 0.6 mmol/l (95% CI 0.2 to 1.1, p=0.06). Plasma concentration of HDL-C did not differ following treatment in the proteinuric patients. In the control group, no significant change in plasma concentration of total cholesterol, triglyceride, VLDL-C, LDL-C, or HDL-C was observed following treatment with omega-3 fatty acids (table 4.2).

4.4.2 LDL Subfractions

In the patient population, there was a significant reduction in the proportion (percent LDL III) and concentration of LDL III. The overall percentage of LDL that was in the form of LDLIII (LDLIII %) fell by a mean of 8.6 (95% CI: 0.8 to 16.4 p=0.01) and LDL III concentration fell by a mean of 26.4 mg/dl (95% CI –14.5 to 67.4, p=0.05). In the patient group, the percentage and concentration of large, light, cholesterol-rich LDLI increased following treatment, with a mean increase in LDLI% of 2.6 (95%CI 0.8 to 4.3 p=0.03 and a mean increase in LDL I concentration of 13.4mg/dl (95%CI 5.2 to 21.6), p=0.001. No change in LDL II concentration or percentage was observed. Total LDL lipoprotein concentration only rose marginally not reaching statistical

significance despite an increase in LDL-C following treatment with omega-3 fatty acids.

Changes in the LDL subfraction profile of the controls were less marked, with no observed difference in LDL III concentration or LDL III %. There was, however, an increase in larger, lighter, less atherogenic LDL with an increase in LDL II concentration of 33.6mg/dl (95% CI 2.1 to 65.0, p=0.02). A significant increase in total LDL concentration was also observed in the controls (31.9 mg/dl: 95% CI 0.6 to 63.2, p=0.04)

Of the patients who completed the study, 13 out of 17 patients (72%) possessed LDL III concentrations above 100mg/dl at baseline falling to 7 out of 17 (41%) after treatment with omega-3 fatty acids. 8 out of 17 controls (47%) had LDL III concentration above 100mg/dl prior to treatment falling to 6 (35%) following treatment.

The effect of omega-3 fatty acids on LDL subfractions was very heterogeneous in both the patient and control populations, ranging from a reduction of 164 mg/dl to an increase of 150 mg/dl in LDL III concentration in the patients group and from a reduction of 78mg/dl to an increase of 92mg/dl in the control group. Figure 4.1 illustrates the effect on LDL subfraction profile before and after treatment with omega-3 fatty acids in one individual patient with a marked reduction in the % LDL III.

4.4.3 Remnant Lipoproteins

Following treatment with omega-3 fatty acids, RLP-C concentration fell in patients by a mean of 3.5mg/dl (95% CI 0.1 to 6.9), p=0.05. Similarly RLP-TG concentration fell by a mean of 12.4mg/dl (95% CI 2.6 to 22.2), p=0.03. In the

control group, RLP-C and RLP-TG were unchanged following treatment with omega-3 fatty acids.

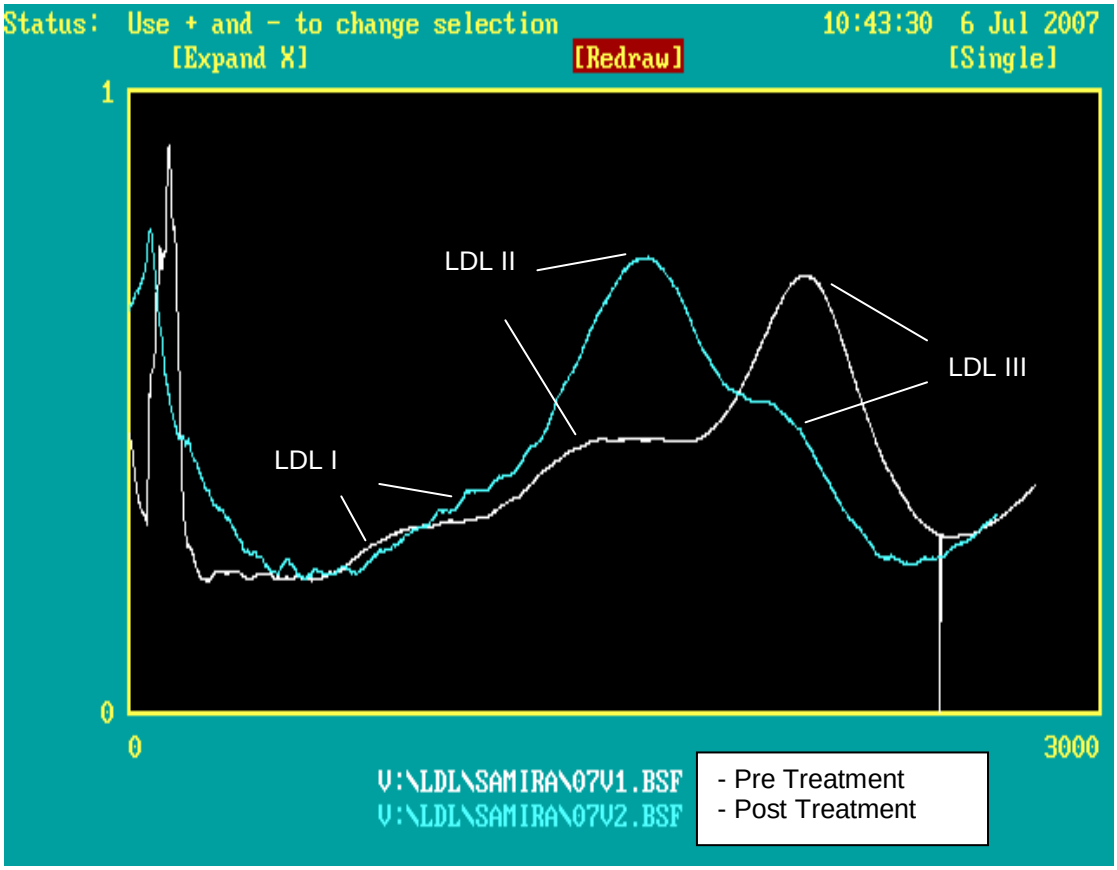

Fig 4.1 Effect on LDL profile in one Patient

Shift in LDL Subfraction profile in an individual patient before and following treatment with Omega-3 fatty acids. Profile changes form predominantly LDL III to LDL II.

4.4.4 Factors determining change in LDLIII and Remnant Lipoproteins

In the patient group, there was no association between either the change in LDL III concentration or the proportion of LDL III (expressed as a percentage) with change in triglyceride, $VLDL_1$, $VLDL_2$, RLP-C or RLP-TG (table 4.5 & figure 4.2). In contrast, a correlation was demonstrated in the control group between the change in both LDL III concentration and LDLIII% and the change in plasma triglyceride, $VLDL_2$ and RLP-TG. As a result of these contrasting results, we proceeded to further statistical analysis using the subject group as an independent variable. This did not show patient group to be an independent predictor of the change in LDLIII concentration or LDLIII% following treatment.

The relationship between remnant lipoprotein reduction and reduction in TG and TG rich lipoproteins were similar in the two groups studied. In the patients, the reduction in RLP-C was associated with the reduction in triglyceride and $VLDL_1$ (r^2 47.5, p=0.001 and r^2=40.2, p=0.004). Whilst the change in RLP-TG correlated with change in $VLDL_1$ (r^2=27.6, p=0.02) but not triglyceride concentration. In the controls, the change in RLP-C and RLP-TG strongly correlated with change in triglyceride, VLDL1 and VLDL2 (table 4.6 & figure 4.3). Again, when subject group was included as an independent variable in multivariate analysis, it was not found to be an independent predictor of change in remnant lipoproteins.

4.5 Discussion

In this study, we have demonstrated that treatment with omega-3 fatty acids in patients with nephrotic range proteinuria has a beneficial effect on atherogenic

lipoproteins. Treatment significantly reduced LDL III concentration and caused a reduction in remnant lipoproteins. Furthermore, they decreased plasma triglyceride and VLDL levels particularly $VLDL_1$. We also observed an increase in plasma LDL- C with omega-3 fatty acid treatment although this was potentially offset by a redistribution to larger, lighter, less atherogenic LDL These effects were not observed in the control group as omega-3 fatty acids neither significantly affected the proportion of, or concentration of LDL III nor did treatment influence plasma triglyceride or VLDL concentration.

The cardioprotective effects of omega-3 fatty acids were first noted following the observation that the Greenland Inuit had a low mortality from CHD despite a high incidence of smoking and obesity. A number of epidemiological and population studies have since demonstrated the cardioprotective effects of omega-3 fatty acids. The Nurses' Health Study found that CHD deaths were 50% lower in those women who consumed fish five times a week. A subgroup of these women with diabetes showed a stronger relationship with a 60% reduction in CHD death (106). The diet and reinfarction trial (DART) (111) found that patients who received dietary advice on increasing fish intake had a relative reduction in mortality of 29% during the 2 year follow-up mainly because of reductions in deaths from CHD. The Physician's Health Study (112) found a reduced risk of sudden cardiac death in subjects with increased levels of omega- 3 fatty acids. Furthermore, the Italian GISSI-Prevenzione trial found that patients randomised to a daily capsule of omega 3 fatty acid (1g) had a reduction in relative risk of 15% in the composite end-point of total mortality, non-fatal myocardial infarction and stroke (113). As a result of these studies, the American Heart Association now recommends that high risk

patients or patients with cardiovascular disease eat fish (particularly fatty fish) at least twice a week.

The cardioprotective effects of omega-3 fatty acids may be as a result of various different mechanisms. These include their effects on atherogenic lipoproteins. In normal controls, omega-3 fatty acids are known to exert a number of effects on plasma lipoproteins. They reduce plasma triglyceride concentration in a dose dependant manner with doses of 4g causing a reduction of 25-30% (191). This is thought to be through suppression of hepatic VLDL and triglyceride production. It has also been suggested that there is increased catabolism of VLDL to LDL through enhanced binding of omega-3 fatty acid enriched VLDL to lipoprotein lipase (192). A review by Harris of human trials concluded that omega-3 fatty acids had no clinically significant effect on total cholesterol with an increase in LDL-C which was more marked in hypertriglyceridaemic patients and a minimal, inconsistent effect on HDL levels(191). At higher doses (3-5g) the LDL-C raising effect of omega-3 fatty acids are well recognized with a redistribution of LDL phenotype to one that is larger, lighter and potentially less atherogenic particles(126).

We observed a fall in LDL III proportion and concentration following treatment with omega-3 fatty acids in the patients with nephrotic range proteinuria. This was not observed in the normal controls. In that context, the mean reduction of triglyceride concentration was 17.5% in the patient group and 5.9% in the control group. However, there was a strong relationship between change in

plasma triglyceride and change in LDL III reduction in the control group, which was not observed in the nephrotic group. We did not find subject group to be an independent factor responsible for these observed differences and so it is likely that these differences are due to a difference in baseline parameters, particularly the baseline levels of plasma triglyceride. It has been hypothesised that in males, in order to produce atherogenic levels of LDL III, adequate hepatic lipase activity and a plasma triglyceride level of >1.5 mmol/l is required. In this study, the majority of the patient group were male (82%) and in 15 out of the 17 patients who completed the study, triglyceride concentration was >1.5 mmol/l at baseline. This fell to 10 out of 17 at the end of treatment. We propose that this explains the lack of relationship between triglyceride reduction and LDL III reduction in the patients group, as the triglyceride reduction was not enough to make plasma triglyceride a rate-limiting factor in the production of small dense LDL with the mean triglyceride in the patients following treatment remaining elevated at 1.8mmol/l. In contrast, 8 out of 17 controls had a baseline triglyceride concentration of >1.5 mmol/l falling to 6 at the end of treatment with a mean triglyceride of 1.5mmol/l. Thus, we suggest the difference in effect of omega-3 fatty acids on LDL III concentration in the 2 populations is due to the difference in baseline triglyceride levels rather than the 2 groups responding differently to treatment.

Remnant lipoproteins were significantly reduced by omega-3 fatty acids in the patient group but not in the control group. Once again, we did not find this to be a group effect and this might be due to the higher baseline levels of triglyceride and $VLDL_1$ in the patient group. We know that triglyceride is a

major determinant of remnant lipoprotein production and we have already shown that they are strongly related to plasma triglyceride and $VLDL_1$ levels. Therefore, both groups display a similar response to the omega-3 fatty acids but as there are higher baseline levels of triglyceride, remnant lipoproteins and a significant triglyceride reduction in the patient group, this results in a marked fall in remnant lipoproteins.

A concerning effect of omega-3 fatty acids was the observed increase in LDL-C in the patient group. This LDL raising effect is a well recognized effect of omega-3 fatty acids (123;191) but is poorly understood. Possible mechanisms include down regulation of the LDL apolipoprotein B/E receptor or a preferential conversion of synthesized VLDL to LDL. This effect on LDL represents one of the controversial aspects of omega-3 fatty acids. Several epidemiological and clinical trials (193-195) have established that the relationship between LDL-C and relative risk for CHD is log-linear. Thus, at any level of LDL-C, for any given mg/dl change in LDL-C level, the change in relative risk is the same (196). This reduction of LDL-C levels, irrespective of how, has been shown to reduce the risk of CHD (197). As discussed in chapter 1, patients with nephrotic range proteinuria have an increased cardiovascular risk, and so increasing levels of LDL-C in these patients could potentially increase this risk. However, it is not known whether the increase in LDL-C caused by omega-3 fatty acids coupled with the redistribution to larger, lighter and therefore potentially less atherogenic LDL would result in an increased cardiovascular risk or whether the other beneficial effects of omega-3 fatty acids (discussed in chapter 1) outweigh this. It has been shown that

cerivastatin reduces LDL III concentrations by 27% in patients with nephrotic range proteinuria(198), therefore it may be useful to combine a statin with omega-3 fatty acids to offset the LDL raising effect.

Omega-3 fatty acids in the form of Omacor were generally well tolerated by both patients and controls. One patient withdrew during the study as he suffered gastrointestinal upset in the form of abdominal pain and diarrhoea. Other reported minor effects were a fishy after taste. Anecdotally, several patients reported that their arthritis had improved on treatment with one patient requesting to continue on treatment after completion of the study.

In conclusion, in patients with nephrotic range proteinuria omega-3 fatty acids reduce LDL III remnant lipoproteins and triglyceride rich lipoproteins thereby reducing cardiovascular risk. However these beneficial effects are offset by an increase in LDL-C. It is not clear whether this increase in LDL-C with redistribution in LDL size confers an increase in cardiovascular risk. One potential role for omega-3 fatty acids could be in conjunction with a statin in order to combine LDL-C lowering with a reduction in atherogenic triglyceride rich lipoproteins. Further work is required in this area to clarify this.

Patients (n=17)	**Baseline**	**Post Treatment**	**p**
Cholesterol (mmol/l)	6.2 (5.5- 7.3)	6.4 (5.5- 7.5)	0.05
Triglyceride (mmo/l)	2.2 (1.7- 2.6)	1.8 (1.2- 2.2)	0.005
VLDL-C (mmol/l)	0.7 (0.6- 1.3)	0.6 (0.4- 0.9)	0.04
LDL-C (mmol/l)	4.1 (3.2- 5.4)	4.6 (4.0- 5.8)	0.06
HDL-C (mmol/l)	1.10 (0.9- 1.2)	1.2 (0.9- 2.4)	ns
IDL-C (mg/dl)	58.2 (43.0- 79.2)	62.3 (48.0- 130.3)	ns
$VLDL_1$ concentration (mg/dl)	106.1 (60.8- 141.1)	70.9 (35.8- 108.0)	0.03
$VLDL_2$ concentration (mg/dl)	87.1 (43.4- 107.9)	73.2 (47.7- 95.2)	ns
RLP-C (mg/dl)	14.6 (5.9- 22.0)	8.8 (6.1- 16.9)	0.05
RLP-TG (mg/dl)	46.0 (21.8- 52.2)	26.4 (19.6- 46.9)	0.03

Table 4.1 The Effect of Omega-3 Fatty Acids on Lipoproteins in Patients

Controls (n=17)	**Baseline**	**Post Treatment**	**p**
Cholesterol (mmol/l)	5.6 (4.7- 5.9)	5.4 (5.0- 6.2)	ns
Triglyceride (mmol/l)	1.5 (1.1- 2.0)	1.3 (1.0- 1.8)	ns
VLDL-C (mmol/l)	0.6 (0.3- 0.7)	0.4 (0.3- 0.7)	ns
LDL- C (mmol/l)	3.8 (3.0- 4.3)	3.9 (3.4- 4.4)	0.07
HDL- C (mmol/l)	1.1(0.9- 1.4)	1.2 (1.0- 1.5)	ns
IDL (mg/dl)	52.7 (43.4- 63.6)	53.8 (43.8- 62.7)	ns
$VLDL_1$ concentration (mg/dl)	64.6 (35.2- 114.8)	59.6 (25.4- 96.1)	ns
$VLDL_2$ concentration (mg/dl)	43.4 (34.8- 60.6)	43.2 (31.7- 100.0)	ns
RLP-C (mg/dl)	5.6 (4.1- 12.7)	5.2 (3.7- 12.4)	ns
RLP-TG (mg/dl)	23.8 (20.8- 49.2)	22.0 (19.4- 40.6)	ns

Table 4.2 The Effect of Omega-3 Fatty Acids on Lipoproteins in Controls

Patients	Baseline	Post Treatment	p
LDL I %	8.4 (5.4- 17.2)	11.5 (6.5- 19.3)	0.03
LDL II%	40.4 (33.6- 62.4)	55.2 (34.2- 64.8)	ns
LDL III %	49.9 (17.4- 61.2)	29.8 (14.9- 58.5)	0.01
LDL I conc (mg/dl)	41.3 (20.6- 59.4)	54.9 (24.9- 81.2)	0.001
LDL II conc (mg/dl)	184.8 (104.9- 248.4)	215.9 (138.2- 309.2)	ns
LDL III conc (mg/dl)	178.8 (61.6- 231.0)	96.1 (49.3- 204.5)	0.05
Total LDL conc (mg/dl)	370.5 (270.8- 480.7)	397.8 (313.8- 527.3)	ns

Table 4.3 The Effect of Omega-3 Fatty Acids on LDL Subfractions in Patients

Controls	Baseline	Post Treatment	p
LDL I %	8.1 (3.8- 16.1)	13.2 (7.5- 54.6)	0.07
LDL II%	65.2 (27.3- 68.3)	66.1 (39.4- 72.0)	ns
LDL III %	24.7 (12.0- 68.4)	16.9 (11.6- 38.8)	ns
LDL I conc (mg/dl)	28.9 (14.8- 57.3)	41.0 (25.2- 62.2)	ns
LDL II conc (mg/dl)	167.0 (72.8- 260.6)	213.7(132.5- 258.3)	0.02
LDL III conc (mg/dl)	53.3 (29.8- 182.0)	50.3 (36.9- 142.6)	ns
Total LDL conc (mg/dl)	317.5 (253.9- 374.2)	338.0 (299.1- 400.8)	0.04

Table 4.4 The Effect of Omega-3 Fatty Acids on Lipoproteins in Controls

	ΔLDL III conc	ΔLDL III% (r^2%)	ΔRLP-C	ΔRLP-TG
ΔTriglyceride	0.7	11.9	47.5[a]	0
Δ$VLDL_1$ conc	0	2.3	40.2[b]	27.6[c]
Δ$VLDL_2$ conc	5.1	12.4	0	8.9
ΔRLP-C	0	13	-	-
ΔRLP-TG	0	3.5	0	-

Table 4.5 Factors Associated with Change in LDL III and Remnant Lipoprotein Concentration in Patients

Linear regression analysis of change in LDL III and remnant lipoproteins with change in triglyceride, $VLDL_1$, $VLDL_2$ and remnant lipoproteins in the patient group. r^2% = coefficient of determination. Level of statistical significance is indicated: [a] p=0.001, [b]p=0.004, [c]p=0.02.

	ΔLDL III conc	ΔLDL III% (r^2%)	ΔRLP-C	ΔRLP-TG
ΔTriglyceride	50.2[b]	38.5[c]	74.9[a]	80.7[a]
Δ$VLDL_1$ conc	4.6	9.4	69.7[a]	56.0[a]
Δ$VLDL_2$ conc	41.1[b]	41.5[b]	35.3[c]	51.3[a]
ΔRLP-C	17.1	16.3	-	-
ΔRLP-TG	30.7[d]	41.4[b]	83.5[a]	-

Table 4.6 Factors associated with Change in LDL III and Remnant Lipoprotein Concentration in Controls

Linear regression analysis of change in LDL III and remnant lipoproteins with change in triglyceride, $VLDL_1$, $VLDL_2$ and remnant lipoproteins in the control group. r^2% = coefficient of determination. Level of statistical significance is indicated:
[a] $p<0.001$, [b] $p<0.005$, [c] $p<0.01$, [d] $p<0.05$.

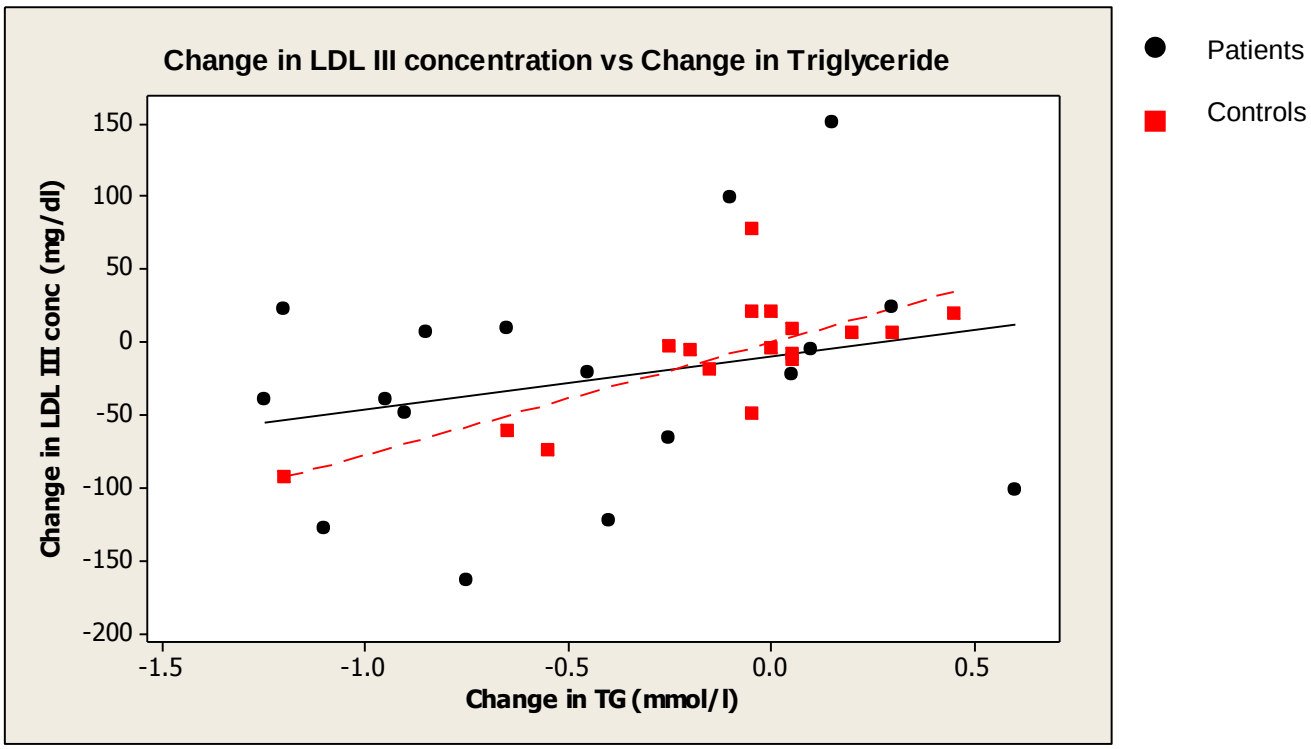

Figure 4.2 Change in LDL III concentration vs Change in Triglyceride

Linear Regression analysis of change in LDL III concentration with change in triglyceride concentration in patients and controls as indicated.
(Patients r^2=0.7%, p=ns & Controls r^2=50.2%, p<0.005)

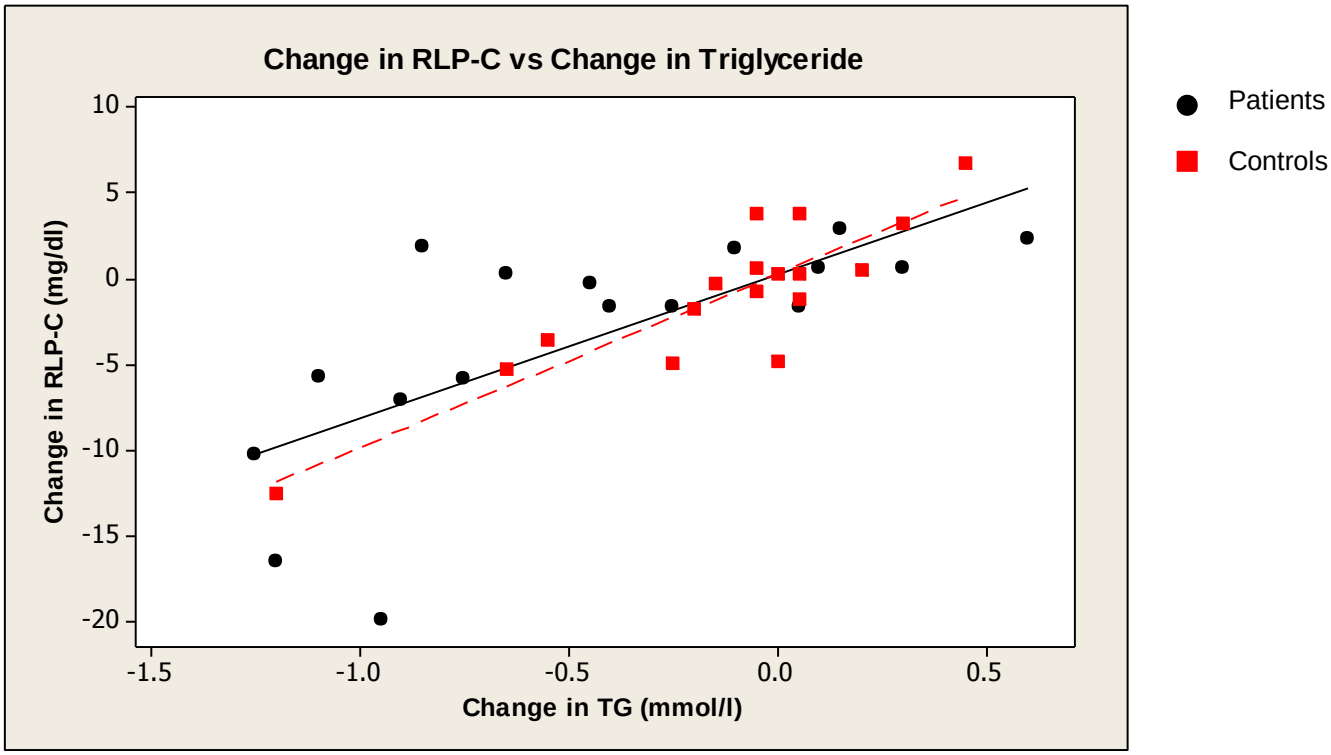

Figure 4.3 Change in RLP-C vs Change in Triglyceride Concentration

Linear Regression analysis of change in RLP-C with change in triglyceride concentration in patients and controls as indicated.
(Patients r^2= 47.5%, p=0.001 & Controls r^2= 74.9, p<0.001)

Chapter 5: Omega-3 Fatty Acids and Postprandial Lipaemia

5.1 Introduction

The importance of postprandial lipid metabolism in the development of coronary artery disease is well established.

Chylomicrons are the largest and lightest triglyceride-rich lipoproteins. They are plentiful postprandially but almost absent when fasted. They are synthesised in response to dietary lipid intake and are responsible for transporting dietary triglyceride and cholesterol. They are composed mainly of triglyceride with apo B48 as the structural protein. VLDL are also triglyceride-rich but have apo B100 as the structural protein. They are responsible for the transport of endogenously synthesised triglyceride. VLDL particles can be subdivided into two subfractions according to their flotation coefficients using cumulative gradient centrifugation (described in chapter 2). $VLDL_1$ (Sf 60-400) is larger, lighter and more triglyceride-rich. $VLDL_2$ (Sf 20-60) is smaller, denser and more enriched in cholesteryl ester. These two subfractions appear to be independently regulated. $VLDL_1$ is associated with raised plasma triglyceride and overproduced in patients with insulin resistance (199). $VLDL_2$ is overproduced in patients with raised LDL cholesterol. $VLDL_1$ is inefficiently converted to LDL in contrast to $VLDL_2$ which is rapidly metabolised to LDL. Patients with nephrotic range proteinuria have been found to have an increase in plasma concentrations of both these VLDL subfractions (162;163).

The effects of omega-3 fatty acids on postprandial lipaemia is not completely understood but they are known to decrease hepatic VLDL production, increase VLDL to LDL conversion, reduce the magnitude of the postprandial triglyceride response (119;200;201) and increase chylomicron clearance. We

aimed to examine: (1) chylomicron and $VLDL_1$ metabolism in patients with nephrotic range proteinuria compared with controls (2) The effect of omega-3 fatty acids on chylomicron and $VLDL_1$ metabolism in patients compared with controls.

5.2 Methods

The same study population described in the methods section were used for this study. Patients and controls were treated with 4 g daily of omega-3 fatty acids (Omacor) for a period of eight weeks and attended on 2 occasions: once prior to treatment and also at the end of the treatment period. They ingested a 90g fat load in the form of a calogen drink. Chylomicrons and $VLDL_1$ were isolated by density ultracentrifugation from EDTA plasma (as described in chapter 2) in fasting and at 2 hour, 4 hour, 6 hour and 8 hour samples after the fat load. Separation of lipoproteins was performed immediately after sampling to prevent degradation of the chylomicrons. Chylomicron and $VLDL_1$ concentrations were measured also as described in chapter 2. Incremental area under the curve (AUC) was calculated by standard methods to quantify postprandial lipoprotein concentrations.

5.3 Statistics

Statistical analyses were performed using MINITAB 14X for Windows. Any factors that were not normally distributed were subject to log transformation. These included chylomicron incremental area under the curve. Results are shown as median and interquartile range (IQR). Paired data were compared using a paired t-tests and comparison between patients and controls were

carried out using 2 sample t-tests. Simple regression analyses were performed to elucidate associations. A General Linear Model was used to perform multivariate analysis if a categorical variable was included.

5.4 Results

5.4.1 Postprandial Chylomicron Concentration

AUC (mmol/l.h)	Baseline	Post Treatment	p
Patients	18.5 (8.9- 32.6)*	13.5 (7.4-22.9)	0.05
Controls	9.2 (4.8- 14.4)	7.2 (4.6- 14.5)	ns

Table 5.1 Chylomicron AUC in Patients and Controls at baseline and following treatment
*p=0.05 when compared with controls

At baseline, postprandial chylomicron AUC was greater in patients compared with controls (Median 18.5mmol/l.h: IQR 8.9-32.6 vs 9.2 mmol/l.h:4.8-14.4) p=0.05. Following treatment we observed a fall in patient chylomicron AUC with a mean reduction of 6.8mmol/l.h (95% CI 0.1-13.6, p=0.05). However, in the control population, the reduction in chylomicron AUC was not significant with a mean decrease of 3.9mmol/l.h (95% CI –3.6 to 11.5, p=ns). As a result of the decrease in patient chylomicron AUC, at the end of 8 weeks treatment, patient and control chylomicron AUC concentrations were no longer significantly different (Table 5.1). Figure 5.1 shows chylomicron triglyceride

concentration in patients and controls before and after treatment with omega-3 fatty acids.

In patients, peak postprandial chylomicron triglyceride concentration fell following treatment with omega-3 fatty acids from 5.5mmol/l (95% CI 2.5- 7.4) to 3.8mmol/l (95% CI 1.7- 5.3, p=0.04). This reduction was not observed in the control group 3.7mmol/l (95% CI 1.0- 4.1) at baseline to 2.7 (95% CI 1.2- 4.1), p=ns.

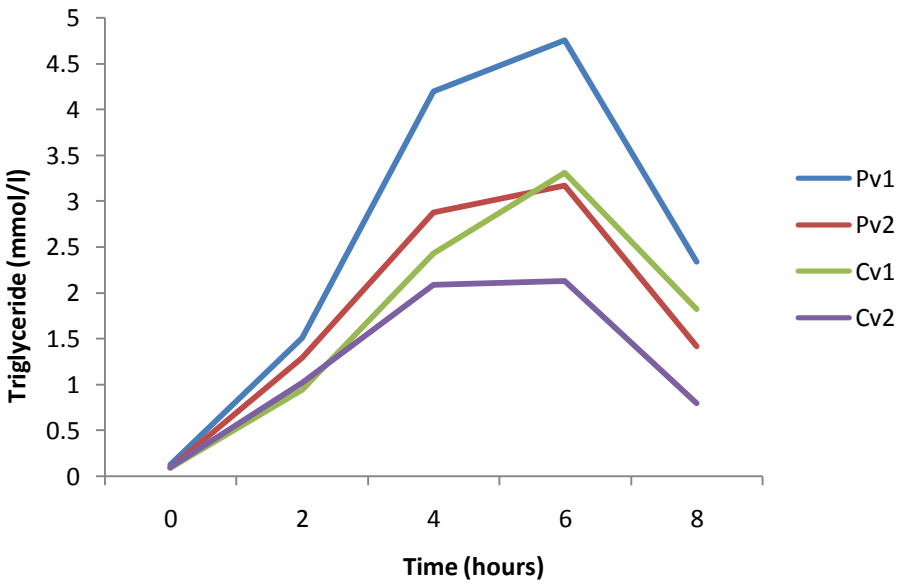

Figure 5.1 Postprandial Chylomicron Triglyceride Concentration

Mean chylomicron triglyceride concentration over 8 hours following a fat load in patients and controls at baseline and following treatment
(Pv1: patients at baseline, Pv2: patients post treatment, Cv1: controls at baseline Cv2: controls post treatment)

5.4.2 Relationship Between Chylomicron AUC and Baseline Parameters

The relationship between baseline chylomicron AUC and baseline parameters are shown in table 5.2. There was no relationship between baseline chylomicron AUC and baseline urinary proteinuria or renal function in patients. In the patient group, chylomicron AUC correlated with baseline triglyceride, $VLDL_1$, $VLDL_2$, remnant lipoproteins and LDL III concentrations. Similar associations were observed in the control group with tighter correlations.

5.4.3 Relationship Between Change in Chylomicron AUC

No association was identified between any of the baseline parameters and the change in chylomicron AUC in the patient group. In the control population only the baseline levels of remnant lipoproteins correlated with change in chylomicron AUC (RLP-C r^2 27.2, p=0.02 and RLP-TG r^2 20.6, p=0.04) (figures 5.3 and 5.4).

Associations between change in chylomicron AUC and change in other lipid and lipoprotein parameters following treatment with omega-3 fatty acids are outlined in table 5.3. The only factors, which were associated with the change in chylomicron AUC in the patient group, were the change in LDL III concentration and the change in LDL III %. However, in the control group, extensive associations were seen with change in chylomicron AUC related to change in triglyceride, $VLDL_1$, $VLDL_2$, LDL III%, LDL III concentration, RLP-C and RLP-TG (figures 5.5 and 5.6).

In view of the apparent differences between the two groups, we proceeded to multivariate analysis using patient group as an independent categorical variable. This demonstrated that the difference between the 2 groups was not related to differences in either baseline chylomicron AUC or baseline triglyceride, but that the categorical variable of patient group appeared to be an independent predictor of the change in chylomicron AUC following treatment with omega-3 fatty acids ($p=0.03$).

Baseline Parameters	All		Patients		Controls	
	r^2	p	r^2	p	r^2	p
Triglyceride	35.7	<0.001	20.8	0.04	74.4	<0.001
$VLDL_1$	37.0	<0.001	40.6	0.004	76.2	<0.001
$VLDL_2$	9.9	0.04	25.3	0.02	77.6	<0.001
RLP-C	39.2	<0.001	31.2	0.01	61.3	<0.001
RLP-TG	37.9	<0.001	27.8	0.02	37.3	0.005
LDLIII conc	31.0	<0.001	19.9	0.04	65.6	<0.00

Table 5.2: Association Between Baseline Chylomicron AUC and Baseline Parameters

Linear regression analysis of baseline chylomicron AUC with baseline parameters in all subjects as a whole, patients and controls. (r^2= coefficient of determination).

Parameters	All		Patients		Controls	
	r^2	p	r^2	p	r^2	p
Δ Triglyceride	2.7	ns	0	0	49.7	0.001
Δ $VLDL_1$	1.3	ns	0	0	30.3	0.01
Δ $VLDL_2$	0	ns	0	0	41.6	0.003
Δ LDL III conc	0.3	ns	28.1	0.02	28.4	0.02
Δ LDL III %	0	ns	21.9	0.03	41.7	0.03
Δ RLP-C	0.7	ns	0	0	43.3	0.002
Δ RLP-TG	0	ns	0	0	59.7	<0.001

Table 5.3: Association Between Change in Chylomicron AUC and Change in Parameters
Linear regression analysis of change in chylomicron AUC with change in parameters in all subjects as a whole, patients and controls. (r^2= coefficient of determination).

5.4.4 Postprandial VLDL Concentration

AUC (mmol/l.h)	Baseline	Post Treatment	p
Patients	7.4 (4.2- 11.8)	5.6 (4.2- 10.1)	ns
Controls	4.6 (1.1- 9.8)	3.2 (1.5- 5.9)	ns

Table 5.4 $VLDL_1$ AUC in Patients and Controls at Baseline and Following Treatment

At baseline, postprandial $VLDL_1$ AUC did not differ between patients and controls [Median 7.4mmol/l.h (IQR 4.2- 11.8) vs 4.6mmol/l.h (1.1- 9.8), p=ns]. Following treatment with omega-3 fatty acids, there was a mean reduction of 1.7mmol/l.h (95% CI -1.0 to 3.4) in patients and 1.5mmol/l.h (95% CI -1.9 to 3.2) in control, both p=ns.

At the end of treatment, patients and control postprandial $VLDL_1$ AUC remained similar (Median 5.6mmol/l.h (4.2- 10.1) vs 3.2mmol/l.h (1.5- 5.9), p=ns). Figure 5.2 shows $VLDL_1$ triglyceride concentration in patients and controls before and after treatment with omega-3 fatty acids. Patient peak postprandial $VLDL_1$ triglyceride concentration fell from 3.3mmol/l (IQR 2.0- 4.2) to 2.4mmol/l (1.5- 3.4), p=0.04. Similarly, in the control group, peak postprandial $VLDL_1$ triglyceride concentration fell from 2.7mmol/l (IQR 1.1- 3.5) to 1.7mmol/l (0.8- 2.7), p=0.03.

5.4.5 Relationship Between $VLDL_1$ AUC and Baseline Parameters

The association between baseline $VLDL_1$ AUC and baseline parameters in patients and controls are shown in table 5.5. Baseline $VLDL_1$ AUC did not correlate with baseline serum creatinine, eGFR or urinary proteinuria in the patient group. In the patient group, the lack of relationship between baseline $VLDL_1$ AUC and baseline triglyceride is noteworthy. A clear outlier who is behaving differently from the other patients can explain this. Once this outlier is removed, a strong correlation is present (r^2=31%, p=0.015). On multivariate analysis, subject group was not an independent predictor of baseline $VLDL_1$ AUC.

Change in $VLDL_1$ AUC did not correlate with any baseline parameter or change in triglyceride, $VLDL_1$, $VLDL_2$, LDL III%, LDL III concentration, RLP-C and RLP-TG in either patients or controls.

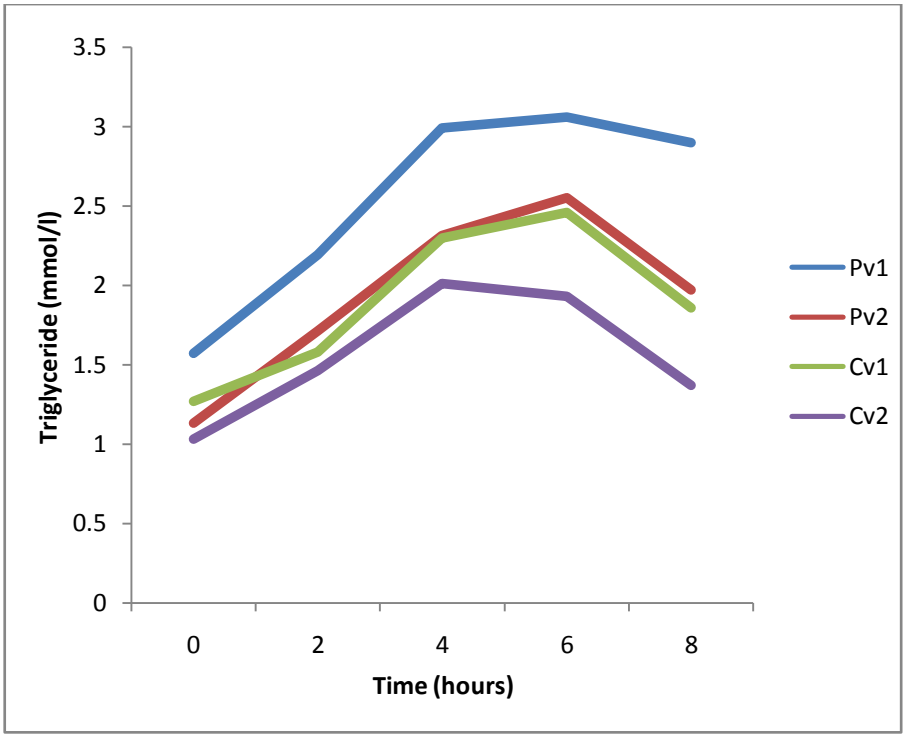

Figure 5.2 Postprandial $VLDL_1$ Triglyceride Concentration

Mean $VLDL_1$ triglyceride concentration over 8 hours following a fat load in patients and controls at baseline and following treatment
(Pv1: patients at baseline, Pv2: patients post treatment, Cv1: controls at baseline Cv2: controls post treatment).

Baseline Parameters	Patients		Controls	
	r^2	p	r^2	p
Triglyceride	14.6	0.07	82.9	<0.001
$VLDL_1$	36.7	0.006	91.6	<0.001
$VLDL_2$	30.3	0.01	74.0	<0.001
RLP-C	15.0	0.07	65.0	<0.001
RLP-TG	16.8	0.06	49.4	0.001
LDLIII %	10.9	0.1	39.1	0.004
LDLIII mass	23.7	0.03	67.0	<0.001

Table 5.5: Association Between $VLDL_1$ AUC and Baseline Parameters
Linear regression analysis of $VLDL_1$ AUC with baseline parameters in patients and controls, r^2= coefficient of determination.

5.5 Discussion

The aim of this study was to investigate chylomicron and $VLDL_1$ metabolism in patients with nephrotic range proteinuria, compare them to normal controls and to examine the effect of treatment with omega-3 fatty acids on their metabolism. By separating chylomicrons and $VLDL_1$ from plasma through density gradient ultracentrifugation, we have shown that postprandial chylomicron concentration is significantly greater in patients with nephrotic range proteinuria compared to normal controls. We demonstrated that baseline triglyceride and triglyceride rich lipoproteins were associated with baseline chylomicron concentration and that treatment with omega-3 fatty acids led to a significant reduction in chylomicron AUC in the patient group but not the controls. In patients, change in chylomicron concentration was associated with change in LDL III proportion and concentration. There was no association between change in chylomicron concentration and change in triglyceride, $VLDL_1$, $VLDL_2$ and remnant lipoproteins in the patient group. However, in the control group there was a strong association with all these parameters.

$VLDL_1$ AUC did not differ at baseline between patients and controls. Furthermore, there was no significant effect on $VLDL_1$ AUC following treatment in patients or controls. However, peak postprandial $VLDL_1$ triglyceride concentration was significantly lowered by treatment with omega-3 fatty acids in all subjects. Baseline $VLDL_1$ AUC correlated with baseline $VLDL_1$, $VLDL_2$ and LDL III mass in patients. In controls, there was a strong correlation between $VLDL_1$ AUC and triglyceride-rich lipoproteins.

There is little information in the literature on chylomicron metabolism in humans with nephrotic syndrome. Animal studies have shown changes in chylomicron synthesis and catabolism as well as qualitative compositional changes (202;203). Warwick et al examined chylomicron metabolism in 9 patients with nephrotic range proteinuria by measuring changes in triglyceride and retinyl palmitate concentration in the d<1.006g/ml fraction of plasma following Vitamin A loaded standard fat meal. They found no significant difference in the time course of the lipaemic response, which was confirmed by comparing AUC curves for controls and patients. They concluded that chylomicron metabolism did not differ between patients and controls (204). This was a small study, which has not been confirmed.

As discussed earlier, the effects of omega-3 fatty acids on lipid metabolism is not completely understood. There is evidence to suggest that omega-3 fatty acids reduce triglyceride levels after a postprandial challenge(130) and they have also been shown to lower postprandial chylomicron and chylomicron remnants (122). This is thought to be through acceleration of chylomicron lipid clearance by facilitation of lipoprotein lipase-mediated lipolysis (120). We observed a reduction in chylomicron AUC in the patient group but not the controls and found this reduction was independent of change in triglyceride (figures 5.5 & 5.6) and baseline chylomicron AUC. Multivariate analysis showed that subject group was an independent predictor of change in chylomicron AUC following treatment with omega-3 fatty acids. The reasons behind this are unclear but may be related to structural or compositional change in the chylomicrons of patients with nephrotic range proteinuria. Westphal et al examined the effect of 4g of omega-3 fatty acids (Omacor) on

hypertriglyceridaemic men following a fat load. They found chylomicron concentration to fall by 49-64% over 4-8 hours(205). Thus, the effect of omega-3 fatty acids on chylomicrons is more marked at higher baseline triglyceride levels.

Treatment with omega-3 fatty acids has been shown to reduce VLDL by reducing their synthesis and secretion. They inhibit 1,2-diacylglycerol-sterol O-acyltransferase(206) or phosphatidate phosphatase (207) thereby resulting in a substantial reduction in synthesis of VLDL. This reduced synthesis would be in keeping with our finding that peak postprandial $VLDL_1$ was reduced but $VLDL_1$ AUC was unchanged by treatment with omega-3 fatty acids.

Zilversmit described atherosclerosis as a 'postprandial phenomenon' over 20 years ago (42). Since then, a number of studies have linked disturbed metabolism of postprandial lipoproteins with increased cardiovascular risk. A meta-analysis of 17 prospective studies found that a 1 mmol/l rise in fasting plasma triglyceride was associated with a 30% risk of total CVD in man and 75% increase in women (208). Postprandial lipaemia is defined as the physiological transitory alteration in lipoprotein metabolism lasting from 6 to 12 hours after ingesting a fatty meal. Fasting plasma triglycerides are known to predict the duration and magnitude of postprandial lipaemia (119). The hypertriglyceridaemia, which occurs following a meal, reflects the accumulation of triglyceride rich lipoproteins such as chylomicrons and their remnants (early response) plus VLDL and its remnants (late response). These triglyceride rich lipoproteins are important in the pathogenesis of atherosclerosis as they allow the accumulation of lipids within the arterial wall

through the generation of small dense LDL and reduction in HDL(209). Patients with nephrotic range proteinuria are known have an increased cardiovascular risk. They are known to have higher levels of fasting triglycerides at baseline than normal controls(164) therefore it would follow that they would have higher levels of postprandial triglyceride rich lipoproteins contributing to their higher cardiovascular risk. The recognition of these abnormalities is important as it allows us to target treatment thereby reducing this excess cardiovascular risk in this population.

The effect of omega-3 fatty acids on postprandial lipaemia in patients with nephrotic range proteinuria has not, to our knowledge, been studied before. Statins are currently the mainstay of treatment for these patients as they reduce cholesterol and LDL-C concentration. Fibrates are effective in reducing triglyceride levels particularly in hypertriglyceridaemic patients but are poorly tolerated in patients with renal disease. Monotherapy with statins alone may not be sufficient in this group of patients to have an adequate effect on both cholesterol and postprandial lipaemia. We have shown that omega-3 fatty acids are effective in reducing postprandial lipaemia in patients with nephrotic syndrome. This effect is not related to the change in triglyceride level but is related to higher baseline triglyceride levels in these patients.

In summary, we have shown that there is increased postprandial lipaemic response in patients with nephrotic range proteinuria and that this is reduced by treatment with omega-3 fatty acids. These differences may be due to structural and compositional differences in chylomicrons and VLDL in patients

with nephrotic range proteinuria compared with the controls. Further analysis of the composition and structure of these particles may provide us with more information on how to interpret these results.

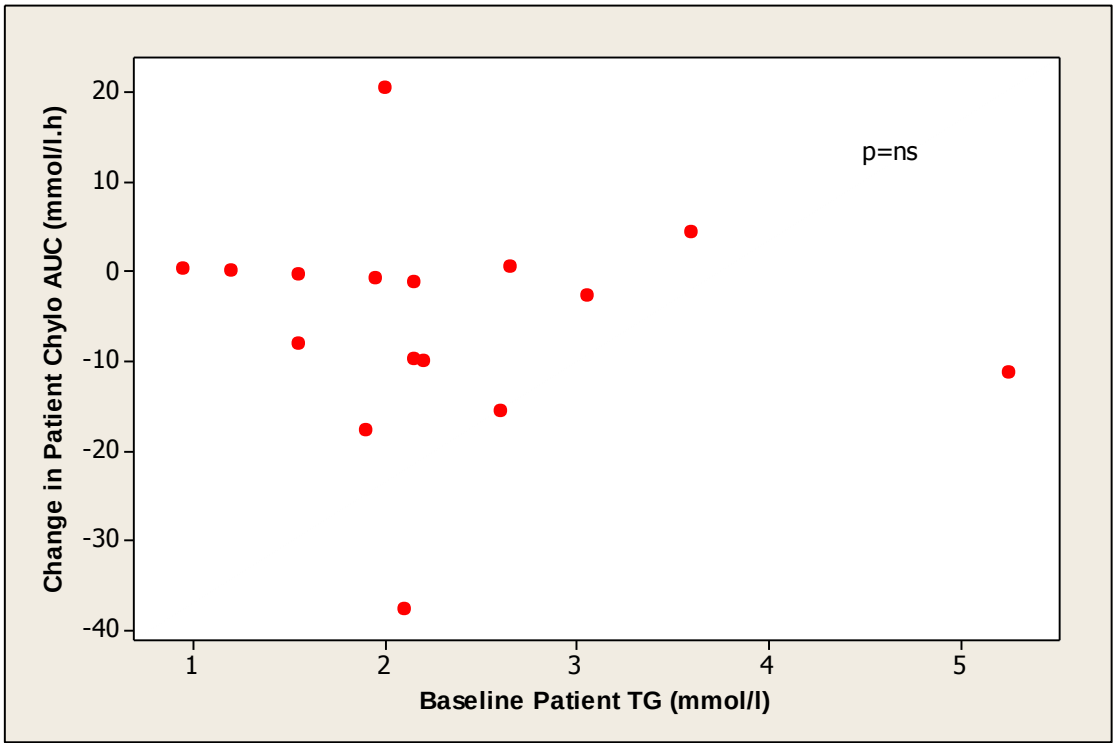

Figure 5.3: Patient Change in Chylomicron AUC vs Baseline Triglyceride Concentration

Linear regression analysis of change in chylomicron concentration with baseline triglyceride concentration in patients.

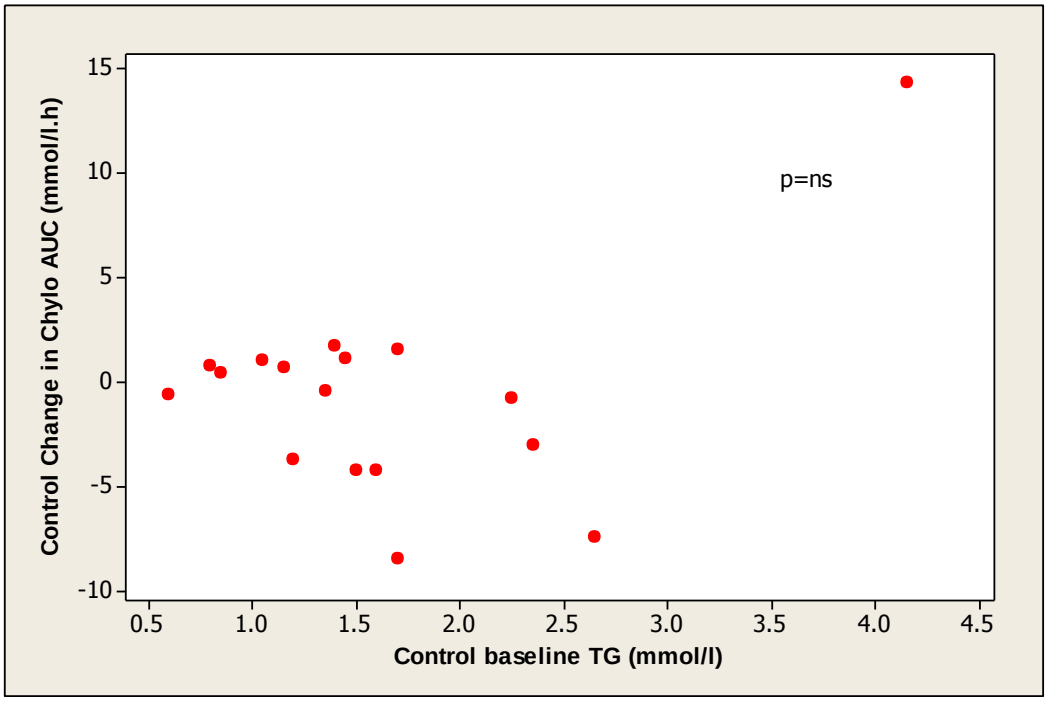

Figure 5.4: Control Change in Chylomicron AUC vs Baseline Triglyceride Concentration
Linear regression analysis of change in chylomicron concentration with baseline triglyceride concentration in controls.

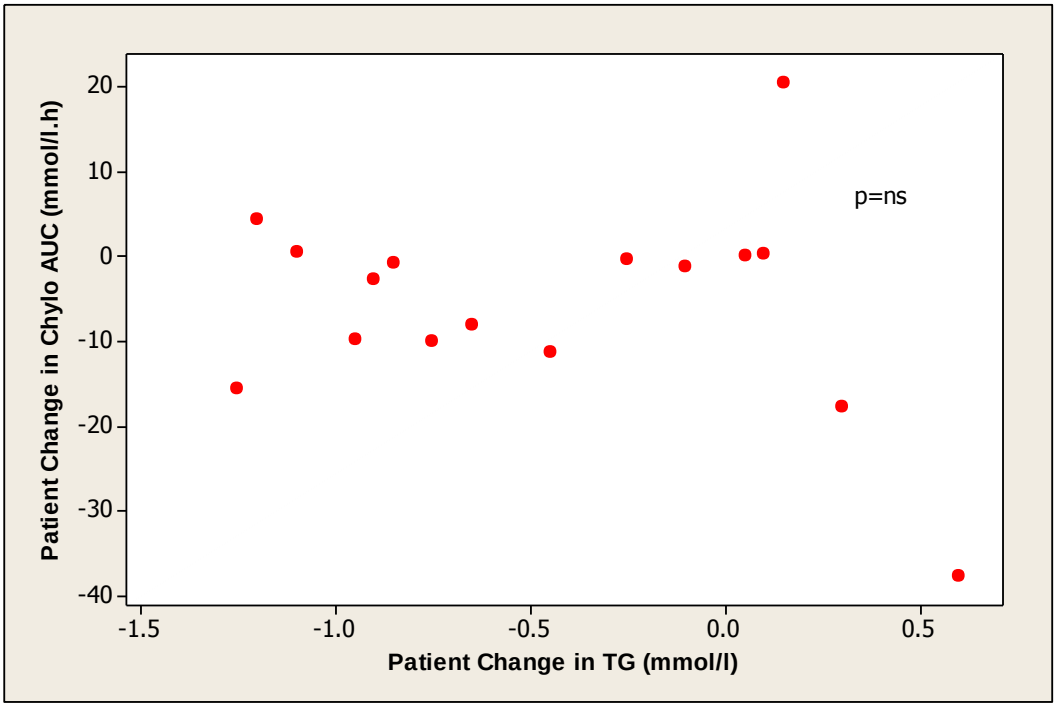

Figure 5.5: Patient Change in Chylomicron AUC vs Patient Change in Triglyceride Concentration
Linear regression analysis of change in chylomicron concentration with change in triglyceride concentration in patients.

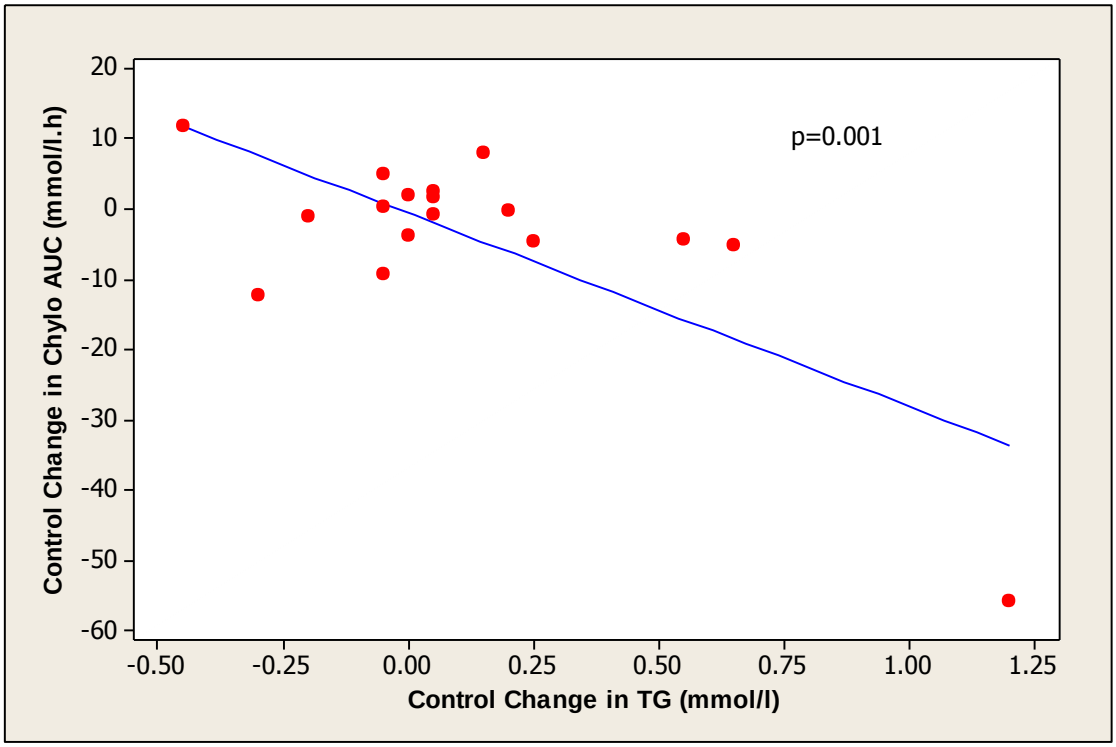

Figure 5.6: Control Change in Chylomicron AUC vs Control Change in Triglyceride Concentration

Linear regression analysis of change in chylomicron concentration with change in triglyceride concentration in controls, r^2= 49.7%, p=0.001.

Chapter 6: The Effect of Omega-3 Fatty Acids on Structure and Composition of Chylomicron and $VLDL_1$ Particles

6.1 Introduction

Patients with nephrotic range proteinuria have an excess of triglyceride rich lipoproteins such as chylomicrons and VLDL. In the previous chapter, we have demonstrated that there is delayed postprandial chylomicron clearance in patients with proteinuria. Several studies have shown that the increase in the $VLDL_1$ subgroup of VLDL in patients with nephrotic range proteinuria is as a result of delayed lipoprotein clearance and increased hepatic production although our data from chapter 5 did not confirm the presence of impaired clearance(162;165;168;210).

Our laboratory has previously shown abnormalities in the structure of $VLDL_1$ in patients with nephrotic range proteinuria (175) with a smaller more crystalline structure and we postulated that this abnormal structure contributed to the impaired clearance of VLDL particles. The evidence regarding the structure of chylomicrons in humans is sparse.

Therefore, the aim of our study was firstly to examine the apolipoprotein and lipid components of chylomicrons and $VLDL_1$ in patients with nephrotic range proteinuria thereby establishing the structure of these particles in both patient and control populations. Thereafter, we examined the effect of omega-3 fatty acids on the structure of these lipoproteins in patients and controls.

6.2 Subjects and Methods

The patients and controls described in the method section were studied in this chapter. Chylomicrons and $VLDL_1$ fractions were separated as described previously. Apolipoproteins B, AI, CII, CIII and E were measured on fasting EDTA plasma samples from patients before and following treatment with omega-3 fatty acids. Apolipoproteins B, CII, CIII and E were measured on fasting VLDL and peak chylomicrons in both patient and control groups pre and post treatment.

6.3 Results

6.3.1 Baseline Plasma Apolipoproteins

The plasma content of apolipoproteins is shown in table 6.1. There was significantly less apolipoprotein CIII in the controls with a trend towards less apolipoprotein B and CII.

Each VLDL, IDL and LDL particle carries one moiety of apo B therefore, using this as a reference, a crude estimate of the amount of apo CII, CIII and E moieties per lipoprotein particle in the plasma was obtained. As a result, we found no difference between the amount of CII, CIII and E per lipoprotein particle (table 6.1).

Apolipoprotein	Patients	Controls	p
B (mg/dl)	122.5 (100.9- 139.2)	112.1 (96-0- 119.4)	ns
AI (mg/dl)	125.6 (115.0- 138.9)	137.4 (116.0- 167.9)	ns
CII (mg/dl)	5.6 (4.4- 7.1)	4.29 (3.3- 5.5)	ns
CIII (mg/dl)	15.1 (13.1- 18.2)	12.6 (10.1- 14.3)	0.02
E (mg/dl)	4.7 (4.1- 5.6)	4.0 (3.5- 5.0)	ns
Apo CII/B*	4.4 (3.5- 5.9)	4.2 (2.9- 4.8)	ns
Apo CIII/B*	12.2 (10.6- 15.5)	11.7 (9.6- 12.8)	ns
Apo E/B*	3.8 (3.0- 5.1)	3.8 (3.3- 4.4)	ns

* (mg/mg x 10^{-2})

Table 6.1: Plasma Apolipoproteins (n=17)
Plasma apolipoproteins in patients compared with controls. Prior to statistical analysis, any parameter which was not normally distributed was normalised by log transformation. All data were compared using unpaired t-tests.

6.3.2 The Effect of Treatment on Plasma Apolipoproteins

Tables 6.2 and 6.3 represent the effect of treatment with omega-3 fatty acids on plasma apolipoprotein concentration in patients and controls. The plasma apolipoproteins of patient and controls were not affected by treatment with omega-3 fatty acids. There was, however, a significant reduction of CII per lipoprotein particle in the patient group. This was not seen in the control population

Apolipoprotein	Baseline	Post Treatment	p
B (mg/dl)	122.5 (100.9- 139.2)	123.7 (114.8- 154.8)	ns
AI (mg/dl)	125.6 (115.0- 138.9)	124.2 (113.5- 138.9)	ns
CII (mg/dl)	5.6 (4.4- 7.1)	5.0 (4.1- 6.9)	ns
CIIII (mg/dl)	15.1 (13.1-18.2)	14.4 (13.2- 18.8)	ns
E (mg/dl)	4.7 (4.1- 5.6)	5.0 (4.3- 5.6)	ns
Apo CII/B*	4.4 (3.5- 5.9)	4.1 (3.1- 5.3)	0.01
Apo CIII/B*	12.2 (10.6- 15.5)	11.6 (10.0- 12.6)	ns
Apo E/B*	3.8 (3.0- 5.1)	3.7 (3.3- 4.5)	ns

All values median & IQR
* (mg/mg x 10^{-2})

Table 6.2: Effect of Treatment on Patient Plasma Apolipoproteins (n=17)

The effect of treatment on plasma apolipoproteins in patients. Prior to statistical analysis, any parameter which was not normally distributed was normalised by log transformation. All data were compared using paired t-tests.

Apolipoprotein	Baseline	Post Treatment	p
B (mg/dl)	112.1 (96.0- 119.4)	110.0 (96.2- 122.1)	ns
AI (mg/dl)	137.4 (116.0- 167.9)	138.8 (114.6- 172.0)	ns
CII (mg/dl)	4.3 (3.3- 5.5)	4.3 (3.1- 5.6)	ns
CIIII (mg/dl)	12.6 (10.1- 14.3)	12.7 (9.1- 13.9)	ns
E (mg/dl)	4.0 (3.5- 5.0)	4.3 (3.6- 5.4)	ns
Apo CII/B*	4.2 (2.9- 4.8)	4.1 (2.9- 4.7)	ns
Apo CIII/B*	11.7 (9.6- 12.8)	11.2 (9.0- 13.3)	ns
Apo E/B*	3.8 (3.3- 4.4)	3.8 (3.4- 4.5)	ns

Table 6.3: Effect of treatment on Control Plasma Apolipoproteins (n=17)
The effect of treatment on plasma apolipoproteins in controls. All values expressed as median & IQR. * (mg/mg x 10^{-2})

6.3.3 Chylomicron Apolipoproteins & Compositions: Patients vs Controls

The concentration of chylomicron apolipoproteins in both patient and control groups are shown in table 6.4. The analysis of the chylomicron apolipoproteins was performed in 10 patients and 6 controls as the concentration of chylomicrons were too low in some patients and controls to allow accurate measurement of either apo CII, CIII or E. If one of the chylomicron apolipoproteins was undetectable, then all of the apolipoprotein and composition results for that subject were disregarded to avoid biasing the result. There was twice as much apo B present in the control chylomicrons (p=0.03) compared with patients, but the quantities of other chylomicron

apolipoproteins did not differ significantly. The molar ratio of apolipoprotein CII, CIII and E to apolipoprotein B was calculated in order to assess the quantity of apolipoprotein CII, CIII and E on each chylomicron particle (table 6.5). This showed that the patient group had significantly higher levels of apo CII, III and E per lipoprotein particle. In addition, the patients' chylomicron particles contained greater quantities of triglyceride, total cholesterol, free cholesterol and phospholipids compared to the controls. Given that the triglyceride is the greatest constituent of chylomicron particles, the 3 fold increase in chylomicron triglyceride strongly suggests that larger chylomicron particles are present in the patients with proteinuria. However, surface structure of chylomicrons did not differ between the patient and control group as outlined in table 6.6. In the control group, we observed a strong relationship between chylomicron size and baseline fasting plasma triglyceride (r^2 85.9%, p=0.005). This was not observed in the patient group (r^2 0, p=0.6). On multivariate analysis, subject group was not an independent predictor of particle size.

Apolipoprotein	**Patients (n=10)**	**Controls (n=6)**	**p**
B (mg/dl)	0.25 (0.20- 0.39)	0.51 (0.45- 0.83)	0.04
CII (mg/dl)	0.34 (0.26- 0.62)	0.19 (0.13- 0.74)	ns
CIIII (mg/dl)	0.90 (0.52- 1.82)	0.32 (0.18- 1.83)	ns
E (mg/dl)	0.13 (0.09- 0.28)	0.09 (0.05- 0.34)	ns

Table 6.4: Plasma Concentration of Chylomicron Apolipoproteins: Patients vs Controls

(All values median & IQR)

Moles per Particle	Patients (n=10)	Controls (n=6)	p
Apo CII	68.3 (56.9- 95.2)	24.6 (13.8- 60.4)	0.03
Apo CIII	179 (145- 248)	58.3 (16.4- 134.8)	0.03
Apo E	7.3 (5.7- 9.8)	2.8 (1.7- 6.6)	0.02
Triglyceride*	491000 (406000-740000)	156000 (89000-312000)	0.008
Total Cholesterol*	58000 (42000- 85000)	19000 (9500- 40000)	0.01
Free Cholesterol*	29000 (21000- 44000)	10000 (4000- 25000)	0.03
Phospholipid*	46000 (36000- 79000)	19000 (9000- 39000)	0.02

Table 6.5: Apolipoproteins and Lipid Content per Chylomicron Particle: Patients vs controls

Prior to statistical analysis, any parameter which was not normally distributed was normalised by log transformation. All data were compared using unpaired t-tests. * Expressed to the nearest 1000.
(All median & IQR)

mg/mg	Patients (n=10)	Controls (n=6)	p
FC:PL	0.58 ± 0.13	0.50 ± 0.15	ns
CII:PL	$1.5 \times 10^{-3} \pm 0.6 \times 10^{-3}$	$1.4 \times 10^{-3} \pm 0.3 \times 10^{-3}$	ns
CIII:PL	$3.6 \times 10^{-3} \pm 1.5 \times 10^{-3}$	$2.7 \times 10^{-3} \pm 1.1 \times 10^{-3}$	ns
E:PL	$0.15 \times 10^{-3} \pm 0.07 \times 10^{-3}$	$0.18 \times 10^{-3} \pm 0.08 \times 10^{-3}$	ns

Table 6.6 Surface Structure of Chylomicrons
(All mean & SD)

6.3.4 Chylomicron Apolipoproteins & Compositions: Treatment Effect in Patients & Controls

The effect of treatment with 4 g daily of omega-3 fatty acids on chylomicron apolipoproteins and compositions in both patients and controls is shown in tables 6.7 and 6.8. No significant change in chylomicron apolipoprotein content or chylomicron lipid composition was observed in either population following treatment.

Moles per particle	**Baseline**	**Post Treatment**	**p**
Apo CII	68.3 (56.9- 95.2)	87.5 (41.7- 158.3)	ns
Apo CIII	179 (145- 248)	184 (122- 326)	ns
Apo E	7.3 (5.7- 9.8)	8.7 (6.2- 16.2)	ns
Triglyceride*	491000 (406000-740000)	455000(196000-701000)	ns
Total Cholesterol*	58000 (42000- 85000)	53000 (24000- 89000)	ns
Free Cholesterol*	29000 (21000- 44000)	22000 (15000- 39000)	ns
Phospholipid *	46000 (36000- 79000)	44000(20000- 98000)	ns

Table 6.7: Apolipoproteins and Lipid content per chylomicron particle: Treatment Effect in Patients (n=10)

Prior to statistical analysis, any parameter which was not normally distributed was normalised by log transformation. All data were compared using unpaired t-tests. * Expressed to the nearest 1000.
(All median & IQR)

Moles per particle	Baseline	Post Treatment	p
Apo CII	24.6 (13.8-60.4)	40.0(6.6-146.7)	ns
Apo CIII	58.3(16.4-134.8)	222(18-848)	ns
Apo E	2.8 (1.7-6.6)	5.3 (2.7-13.5)	ns
Triglyceride*	156000 (89000-312000)	157000(43000-684000)	ns
Total Cholesterol*	19000 (9500-40000)	9000(4000-98000)	ns
Free Cholesterol*	10000(4000-25000)	15000(1000-41000)	ns
Phospholipid *	19000 (9000-39000)	18000 (7000-83000)	ns

Table 6.8: Apolipoproteins and Lipid Content per Chylomicron Particle: Treatment Effect in Controls (n=6)

* Expressed to the nearest 1000. (All values median & IQR)

6.3.5 $VLDL_1$ Apolipoproteins & Compositions: Patients vs Controls

The concentration of $VLDL_1$ apolipoproteins are shown in table 6.9. Once again as with the chylomicrons, complete data was only available for 15 patients and 13 controls therefore only these subjects were included in the analysis. No difference in apolipoprotein concentration of $VLDL_1$ between patients and controls was seen.

Furthermore, after calculating the molar ratio of apolipoprotein CII, CIII and E to apo B, there was no difference identified between patients and controls (table 6.10). The triglyceride, cholesterol and phospholipids content of the

$VLDL_1$ particles were also similar. In addition, surface structure did not differ between patients and controls (table 6.11).

Apolipoprotein	Patients (n=15)	Controls (n=13)	p
B (mg/dl)	3.33(1.79-5.12)	2.82 (1.15-3.71)	ns
CII (mg/dl)	1.14 (0.50-1.77)	0.78 (0.34-2.0)	ns
CIII (mg/dl)	3.10 (1.68-4.50)	1.65 (1.04-3.54)	ns
E (mg/dl)	0.12 (0.09-0.34)	0.17 (0.11-0.29)	ns

Table 6.9: Plasma Concentration of $VLDL_1$ Apolipoproteins: Patients vs Controls
(All values median & IQR)

Moles per particle	Patients (n=15)	Controls (n=13)	p
Apo CII	17.8 (12.9-23.6)	24.7 (15.6-36.3)	ns
Apo CIII	46.4 (39.3-67.0)	47.7 (30.7-81.1)	ns
Apo E	0.87 (0.54-1.63)	1.00 (0.77-1.63)	ns
Triglyceride*	23000 (20000-2000)	26000 (22000-40000)	ns
Total Cholesterol*	9000 (8000-10000)	10000 (8000-13000)	ns
Free Cholesterol*	5000 (4000-6000)	5000 (4000-7000)	ns
Phospholipid *	7000 (6000-8000)	7000 (6000-11000)	ns

Table 6.10: Apolipoproteins and Lipid Content per $VLDL_1$ Particle: Patients vs controls
* Expressed to nearest 1000. (All values median & IQR)

mg/mg	Patients (n=15)	Controls (n=13)	p
FC:PL	0.7 ± 0.08	0.7 ± 0.07	ns
CII:PL	2.6. x 10^{-3} ± 1.1	2.9 x 10^{-3} ± 1.2	ns
CIII:PL	7.0 x 10^{-3} ± 2.4	6.0. x 10^{-3} ± 1.3	ns
E:PL	0.14 x 10^{-3} ± 0.08	0.16 x 10^{-3} ± 0.02	ns

Table 6.11: Surface Structure of $VLDL_1$

(All mean & SD)

6.3.6 $VLDL_1$ Apolipoproteins & Compositions: Treatment Effect in Patients and Controls

Tables 6.12 and 6.13 display the apolipoprotein and lipid content per $VLDL_1$ particle following treatment with omega-3 fatty acids in patients and controls. Treatment with omega-3 fatty acids did not alter the apolipoprotein or lipid content of $VLDL_1$ particles in either patients or controls.

Moles per particle	Baseline	Post Treatment	p
Apo CII	17.8 (12.9-23.6)	20.4 (13.3-30.7)	ns
Apo CIII	46.4 (39.3-67.0)	50.0 (40.3-58.2)	ns
Apo E	0.87 (0.54-1.63)	0.71 (0.44-1.62)	ns
Triglyceride*	23000 (20000-27000)	25000 (24000-30000)	ns
Total Cholesterol*	9000 (8000-10000)	10000 (8000-11000)	ns
Free Cholesterol*	5000 (4000 -6000)	5000 (4000 -6000)	ns
Phospholipid *	7000 (6000-8000)	8000(6000-8000)	ns

Table 6.12: Apolipoproteins and Lipid Content per VLDL particle: Treatment Effect in Patients (n=15)
*Expressed to nearest 1000. (All values median& IQR)

Moles per particle	Baseline	Post Treatment	p
Apo CII	24.7 (15.6-36.3)	18.2 (8.8-31.8)	ns
Apo CIII	47.7 (30.7-81.1)	42.3 (24.5- 62.0)	ns
Apo E	1.00 (0.77-1.63)	1.2 (0.68-2.88)	ns
Triglyceride	26000 (22000-40000)	27000 (19000-341000)	ns
Total Cholesterol	10000 (8000-13000)	10000 (8000-11000)	ns
Free Cholesterol	5000 (4000-7000)	5000 (4000-6000)	ns
Phospholipid	7000 (6000-12000)	7000 (6000-8000)	ns

Table 6.13: Apolipoproteins and Lipid Content per VLDL Particle: Treatment Effect in Controls (n=13)
*Expressed to nearest 1000. (All values median & IQR)

6.4 Discussion

In this study, we have demonstrated that chylomicrons in patients with nephrotic range proteinuria differ in structure and composition compared with normal controls. Patients had significantly higher levels of apolipoprotein CII, CIII and E per chylomicron particle with greater quantities of triglyceride, total cholesterol, free cholesterol and phospholipids. The quantity of triglyceride per chylomicron particle can be used as a marker of particle size and so this strongly suggests that patient chylomicron particles are significantly larger than the control population. Treatment with omega-3 fatty acids did not alter the structure or composition of chylomicrons in patients or controls. In contrast to previous data, however, we found no difference in the structure or composition of $VLDL_1$ particles when comparing patients and controls(175). Once again, treatment with omega-3 fatty acids did not alter the composition or structure of $VLDL_1$ particles in neither patients nor controls.

There is little in the literature regarding the structure of chylomicrons in human nephrotics. Levy et al examined chylomicron structure in nephrotic rats. They concluded that chylomicrons of nephrotic rats were larger in size with a higher triacyglycerol to protein ratio(202). A further study by this group once again showed that chylomicrons of nephrotic rats were larger, deficient in apo E and apo AI, rich in triacylglycerol and cholesterol but poor in phospholipid (203). To our knowledge, there are no human studies examining chylomicron structure in nephrotic humans.

Our data suggests that patients with nephrotic range proteinuria have larger chylomicrons than the control group with decreased apolipoprotein to triglyceride ratio. This size discrepancy may be responsible for less efficient

lipolysis and therefore clearance of these larger particles. In the previous chapter, we demonstrated that treatment with omega-3 fatty acids improved chylomicron clearance in patients with nephrotic range proteinuria. However, treatment did not alter the structure of chylomicrons in the patient or control group. Thus, the better clearance observed is not related to structural changes. In the control group, we observed a strong relationship between chylomicron size and baseline fasting plasma triglyceride. This was not observed in the patient group with no correlation between particle size and baseline triglyceride. Therefore differences in baseline triglyceride levels cannot account for the difference in structure of the chylomicron particles. On multivariate analysis, subject group was not an independent predictor of particle size. However, in view of the small numbers, these results must be interpreted with caution.

We did not show any difference in structure or composition of $VLDL_1$ between patients with nephrotic range proteinuria compared with controls. In addition, treatment with omega-3 fatty acids did not alter the structure of these particles in both groups. There are very few studies examining the structure of $VLDL_1$ in nephrotic humans but, in contrast to our findings, data from our laboratory has previously shown that $VLDL_1$ from nephrotic patients were deficient in apo CII, CIII and E. The particles were smaller, with increased triglyceride content per particle with higher free cholesterol to phospholipid ratio(175).

These differences in results can be explained in a number of ways. Firstly, patients with nephrotic range proteinuria are a very heterogeneous group with a range of lipoprotein abnormalities and so the numbers may be too small to be representative of the group as a whole. The apo B assay used in the

laboratory is currently more sensitive at detecting lower levels than the older assay used in the previous study. Also, the triglyceride levels in our control population are higher than the previous study as we attempted to triglyceride match our control group.

Thus, we have demonstrated that chylomicron particles in patients with nephrotic range proteinuria are larger than in normal controls. This structural abnormality may be why their clearance is reduced. We did not observe any difference in $VLDL_1$ structure. Although treatment with omega-3 fatty acids improves chylomicron clearance in patients with nephrotic range proteinuria, they do not seem to do this through altering their structure. Therefore, it is possible that omega-3 fatty acids reduce synthesis of chylomicrons.

Chapter 7: Omega-3 Fatty Acids and HDL Subfractions in Patients with Nephrotic Range Proteinuria

7.1 Introduction

Prospective epidemiological studies have demonstrated that reduced levels of plasma HDL-C are an independent risk factor for coronary heart disease and that increasing HDL-C reduces this risk. This is has been reviewed in detail in chapter 1. Plasma high density lipoprotein (HDL) is heterogeneous, comprising of a number of particles of differing size and composition. There are several ways of separating HDL subfractions. Ultracentrifugation separates HDL into 2 subclasses: HDL_2 and HDL_3. HDL_2 is larger and cholesterol-rich and HDL_3 is smaller and denser. Electrophoretic methods separate HDL on the basis of their charge and size. Blanche et al described 5 subclasses: HDL2a, HDL2b, HDL3a, HDL3b and HDL3c (see chapter 2). It is generally thought that larger cholesterol rich HDL_2 is cardioprotective while smaller dense HDL_3 is associated with an increased risk of CHD. However, there is conflicting data regarding the atherogenicity of these subfractions.

In most studies examining patients with nephrotic syndrome or nephrotic range proteinuria, HDL-C levels have been reported as normal or low(167;170;186;211). Reduced HDL_2 levels and normal HDL_3 levels are also reported(170;212;213). Most of the data regarding the effect of treatment with omega-3 fatty acids on HDL metabolism is favourable.

The aim of this study was two fold. 1. To compare the distribution of HDL subfractions in subjects with nephrotic range proteinuria (patients) and in age and sex matched controls (controls). 2. To study the effect of omega-3 fatty acids on HDL subfraction distribution.

7.2 Subjects & Methods

17 patients and 17 controls as described earlier took part in this study. Fasting EDTA plasma prior to and following an eight week treatment period with omega-3 fatty acids were taken. Analysis was carried out on fresh samples within 5 days of drawing the samples. Samples were kept refrigerated at -4°C. HDL_2 and HDL_3 subfractions were isolated by density gradient ultracentrifugation as described in the methods section. The sizes of HDL_2 and HDL_3 were quantified using gradient gel electrophoresis as described in chapter 2.

7.3 Statistics

Statistical analyses were carried out using MINITAB 13.1 for Windows (Minitab inc). Results are shown as mean and standard deviation (SD), or where data were not normally distributed as median and interquartile ranges (IQR). Prior to statistical analysis, any factor that was not normally distributed was normalised by log transformation. Patient and control results were compared using unpaired t-tests. Results pre and post treatment were compared using paired t-tests. Linear regression analysis was performed to identify significant correlations with a ‘p’ value less than 0.05 considered significant.

7.3 Results

7.3.1 Baseline HDL Concentrations

At baseline, HDL-C, total HDL concentration and HDL_2 did not differ significantly between patients and controls (table 7.1). HDL_3 concentration displayed a trend towards being higher in the patient population but this did not reach statistical significance (p=0.059). Other baseline parameters in patients compared with controls are presented in chapter 3.

	Patients	**Controls**	**p**
HDL-C (mmol/l)	1.0 (0.9-1.1)	1.1 (0.9-1.4)	ns
Total HDL (mg/dl)	196.3 (172.2-231.6)	177.1 (159.3-216.8)	ns
HDL_2 (mg/dl)	66.5 (52.4 –83.5)	80.9 (38.4 –117.4)	ns
HDL_3 (mg/dl)	132.6 (110.3-143.0)	116.7 (93.3-130.1)	0.059

Table 7.1: Baseline HDL Concentrations in Patients vs Controls (n=17)

7.3.2 Treatment Effect in Patients and Controls

The effect of treatment with omega-3 fatty acids on HDL_2 concentrations in patients and controls is outlined in table 7.2. In the patient group, HDL_2 concentration increased by a mean of 11.3 mg/dl (95% CI 0.6 – 22.0, p=0.05). In controls, HDL_2 increased by a mean of 17.2 mg/dl (95% CI 1.4- 33.0,

p=0.02). Although the medians appear similar, the mean results are shown below and statistically differ.

The effect of treatment on HDL_3 and total HDL concentration in patients and controls is shown in tables 7.3 and 7.4. Treatment with omega-3 fatty acids did not alter HDL_3 or total HDL concentration in patients or controls.

HDL_2 (mg/dl)	**Baseline**	**Post Treatment**	**p**
Patients	66.5 (52.4-83.5)	71.0 (52.6-118.2)	0.05
Controls	80.9 (38.4-117.4)*	80.1 (58.6-127.6) **	0.02

Table 7.2: The Effect of Treatment on Patient and Control HDL_2 Concentrations

(All median & IQR)
In controls, although the medians quoted above appear similar, the mean results statistically differ (*mean 84.5 ± 59.4 vs **mean 101.7± 70.3).

HDL_3 (mg/dl)	**Baseline**	**Post Treatment**	**p**
Patients	132.63(110.3-143.0)	124.9 (117.1-140.1)	ns
Controls	116.7 (93.3-130.1)	109.8 (103.1-118.7)	ns

Table 7.3: The Effect of Treatment on Patients and Control HDL_3 Concentrations
(All median & IQR)

Total HDL (mg/dl)	Baseline	Post Treatment	p
Patients	196.3 (172.2- 231.6)	214.8 (195.3-246.0)	ns
Controls	177.1 (159.3- 216.8)	197.8 (168.9- 222.5)	ns

Table 7.4 The Effect of Treatment on Total HDL Lipoprotein Concentrations
(All median & IQR)

7.3.3 Association Between Baseline HDL_2 and Baseline Parameters

Table 7.5 displays the relationship between baseline HDL_2 concentration and baseline parameters for patients and control groups individually and collectively.

For patients and controls collectively, there were strong negative correlations between baseline HDL_2 and baseline triglyceride, LDL III, $VLDL_1$ and RLP-C (table 7.5 and figures 7.1 & 7.2). In patients, there was no association between baseline HDL_2 concentration and baseline renal function and proteinuria. Baseline triglyceride correlated with baseline HDL_2 in patients and controls (r^2 -24.4%, p=0.02 and r^2- 29.5%, p=0.01). Baseline $VLDL_1$ and $VLDL_2$ correlated with baseline HDL_2 in both patients and control with a tighter association in the patient group. When entering the subject group into a general linear model as a variable, it was not an independent predictor.

7.3.4 Association Between HDL_3 and Baseline Parameters

Table 7.6 displays the relationship between baseline HDL_3 concentration and baseline parameters for patients and control groups individually and collectively. For patients and controls as a whole, there were strong correlations between baseline HDL_3 and baseline triglyceride, LDL III, $VLDL_1$ and RLP-C (table 7.6 and figures 7.3 & 7.4). In the patient group there was no association between baseline HDL_3 or baseline parameters including renal function and urinary proteinuria. This is likely to be due to a clear outlier. On removing this outlier, there is a strong correlation between baseline HDL_3 and baseline triglyceride (r^2 36.6%, p=0.008) but the other correlations are not affected. However, in the control group, several baseline parameters correlated with baseline HDL_3. These included cholesterol (r^2 22.5%, p=0.03), triglyceride (r^2 31.8%, p=0.01), LDLIII% (r^2 32.6%, p=0.01), LDLIII mass (r^2 34.2%, p=0.008), $VLDL_1$ (r^2 41.3%, p=0.003), $VLDL_2$ (r^2 26.7%, p=0.02) and RLP-C (r^2 44.2, p=0.002). Once again, when examining the population as a whole, subject group was not an independent predictor.

7.3.5 Change in HDL_2 and HDL_3 Concentrations

There was no association between change in HDL_2 concentration and change in triglyceride, LDL III, $VLDL_1$, $VLDL_2$, RLP-C and RLP-TG in patients and controls.

Similarly, with regards to change in HDL_3 concentration, there was no association with change in triglyceride, LDL III, $VLDL_1$, $VLDL_2$, RLP-C and RLP-TG in patients. In controls, only a decrease in $VLDL_1$ concentration correlated with an increase in HDL_3 (r^2 23.9%, p=0.03).

	All		Patients		Controls	
	r^2	p	r^2	p	r^2	p
Triglyceride	22.7	0.003	24.4	0.025	29.5	0.014
LDL III %	13.0	0.02	20.8	0.038	6.7	ns
LDL III conc	24.8	0.002	27.9	0.017	28.7	0.016
$VLDL_1$	42.1	0.000	46.8	0.001	41.0	0.003
RLP-C	16.7	0.01	14.1	0.076	22.9	0.03

Table 7.5: Association Between Baseline HDL_2 and Baseline Parameters in Patients and Controls
Linear regression analysis of baseline HDL_2 concentration with baseline parameters in all the subjects as a whole, patients and controls. (All correlations negative)

	All		Patients		Controls	
	r^2	p	r^2	p	r^2	p
Triglyceride	17.6	0.008	0	ns	31.8	0.01
LDL III %	16.7	0.009	0	ns	32.6	0.01
LDL III conc	23.8	0.002	0	ns	34.2	0.008
$VLDL_1$	12.5	0.023	0	ns	41.3	0.003
RLP-C	18.6	0.006	0	ns	44.2	0.002

Table 7.6: Association Between Baseline HDL_3 and Baseline Parameters in Patients and Controls

Linear regression analysis of baseline HDL_3 concentration with baseline parameters in all the subjects as a whole, patients and controls.

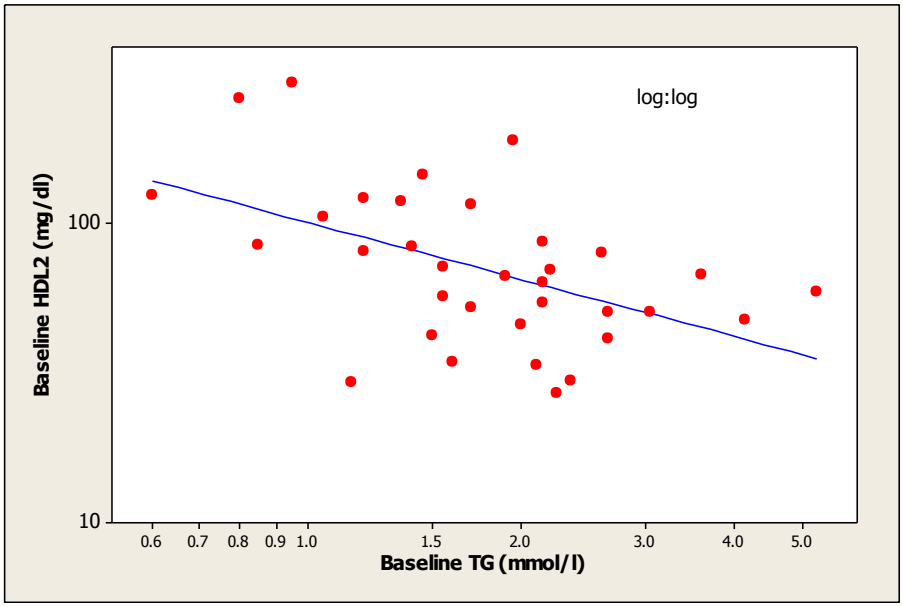

Figure 7.1 Baseline HDL_2 vs Baseline Triglyceride in Patients & Controls

Linear regression analysis of baseline HDL_2 with baseline triglyceride in all subjects (r^2 = -22.7%, p=0.003).

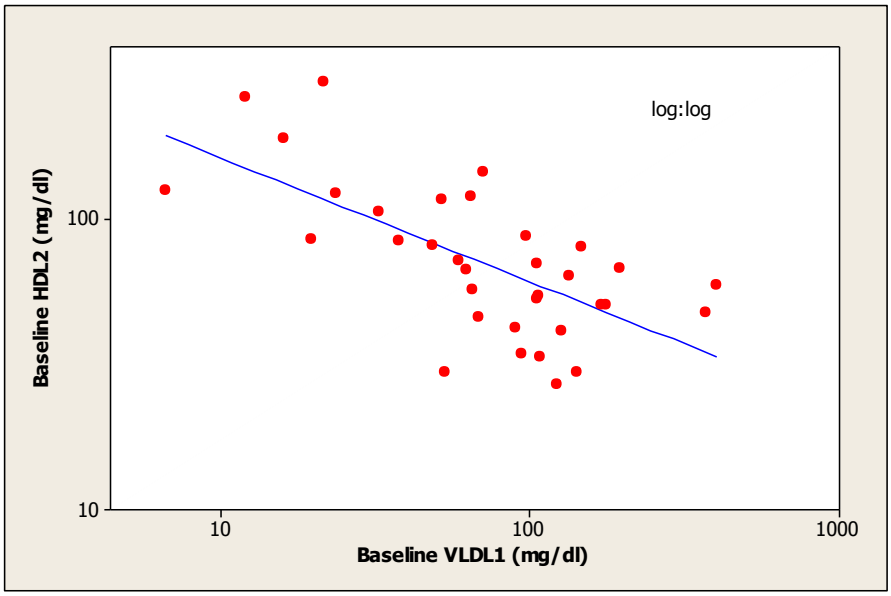

Figure 7.2 Baseline HDL_2 vs Baseline $VLDL_1$ in Patients & Controls

Linear regression analysis of baseline HDL_2 with baseline $VLDL_1$ in all subjects (r^2 = -46.8%, p<0.001).

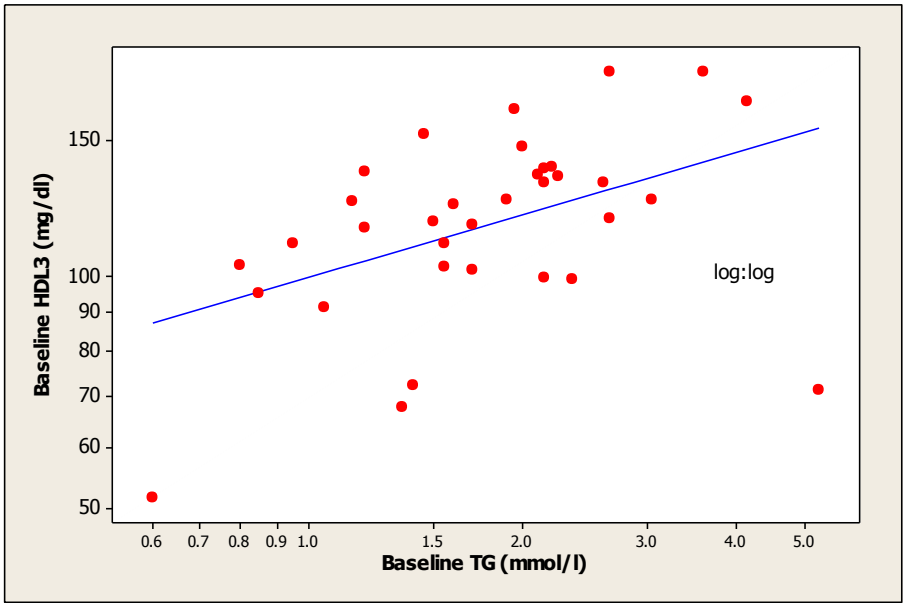

Figure 7.3 Baseline HDL_3 vs Baseline Triglyceride in Patients & Controls

Linear regression analysis of baseline HDL_3 with baseline triglyceride in all subjects (r^2= 17.6%, p=0.008).

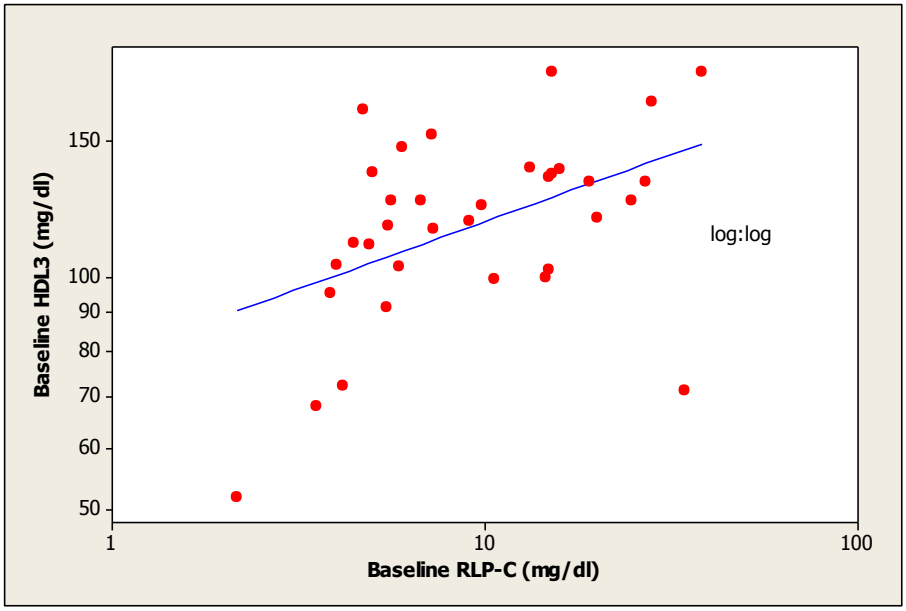

Figure 7.4 Baseline HDL_3 vs Baseline RLP-C in Patients & Controls

Linear regression analysis of baseline HDL_3 with baseline RLP-C in all subjects (r^2= 18.6%, p= 0.006).

HDL Subclass (%)	Patients	Controls	p
2a	24.4 (23.1- 28.4)	25.5 (21.6- 27.9)	ns
2b	24.1 (21.8- 26.1)	24.8 (21.3- 29.1)	ns
3a	25.9 (24.4- 27.2)	26.4 (23.9- 28.4)	ns
3b	15.8 (13.2- 16.7)	14.3 (11.7- 18.3)	ns
3c	9.2 (5.7 - 0.7)	7.7 (6.2- 10.2)	ns

Table 7.7: Baseline Patient and Control HDL Subclasses by Gel Electrophoresis

7.3.6 Baseline Subclasses- Gel Electrophoresis

Patient and control baseline distribution of HDL subclasses are shown in table 7.7. There was no significant difference in all the subclasses between patients and controls.

7.3.7 Treatment Effect on HDL subclasses- Gel electrophoresis

Table 7.8 outlines the effect of treatment with omega-3 fatty acids on % of each HDL subclass as measured by gel electrophoresis in patients and controls.

Omega-3 fatty acids did not affect HDL2a in patients or controls however the percentage of HDL2b was increased in patients and controls (mean increase patient group 3.98 (95%CI 1.58 to 6.39), controls 3.7 (95%CI 2.0 to 5.3). Both percentages of HDL3a and HDL3b subclasses were reduced by treatment with omega-3 fatty acids in patients and controls: HDL3a- patients 2.0 (95%CI

0.6 to 3.4), controls: 1.8 (95%CI 1.1 to 2.6), HDL3b- patients: 1.5 (95%CI 0.4 to 2.6), controls: 1.7 (95%CI 0.9 to 2.6).

The percentage of HDL3c was not significantly affected in patients but in controls fell by mean of 0.8 (95%CI 0.1 to 1.7). Mean HDL particle diameter increased in both patients and controls following treatment with omega-3 fatty acids: patients 9.39nm (IQR 9.34- 9.54) to 9.51nm (9.40- 9.80) p=0.03, controls 9.42nm (IQR 9.36- 9.53) to 9.50nm (9.40- 9.82) p=0.001.

HDL subclass	Patients			Controls		
(%)	Baseline	Post Treatment	p	Baseline	Post Treatment	p
2a	24.4 (23.1- 28.4)	26.6 (23.2 -27.9)	ns	25.5 (21.6 – 27.9)	25.4 (23.7- 28.4)	ns
2b	24.1 (21.8- 26.1)	26.7 (23.9- 35.0)	0.003	24.8 (21.3- 29.1)	26.9 (22.9- 36.6)	<0.001
3a	25.9 (24.4- 27.2)	23.7 (19.5- 27.5)	0.009	26.4 (23.9- 28.4)	24.7 (21.1- 27.4)	<0.001
3b	15.8 (13.2- 16.7)	13.5 (10.9- 15.6)	0.009	14.3 (11.7- 18.3)	14.1 (8.6- 16.2)	<0.001
3c	9.2 (5.7- 10.7)	7.3 (5.9- 10.2)	ns	7.7 (6.2- 10.2)	8.2 (4.6- 9.9)	0.03

Table 7.8: Effect of Treatment on HDL Subclasses Measured by Gel Electrophoresis

Percentage of each HDL subclass in patients and controls at baseline and following treatment.

7.5 Discussion

In this study we have identified no difference in the plasma concentration of HDL subfractions in patients with nephrotic range proteinuria compared with age and sex matched controls analysed either by density ultracentrifugation or gradient gel electrophoresis. There was however a trend towards increased levels of the smaller denser HDL_3 and as mentioned previously, there is a clear outlier in the patient group and once this outlier is omitted, HDL_3 levels are significantly higher in the patient group compared with the controls. Omega-3 fatty acids significantly increased HDL_2 concentration in patients and controls but HDL_3 concentration remained unchanged. On gel electrophoresis, HDL subclass 2a was unaffected by treatment but %2b increased in both patients and controls. Percentage of HDL subclasses 3a and 3b were reduced by treatment but only 3c was reduced in controls. However, mean particle diameter was increased by omega-3 fatty acids in both patients and controls. Baseline lipoprotein concentration of HDL_2 was inversely associated with atherogenic triglyceride rich lipoproteins in patients and controls, whereas lipoprotein concentration of HDL_3 correlated with atherogenic triglyceride rich lipoprotein, at baseline, in the control group only. Subject group was not an independent predictor of baseline HDL_2 or HDL_3 concentrations or change in levels following treatment with Omega-3 fatty acids.

As discussed in the introduction, it is widely recognised that high HDL-C levels are associated with reduced cardiovascular risk and that raising HDL further reduces this risk(29;30;32;33). However, there is conflicting data regarding the

atherogenicity of HDL subfractions. Several studies have suggested a strong inverse relationship between HDL_2 and coronary atherosclerosis. Salonen at al examined HDL subfractions in Finnish men. They found HDL-C and HDL_2 to be inversely related to the risk of myocardial infarction (214). There was also an inverse relationship between HDL_3 and ischaemic heart disease risk but statistical significance was lost when adjusted for HDL_2. Furthermore, data from the VA-HIT study showed that levels of small HDL predicted CHD events suggesting that the larger HDL (HDL_2) particles were more protective(215).

In contrast, data from the Physicians' Health Study (216) and the Caerphilly and Speedwell Collaborative Heart Disease Studies found HDL_3 to be the strongest inverse predictor of IHD(217). While density ultracentrifugation separates HDL into 2 subfractions, gradient gel electrophoresis methods have reported from 5 to 14 subclasses(184;218). Several studies have suggested that reduction in large HDL2b particles are associated with an increased in CHD risk(219;220).The variety of results from studies examining HDL subclasses reflects the heterogeneity of HDL particles. Quantification of these subclasses is useful but the available methods are time consuming and difficult as well as lacking in uniformity and validation.

In patients with nephrotic syndrome, HDL-C levels are usually normal or reduced, often with a reduced HDL_2 subfraction. The mechanism behind this is unclear. It has been shown that levels of cholesteryl ester transfer protein (CETP) are increased in this population. This protein mediates transfer of esterified cholesterol from HDL_2 to VLDL. This increase in CETP activity is likely to be driven by increased levels of triglyceride rich lipoproteins prevalent

in nephrotic range proteinuria thereby leading to decreased HDL_2 and increased HDL_3. Also, reduced activity of another enzyme, Lecithin cholesterol acyltransferase (LCAT) has been documented in human and nephrotic rats. LCAT is the enzyme responsible for the esterification of cholesterol within the plasma maintaining a concentration gradient for unesterified cholesterol between peripheral cells and plasma. The reduced activity of LCAT may be due to hypoalbuminaemia as albumin normally binds lysolecithin (a product of LCAT reaction) and accumulation of unbound lysolecithin impairs enzyme activity (169), or LCAT may be lost in the urine of these patients. The resulting reduced levels of LCAT would impair cholesteryl esterification within the HDL particle and so inhibit conversion of HDL_3 to HDL_2.

Omega-3 fatty acids have been shown to have a favourable effect on HDL. Doses of 3.5 to 4g have been shown to consistently increase HDL-C primarily by increasing HDL_2 (221). They are thought to increase larger HDL (HDL_2) at the expense of HDL_3(129) mediated by a reduction in cholesteryl ester transfer protein activity thereby favouring large cholesterol-rich HDL formation. Using the method of ultracentrifugation to separate HDL, we found that treatment with omega-3 fatty acids increased concentration of HDL_2 but did not affect HDL_3. However, using gradient gel electrophoresis, we found that %HDL2b increased percentages of HDL3a and 3b fell and mean particle diameter of HDL particle increased with treatment in keeping with the above mechanism.

Although we showed that baseline HDL_2 concentration was associated with baseline triglyceride concentration, we did not find any association between

change in HDL_2 concentration and change in triglyceride concentration. This may be explained by the fact that treatment did not actually reduce total HDL or HDL-C but caused redistribution to larger HDL_2. We also did not show a group effect, implying that the mechanism in patients and controls is similar and the differences are related to differences in baseline characteristics.

Other than omega-3 fatty acids, a number of other agents are known to increase HDL-C, primarily statins and fibrates. Treatment with statins has a moderate effect on HDL raising it by about 5% (222). The mechanism behind the HDL raising effect of statins is not fully understood. They increase HDL apo AI production by approximately 15% in the liver (223) thereby increasing HDL_2 production. In addition, they increase expression of LDL receptors causing a reduction in the numbers of potentially atherogenic lipoprotein acceptors of cholesteryl ester via the CETP mechanism from HDL. Fibrates have a greater effect on HDL and can raise HDL concentration by up to 25% through a number of mechanisms but their use is limited in patients with nephrotic range proteinuria as they are poorly tolerated.

In summary, therefore, we have demonstrated that omega-3 fatty acids have a favourable effect on HDL subclasses in both patients and controls which contributes further to their cardioprotective properties.

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Chapter 8: The Effect of Omega-3 Fatty Acids on Novel Cardiovascular Risk Markers and Endothelial function in Patients with Nephrotic Range Proteinuria

8.1 Introduction

As discussed previously, patients with nephrotic range proteinuria have a marked increase in cardiovascular risk. However, the lipid and lipoprotein abnormalities described earlier cannot alone account for the large burden of cardiovascular disease observed in this population.

The endothelium is thought to play a central role in the early development of atherosclerosis(224). The pathological changes associated with proteinuria are believed to cause endothelial dysfunction. Both inflammation and oxidative stress are important mechanisms in the development of an atherosclerotic plaque.

Omega-3 fatty acids have been shown to have beneficial effects on endothelial function and anti-inflammatory properties in the general population as discussed in chapter 1.Therefore, the aim of this study was three-fold. Firstly to examine endothelial function using the non-invasive technique of laser doppler imaging in patients with nephrotic range proteinuria and compare with a control population. This technique is based on the iontophoretic transdermal delivery of acetylcholine (Ach) and sodium nitroprusside (SNP) which are vasodilators(225). Ach induced vasodilatation is ‘endothelium-dependent’ as it requires an intact endothelium with binding to muscarinic receptors and the generation of nitric oxide. SNP induced vasodilatation is termed ‘endothelium- independent’. Nitric oxide acts on vascular smooth muscle leading to vasodilatation therefore, as SNP is a nitric oxide donor, it acts as an ‘endothelium independent’ control. Laser Doppler imaging measures skin perfusion under the influence of these drugs.

Secondly, to assess whether omega-3 fatty acids improve endothelial function in this population. Finally, we aimed to examine novel markers of endothelial function and inflammation in this population both pre and post treatment with omega-3 fatty acids.

8.2 Subjects and Methods

8.2.1 Subjects

The patients and controls described in the methods section were studied in this chapter. Patients and controls attended on 2 occasions as previously described: once at the start of the study prior to commencing omega-3 fatty acids and once following 8 weeks of treatment with 4g daily of Omacor. Endothelial function was measured using the technique of laser doppler imaging described below on both occasions prior to venesection. Fasting EDTA samples were drawn and plasma isolated as previously described on these 2 occasions. Samples were divided into 0.5ml aliquots and frozen at -80°C.Highly sensitive CRP (hsCRP), I-CAM, V-CAM, oxidised LDL and PLA2 assays were performed as described in the methods section on all patients and controls completing the study.

8.2.2 Iontophoresis Protocol

This LDI protocol is based on one developed in Glasgow(225). We carried out the measurements in a temperature-controlled room at 23^{o}C (±2^{o}C) after allowing the patients and controls to acclimatise for 30 minutes before the procedure. A battery-powered constant current iontophoresis controller (MIC-Ie; Moor Instruments Ltd. Axminster, UK) allowed drug delivery with Perspex

chambers (internal diameter 22 mm, area 3.8 cm^2) and an internal platinum wire electrode were used for iontophoresis (ION 6; Moor Instruments Ltd.). Doubled-sided adhesive disks were used to attach the two chambers to the skin of the volar aspect of the forearm avoiding hair, broken skin, and superficial veins, Figure 8.1. The chambers were connected to the anode and cathode connections on the iontophoresis controller.

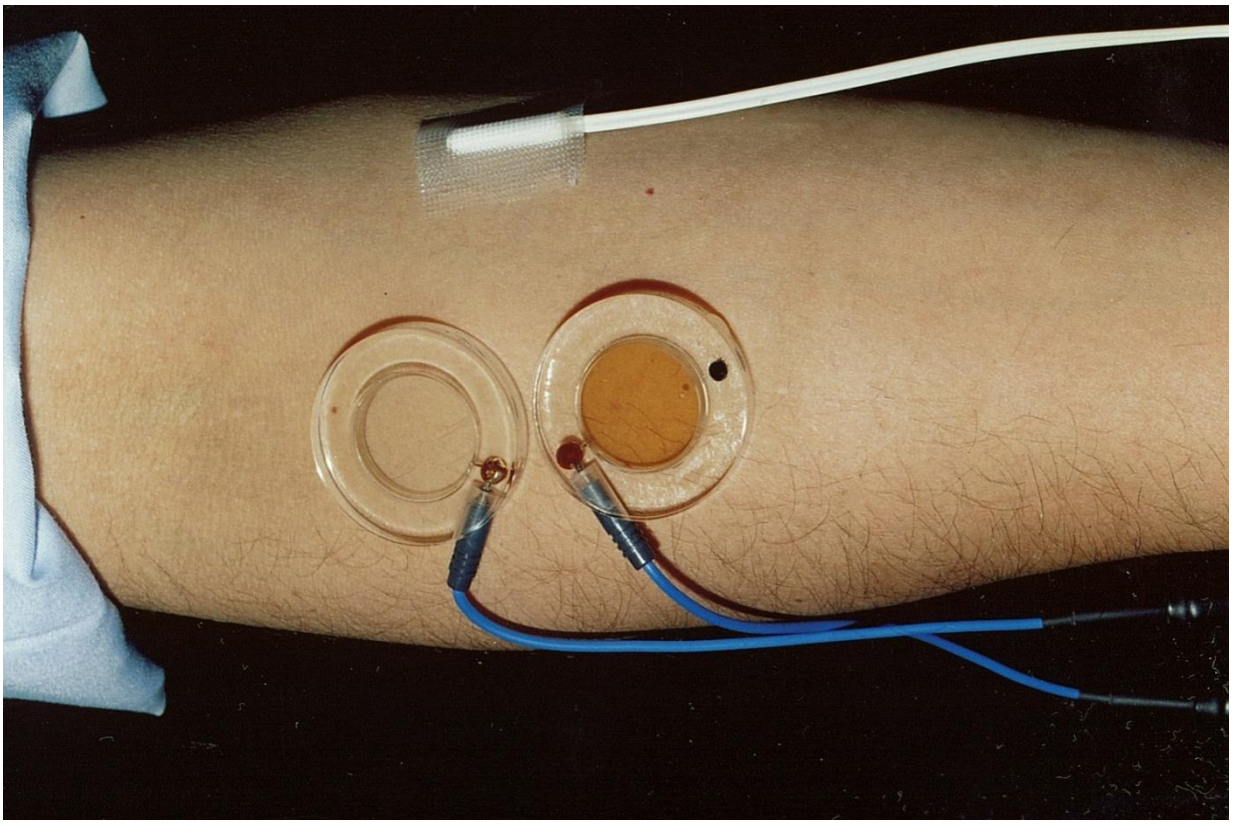

Figure 8.1 Drug Delivery Chambers

Perspex drug delivery chambers with overlying coverslips with a temperature probe attached to the skin.

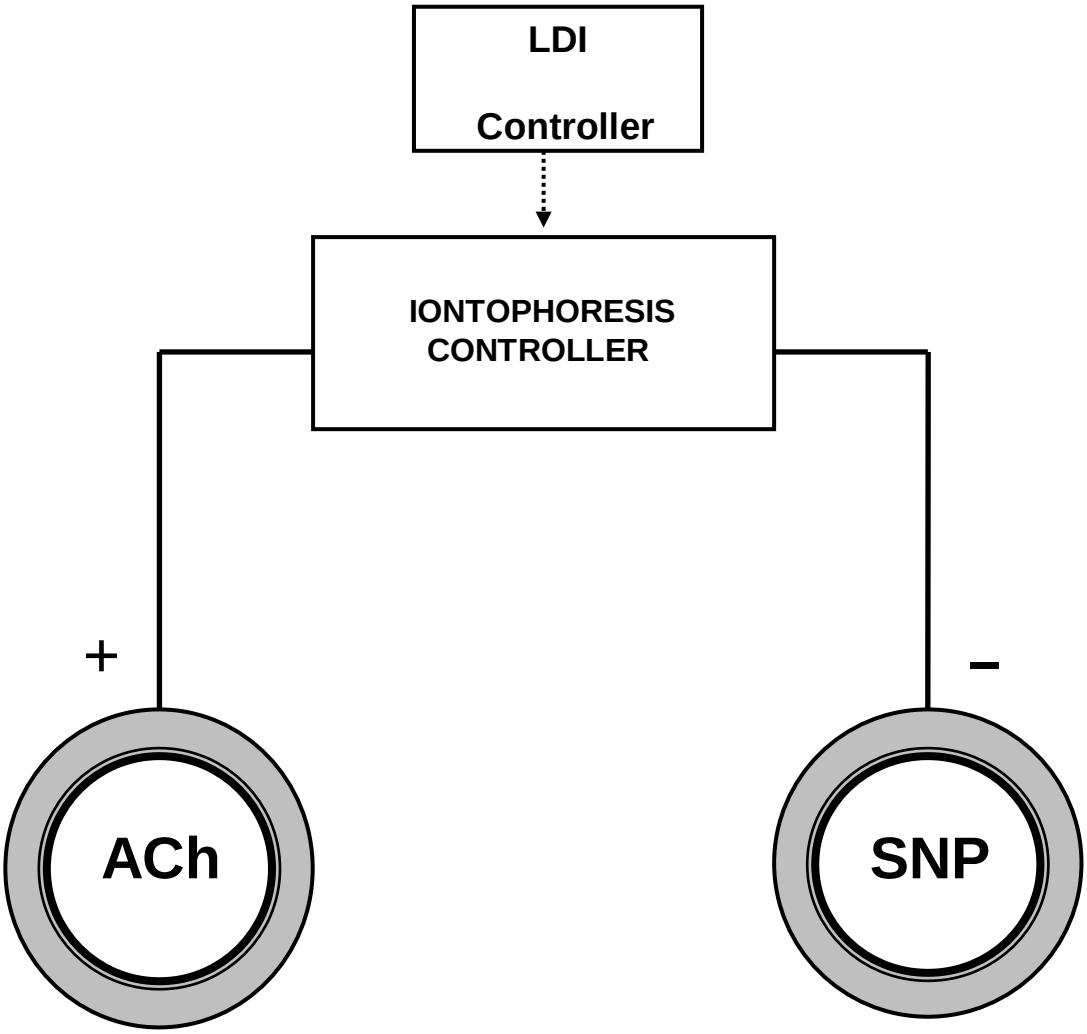

Figure 8.2 LDI Experimental Setup

Block Diagram of the experimental setup. The anodal and cathodal chambers contained acetylcholine (ACh) and sodium, nitroprusside (SNP) respectively**.**

The laser doppler imager software was programmed in order to control current delivery. The current was switched on at the beginning of a scan and remained on until the start of the next scan after which the current was then either left on for the next scan or was switched off after the total charge had been delivered. The duration of the current was the time taken to complete each scan (50 s) multiplied by the total number of scans programmed. Low currents were used to limit the iontophoresis dose. The procedure involved incremental current delivery with four scans at 5 µA, four at 10 µA, four at 15 µA, and two at 20 µA amounting to a total charge of 8 mC. 2.5 ml of 1% ACh (Sigma Chemical Co., St. Louis, MO, U.S.A.) was placed into the anodal chamber and 2.5 ml of 1% SNP (Sigma) was placed in the cathodal chamber therefore both agents were delivered simultaneously during the delivery of current. 0.5% NaCl was used as the vehicle for these agents. Circular coverslips (32mm) over the chambers prevented the leakage of fluid.

8.2.3 Perfusion Measurements

Measurement of skin perfusion was carried out using a laser doppler imager (Moor Instruments Ltd.) with a red laser (wavelength, 633 nm; power, 1 mW; beam diameter, 1 mm). This non-invasive technique is based on the doppler shift imparted by moving blood cells in the underlying tissue to the backscattered light. The laser is scanned over both chambers through the coverslips and so backscattered light is collected by photodetectors which is converted into a signal proportional to perfusion. This is expressed as

arbitrary perfusion (flux) units (PU). The signal is then displayed as a colour-coded image on a monitor. The imager manufacturer's image analysis software allowed perfusion measurements by outlining a region of interest (ROI) around the internal circumference of the chamber. The median flux value across approximately 700 measurement points was calculated through statistical analysis of the ROI. Twenty scans were carried out. The first was a control prior to current administration followed by 14 scans of the incremental current protocol outlined earlier (14 scans) and five further scans without current administration. (Figure 8.4) Skin temperature close to the iontophoresis chambers was monitored continuously throughout. No significant temperature change occurred between the beginning and the end of perfusion measurements, indicating stable conditions.

8.3 Statistics

Analysis was performed using MINITAB 13.1 for Windows (Minitab Inc). Differences in measures of endothelial function (VCAM and ICAM) and other parameters between patients and controls were tested by the Mann-Whitney U test or Students' t-test as appropriate. For measurement of skin perfusion responses an assessment of the overall response to drugs (or vehicle) was obtained by taking the area under the perfusion-current curve (AUC), with comparisons made using the Mann-Whitney U test. All data is expressed as mean ±SD or SEM. A p value of <0.05 was considered as statistically significant throughout.

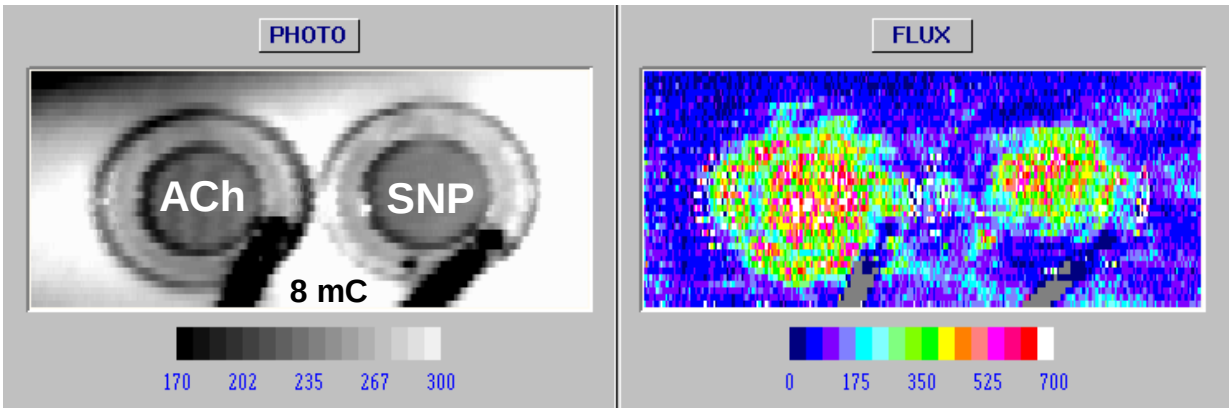

Figure 8.3 LDI Scan Pictures

The photo image is generated by back scattered light and the flux image is the doppler shifted component. Flux is colour-coded with lowest perfusion in dark blue (0 PU) and highest in dark red (500 PU).

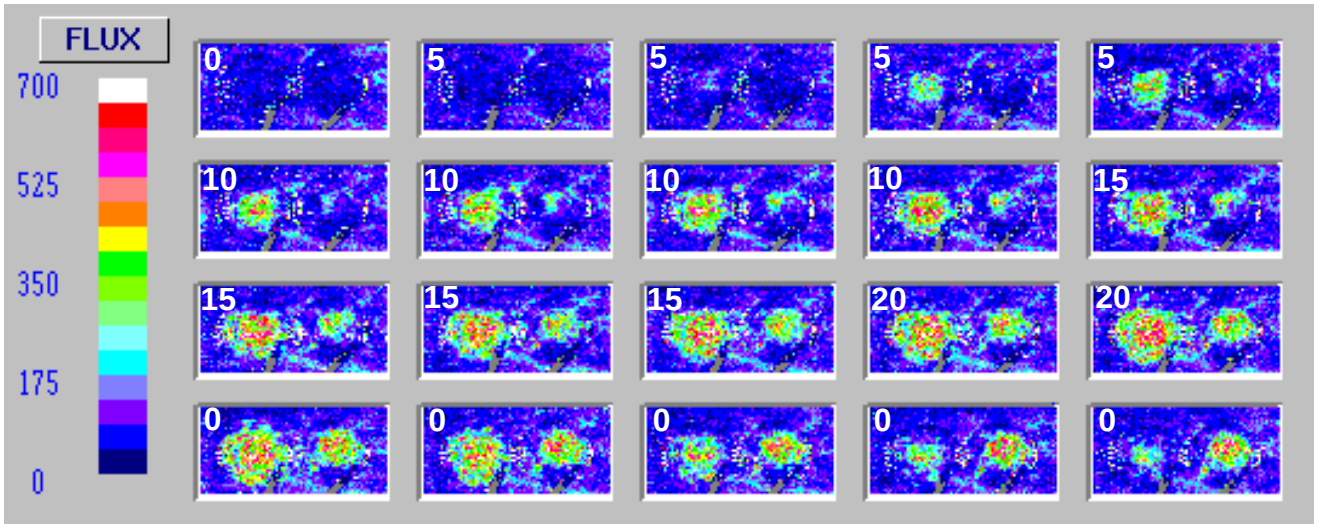

Figure 8.4 LDI Scan Pictures with Incremental Current Administration

Sodium nitroprusside (SNP) is in the upper chamber and acetylcholine (ACh) in the lower chamber. Current was incrementally applied as described in the text. The time courses of the drug-induced vasodilator responses differ.

8.4 Results

8.4.1 Laser Doppler Assessment of Microvascular Endothelial Function

At baseline, the vascular response to both SNP and acetylcholine did not differ significantly between patients and controls: SNP AUC , 1755 ± 490 vs 1372 ± 755, Acetylcholine AUC, 1633 ± 411 vs 1654 ± 436 PU, p=ns). This is portrayed in figures 8.5 and 8.6.

Treatment with omega-3 fatty acids did not improve vascular response to either SNP or acetylcholine in patients or controls. This is outlined in figures 8.7, 8.8, 8.9 and 8.10.

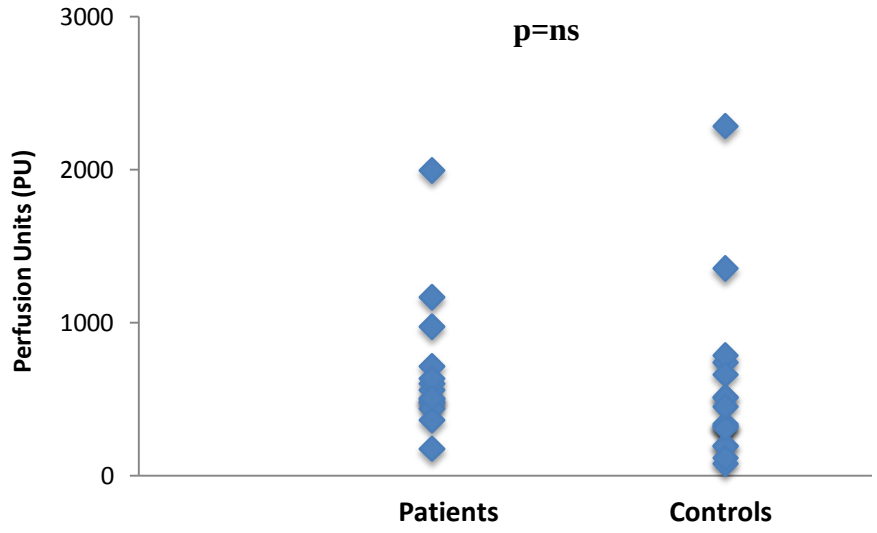

Figure 8.5 Comparison of ACh Responses Between Patients & Controls

Comparison of vascular responses to ACh between patients and controls represented as arbitrary perfusion units under the perfusion current curve. No difference is observed, p=ns.

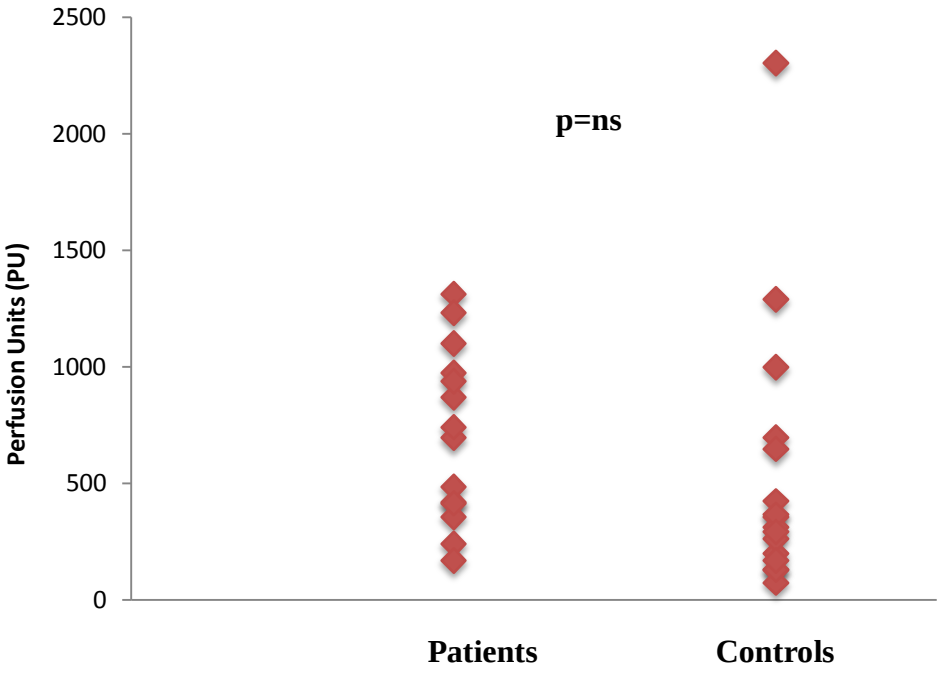

Figure 8.6 Comparison of SNP Response between Patients & Controls

Comparison of vascular responses to SNP between patients and controls represented as arbitrary perfusion units under the perfusion current curve. No difference is observed, p=ns.

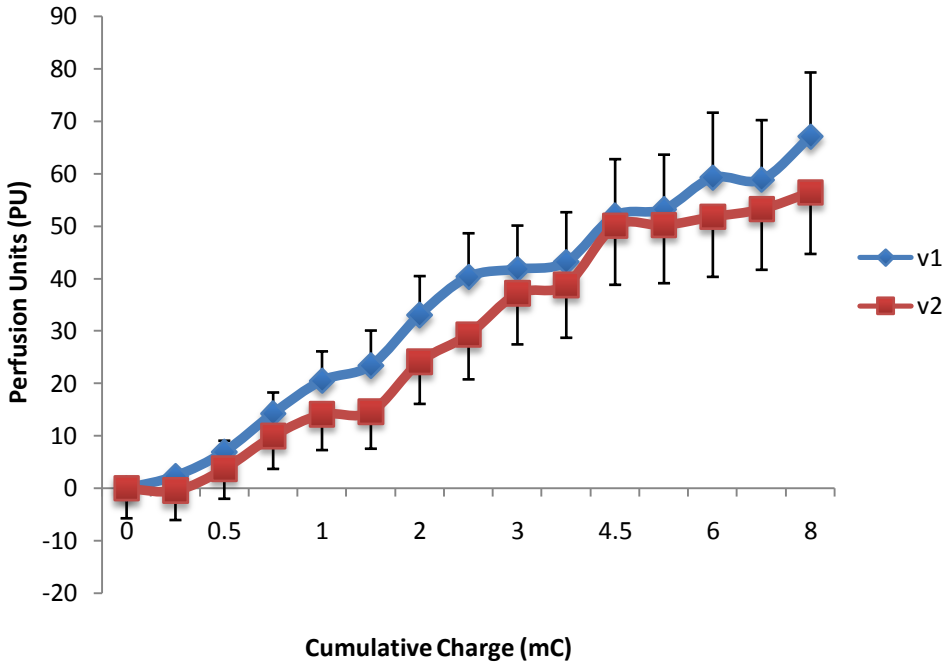

Figure 8.7 Acetylcholine Response in Patients Pre and Post Treatment

Vascular response to ACh in patients pre (v1) and post (v2) treatment with omega-3 fatty acids. Bars represent standard error of mean, p=ns (two way ANOVA).

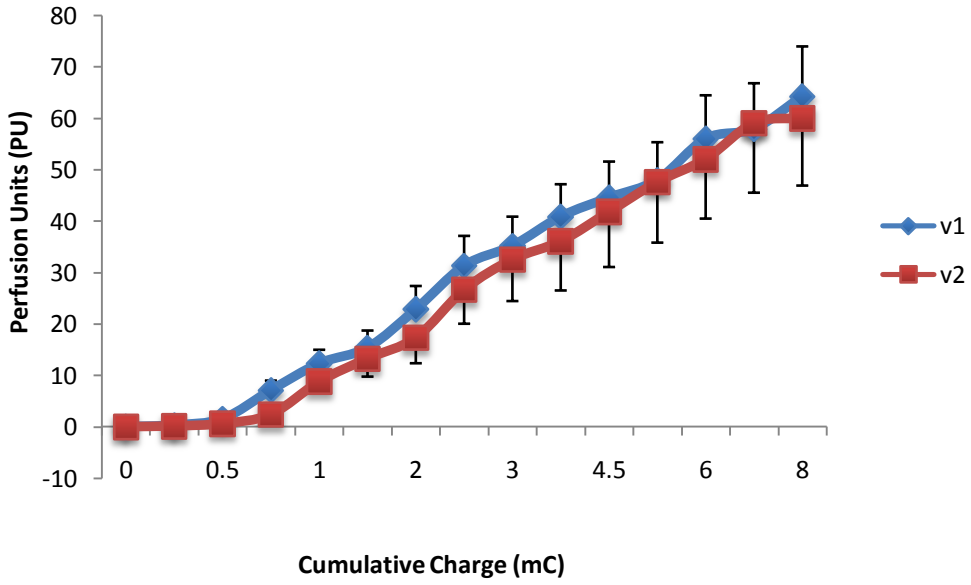

Figure 8.8 SNP Response in Patients Pre and Post Treatment

Vascular response to SNP in patients pre (v1) and post (v2) treatment with omega-3 fatty acids. Bars represent standard error of mean, p=ns (two way ANOVA).

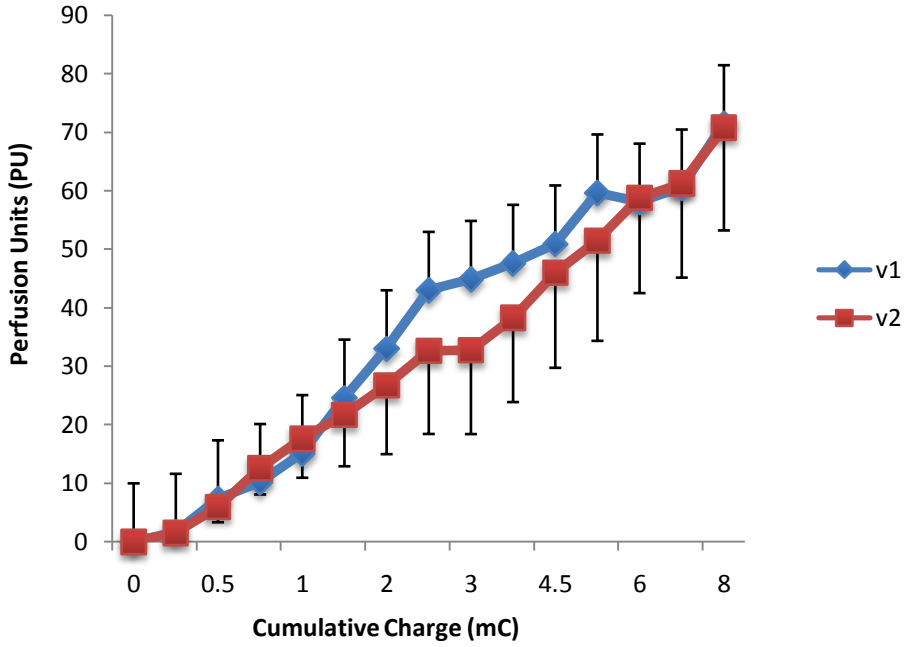

Figure 8.9 Acetylcholine Response in Controls Pre and Post Treatment

Vascular response to ACh in controls pre (v1) and post (v2) treatment with omega-3 fatty acids. Bars represent standard error of mean, p=ns (two way ANOVA).

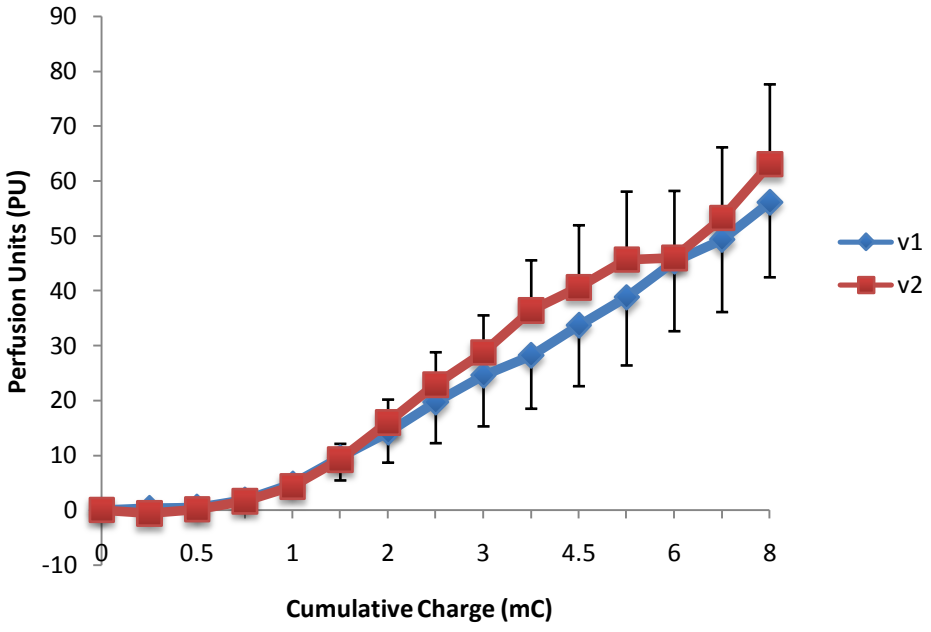

Figure 8.10 SNP Response in Controls Pre and Post Treatment

Vascular response to SNP in controls pre (v1) and post (v2) treatment with omega-3 fatty acids. Bars represent standard error of mean, p=ns (two way ANOVA).

8.4.2 Baseline Cardiovascular Risk Markers

Baseline levels of hsCRP, oxidised LDL, I-CAM, V-CAM and PLA_2 in patients and controls are portrayed in table 8.1.Plasma V-CAM and PLA_2 levels were significantly higher in the patient group at baseline ($p<0.001$ and $p=0.04$). No difference in baseline hsCRP, oxidized LDL and I-CAM was observed.

	Patients (n=17)	**Controls (n=17)**	**p**
hsCRP (mg/l)	2.2 (0.8-6.2)	1.4 (0.9-8.0)	ns
Ox LDL (mU/l)	83.6 (62.2-100.8)	79.4 (54.9-85.6)	ns
I-CAM (ng/ml)	257 (196 -308)	262 (218 -294)	ns
V-CAM (ng/ml)	879 (739-1103)	621 (428-701)	<0.001
PLA_2 (ng/ml)	228 (186-241)	185 (170-217)	0.04

Table 8.1 Cardiovascular Risk Markers in Patients and Controls

(All median & IQR)

8.4.3 The Effect of Treatment on Cardiovascular Risk Markers in Patients & Controls

In the patient group, treatment with omega-3 fatty acids did not significantly affect levels of hsCRP, oxidised LDL, I-CAM, V-CAM or PLA_2 (table 8.2).
In the controls, the only observed difference was in V-CAM concentration with a mean increase of 80 ng/ml (95% CI: 23 -138) (table 8.3). As in the patient group, hsCRP, oxidised LDL, I-CAM and PLA_2 were unchanged.

Patients	Baseline (n=17)	Post Treatment (n=17)	p
hsCRP (mg/l)	2.2 (0.8- 6.2)	1.6 (0.7-6.2)	ns
Ox LDL (mU/l)	84 (63 -101)	95 (70 -107)	ns
I-CAM (ng/ml)	257 (196-308)	247 (207-340)	ns
V-CAM (ng/ml)	879 (739-1103)	912 (1179-2541)	ns
PLA_2 (ng/ml)	228 (186-241)	247 (186-271)	ns

Table 8.2 The Effect of Omega-3 Fatty Acids on Cardiovascular Risk Markers in Patients

(All median & IQR)

Controls	Baseline (n=17)	Post Treatment (n=17)	p
hsCRP (mg/l)	1.4 (0.9- 8.0)	1.6 (0.8-3.4)	ns
Ox LDL (mU/l)	79 (55 - 86)	78 (56 -86)	ns
I-CAM(ng/ml)	262 (218- 294)	276 (229-301)	ns
V-CAM (ng/ml)	621 (428- 701)	630 (539- 732)	0.01
PLA_2 (ng/ml)	185 (170-217)	175 (168- 217)	ns

Table 8.3 The Effect of Omega-3 Fatty Acids on Cardiovascular Risk Markers in Controls

(All median & IQR)

8.5 Discussion

This study has shown no difference between microvascular endothelial function assessed by the technique of laser doppler imaging in patients with nephrotic range proteinuria compared with controls. Furthermore, we showed that omega-3 fatty acids had no demonstrable effect on endothelial function measured directly using LDI or through the measurement of soluble markers of endothelial activation in either population. We demonstrated that patients had higher baseline levels of V-CAM and PLA_2 than controls; however the only change that was observed following treatment with omega-3 fatty acids was an increase in V-CAM levels in the control group.

There have been two studies examining endothelial function using the technique of laser doppler iontophoresis in proteinuric patients. The first failed to show any difference in vascular response between patients and controls. They examined 21 otherwise healthy patients with asymptomatic proteinuria (226). The second examined 39 patients with glomerulonephritis and a mean albumin: creatinine ratio (ACR) of 191mg/mmol. Patients with co-existing vascular disease were excluded. They stopped ACE inhibitors and angiotensin receptor antagonists prior to the study. They demonstrated that the patients with proteinuria had significantly abnormal microvascular endothelial function compared with the controls. The vascular response to both SNP and ACh was impaired in the proteinuric group (227). Other techniques of assessing endothelial function have yielded similar results. Stroes et al demonstrated endothelial dysfunction in nine patients with nephrotic range proteinuria by venous occlusion plethysmography(178) and an Australian group found abnormal ‘endothelium- dependent’ response but

preserved ‘endothelium- independent’ response in patients with nephrotic syndrome using brachial artery ultrasound(179).

There are many factors which can affect endothelial function such as age, gender and smoking status. Drugs such as ACE inhibitors, angiotensin receptor antagonists and statins have all been shown to improve endothelial function(228;229). In this study, all statins were stopped for 4 weeks prior to enrolment, however we did not feel it was ethical to stop either ACE inhibitors or angiotensin receptor blockers in the patients with nephrotic range proteinuria and so all the patients were on either agent with two on both. Clearly, this may have influenced our results and account for some of the differences compared with previously published data. In addition, as we attempted to triglyceride match our controls, these subjects may not have been representative of a normal control population. Furthermore, the numbers in our study were small and the heterogeneous nature of the group may have contributed to the lack of observed difference between the patients with proteinuria and the control population.

Studies have shown a beneficial effect of omega-3 fatty acids on endothelial function. Supplementation with 4g daily of omega-3 fatty acids improved endothelial function measured by brachial artery ultrasound in a group of 15 hypercholesterolaemic subjects compared with controls(151). Using laser doppler imaging, supplementation with omega-3 fatty acids improved ACh mediated vascular response but not SNP response in healthy subjects(230). However, their effect on endothelial function in patients with nephrotic range proteinuria has not, to our knowledge, been studied.

Lipoprotein associated phospholipase A_2 (Lp-PLA_2) is a member of the phospholipase A_2 family of enzymes produced by macrophages, T lymphocytes and mast cells. Lp-PLA2 is present in atherosclerotic plaques (231). It hydrolyses oxidised phospholipids resulting in pro-inflammatory particles(232). This is an emerging marker for vascular events with epidemiological studies suggesting an association between increased Lp-PLA_2 levels and CHD (183;233-235). In addition, increased Lp-PLA_2 levels have been demonstrated in patients with subclinical coronary disease and coronary calcifications(236). More recently, Lp-PLA_2 has been implicated in endothelial dysfunction(232;237) . To our knowledge, no studies have examined the effect of omega-3 fatty acids on PLA_2 levels in patients with nephrotic range proteinuria. There have been a number of studies examining markers of endothelial function in patients with chronic renal failure but few have looked at the association between endothelial markers and the level of proteinuria. Elevated levels of I-CAM and V-CAM have been shown in patients with mild renal impairment(238), advanced renal impairment(239) and those on dialysis(240). MacKinnon et al demonstrated that patients with urinary protein levels greater than 2g in 24 hours had higher levels of I-CAM, V-CAM and CRP(241). Our study showed that patients with nephrotic range proteinuria had higher levels of V-CAM but I-CAM and CRP did not differ. The numbers in our study were small and as described above there were a number of confounding factors.

Omega-3 fatty acids are known to have established ant-inflammatory properties (242). However, the interventional data is inconsistent (243-246). A

recent meta-analysis of four prospective randomised studies showed that omega-3 fatty acids did not affect hs-CRP (247). Similarly, studies examining the effect of omega-3 fatty acids on markers of endothelial activation have displayed varying results. In a study of sixty healthy volunteers, supplementation with 6.6g omega-3 fatty acids had no effect on I-CAM or V-CAM levels(248). However, a study of 563 hyperlipidaemic men showed that I-CAM fell but V-CAM was unchanged following treatment (249). This was a large, long-term study with a treatment duration of 3 years. The data regarding the effect of omega-3 fatty acids on oxidised LDL levels is sparse. There was no change in oxidised LDL levels in a study of hypercholesterolaemic subjects supplemented with alpha-linolenic acid for 2 years(250).

It is difficult to draw conclusions from our data in view of the small numbers and short treatment duration.

In summary, we were unable to demonstrate a difference in endothelial function between patients with nephrotic range proteinuria compared with normal controls. However, to our knowledge, we are the first to observe higher levels of Lp-PLA_2 in this population. Moreover, treatment with omega-3 fatty acids had no beneficial effect in either population.

Chapter 9: General Discussion and Conclusions

9.1 Nephrotic Syndrome and Lipoprotein Abnormalities

The dyslipidaemia associated with nephrotic syndrome is characterized by increased levels of cholesterol, triglyceride, VLDL-C, excess small dense LDL and low HDL-C(162-165). This work confirms these abnormalities.

The mechanism leading to this dyslipidaemia is unclear. Several studies have suggested that there is reduced catabolism of Apo B containing lipoproteins (TG, VLDL, IDL and LDL) rather than increased hepatic synthesis. Demant et al examined metabolism of Apo B containing lipoproteins using radiotracer kinetic studies. They found impaired delipidation of $VLDL_2$ and IDL with reduced LDL catabolism(251). In addition, a further study of hypercholesterolaemic patients with nephrotic syndrome showed that these patients had lower catabolic rates of LDL Apo B but no overproduction of LDL Apo B (252). Impaired triglyceride metabolism rather than increased synthesis is responsible for the increased triglyceride levels observed in these patients. Animal work has shown higher triglyceride levels in nephrotic rats than in rats with analbuminaemia despite similar rates of triglyceride production(253) .

Total HDL levels are usually normal even although HDL_2 to HDL_3 ratio in patients with nephrotic syndrome are often low. Levels of LCAT (the enzyme responsible for the conversion of HDL_3 to HDL_2) have been found to be low due to urinary losses(254). Hepatic mRNA levels were unaffected suggesting increased excretion of LCAT rather than decreased production resulting in increased levels of HDL_2.

Increased levels of remnant lipoproteins and small dense LDL have previously been described in this population (255). Again, our observations have confirmed these abnormalities. Plasma triglycerides were the most important

factor influencing LDL phenotype and remnant lipoprotein concentration in our study. Once again, confirming previous observations. The increase in cholesterol, triglyceride, LDL-C, remnant lipoproteins and small dense LDL all contribute to increasing CV risk.

9.2 Postprandial Lipaemia

There is little published human work examining chylomicron and VLDL metabolism in patients with nephrotic range proteinuria. The existing evidence in this area is conflicting. Studies in nephrotic rats have found changes in the catabolism and composition of chylomicrons(202;203). These studies have been difficult to perform due to technical difficulties in separating the chylomicrons from VLDL. Warwick et al measured TG and retinyl palmitate in the d1.006g/ml fraction of plasma following a vitamin A loaded standard fat meal in nephrotic subjects. They found no difference in the time course of the postprandial response suggesting that chylomicron metabolism did not differ between nephrotic subjects and normal control (204). However, differences were observed between individuals in their postprandial response. This emphasizes the heterogeneity of the population being studied. The same group demonstrated a reduction in clearance of VLDL in nephrotic subjects but others have failed to demonstrate this (256). It is likely that the discrepancy in results is multifactorial with the heterogeneity of this population, the difficulty in separating and measuring chylomicrons and differences in fat load administration all contributing.

There are very few studies examining the structure and composition of chylomicrons and VLDL. Chylomicrons from nephrotic rats have been shown

to be larger and rich in triacylgycerol(202). To our knowledge, there have been no human studies examining this. Our work is in keeping with the animal studies. We observed larger chylomicrons in the patients with nephrotic range proteinuria. These structural abnormalities may account for their less efficient lipolysis that we observed in chapter 5.

Work from our laboratory previously reported abnormalities in the structure of VLDL particles with a smaller more crystalline particle deficient in apo CII, III and E(175). We were unable to replicate these findings in our population. Again, the heterogeneity of the population being studied along with the small numbers involved and advances in the apoB assay may, in part, account for this. However, our findings are in keeping with our results from chapter 5 where we showed delayed postprandial chylomicron clearance but unaltered postprandial VLDL metabolism in the patients with nephrotic range proteinuria. Further studies are required to clarify this.

9.3 Endothelial Function and Inflammation

Proteinuria is increasingly being thought of as a marker of vascular disease. Microalbuminuria has been linked with endothelial dysfunction (10) .However; studies using the technique of laser doppler iontophoresis to assess endothelial function in patients with proteinuria are conflicting. A study of 21 asymptomatic proteinuric patients failed to show any difference in vascular response(226). A further study examined 29 patients with a mean ACR of 191mg/mmol found both impaired SNP (endothelium-independent) and Ach (endothelium independent) response in the patients with proteinuria(241). We failed to replicate this in our population of 17 patients with nephrotic range

proteinuria. Potential confounding factors that may have influenced our results included the small number of patients studied and the continued use ACE inhibitors and Angiotensin receptor blockers throughout the study due to ethical concerns regarding discontinuing these preparations.

Inflammation is known to play an important role in atherosclerosis. A number of biomarkers for endothelial dysfunction and inflammation are emerging which have been shown to be useful for the prediction of coronary heart disease. Several studies have indicated that increased levels of hs CRP are predictive of cardiovascular events in healthy subjects(88). Increased levels of hsCRP have also been observed in patients with proteinuria(241). Expression of adhesion molecules (I-CAM and V-CAM) are increased on endothelial cells overlying atheroma and elevated levels of these molecules have been found in patients with all levels of renal impairment from mild to end stage renal failure and also in patients with proteinuria(238-240). PLA_2 is an emerging marker for vascular events. It is present in atherosclerotic plaques and associated with endothelial dysfunction(232;237). In our study we observed increased levels of PLA_2 and V-CAM but no difference in I-CAM and CRP. We acknowledge that our study was not adequately powered to detect a difference in these parameters and there are a number of confounding factors as mentioned earlier. However, the increase in PLA_2 and V-CAM are noteworthy but further more detailed investigation in a large study population is required to clarify this.

9.4 Omega- 3 Fatty Acids and Nephrotic Syndrome

Prospective randomised controlled trial evidence has demonstrated the benefit of omega-3 fatty acids in different populations. Men without clinical heart disease, patients with angiographic evidence of CHD, patients with recent myocardial infarctions, adults over 65 years old and hypercholesterolaemic subjects are among the groups where the benefit of omega-3 fatty acids have been demonstrated(114;125;257-259). However, there has been little work examining their effect on dyslipidaemia in patients with nephrotic range proteinuria.

The major effect of omega-3 fatty acids on lipids and lipoproteins is a reduction in plasma TG and TRLs. Studies have shown that they inhibit TG synthesis and secretion of VLDL from the liver(118;119;191). They are thought to reduce production of triglyceride rich lipoproteins rather than enhancing their clearance(52;120;121). They are also known to increase LDL-C. The mechanism behind this is unclear but it has been suggested that they down regulate the LDL Apo B/E receptor as well as preferentially converting VLDL to LDL(127;128). This increase in LDL-C is potentially offset by a redistribution to larger less atherogenic LDL but remains a concern to some clinicians. This is thought to be caused by suppression of CETP activity thus reducing the transfer of cholesteryl ester from HDL to VLDL and LDL(129) resulting in triglyceride enrichment of HDL and LDL.

We have shown in chapters 3 and 4 that patients with nephrotic range proteinuria had increased levels of triglyceride rich lipoproteins and excess small dense LDL. We found that treatment with omega-3 fatty acids reduced triglyceride rich lipoproteins and levels of small dense LDL concentration in

the patients with nephrotic range proteinuria but no reduction was observed in the control group. The differing effects are likely to be due to the higher baseline TG levels in patients with nephrotic range proteinuria compared with the controls. These benefits are similar to those seen in hypertriglyceridaemic subject but no underlying renal disease.

Treatment with omega-3 fatty acids have been shown to reduce postprandial lipaemia in the normal population and hypertriglyceridaemic subjects with a greater benefit displayed with higher baseline TG levels(120;130;131). It is thought that they accelerate chylomicron clearance by facilitation of lipoprotein lipase mediated lipolysis(120). However, their effect on postprandial lipaemia has not been studied in nephrotic patients. In our study, we observed a reduction in chylomicron AUC following treatment with omega-3 fatty acids in the patients with nephrotic range proteinuria but not in the control population. We postulated that the differences again were related to the higher levels of baseline triglyceride in the patient group however on multivariate analysis subject group rather than baseline triglyceride concentration was found to be an independent predictor of change in chylomicron AUC. This suggests that the chylomicrons of patients with nephrotic range proteinuria may differ in structure or composition.

Omega-3 fatty acids are thought to reduce VLDL synthesis and secretion (118;119;191). We found a reduction in peak postprandial $VLDL_1$ AUC following treatment. Once again, this has not been previously studied in this population but is in keeping with previous studies suggesting a reduction in synthesis. The effect of omega-3 fatty acids on the structure or composition of

chylomicrons or VLDL has not been studied in any population. We observed no difference in the structure or composition of chylomicron or VLDL particles in patients or controls following treatment. This suggests that the effect we observed of omega-3 fatty acids on postprandial lipaemia is through either increased clearance or reduced the synthesis of chylomicrons and VLDL rather than an alteration in their structure.

Omega-3 fatty acids have been shown to have a beneficial effect on HDL. They are thought to increase HDL_2 at the expense of HDL_3 by reducing CETP activity(129). In keeping with this suggested mechanism, we found that treatment increased levels of HDL_2 with an increase in mean particle diameter of HDL in both patients and controls but HDL-C was unaltered. We did not show a group effect implying a similar mechanism in both groups. Increasing levels of HDL-C reduce CV risk(29-33) but the atherogenicity of the HDL subfractions is less clear. Quantification and measurement of these subclasses is difficult and time-consuming. Thus, the clinical significance of the effect of omega-3 fatty acids on HDL in this population is uncertain.
The evidence regarding the effect of omega-3 fatty acids on endothelial function is conflicting. They have been shown to improve endothelial function in healthy patients when measured by both brachial artery ultrasound and laser doppler imaging(151;230) but their effect on endothelial function in patients with nephrotic range proteinuria has not previously been studied. We were unable to demonstrate an improvement in endothelial function of patients or controls following treatment. It has been suggested that omega-3 fatty acids exert their effect on the endothelium by causing changes in the

composition of the membrane bilipid layer thereby promoting increased release of endothelium derived NO in response to Ach(152). As discussed earlier, our study was limited by a number of confounding factors such as renal dysfunction, co-existing vascular disease, the use of ACE inhibitors and Angiotensin receptor blockers and the short duration of treatment. Further work with larger numbers of patients may help clarify the effect of omega-3 fatty acids but studies may be limited due to the widespread use of ACE inhibitors and angiotensin receptor antagonists. They're proven efficacy in reducing the rate of renal progression makes stopping these preparations difficult to justify.

9.5 Clinical Implications

We observed increased levels of total cholesterol, triglyceride, VLDL-C, sd LDL and remnant lipoproteins in the patients with nephrotic range proteinuria compared with the controls. These abnormalities are associated with increased CV risk and so our data has a number of implications.

Randomised controlled trials have demonstrated the benefit of omega-3 fatty acids. In the Diet and Reinfarction Trial (DART), there was a 29 % reduction in mortality in the patients who received dietary advice on fish consumption. However, no change in cholesterol levels were observed(111). The GISSI-Prevenzione trial showed a 10 % reduction in the relative risk for the primary end-point of total mortality, non-fatal myocardial infarction and stroke with a 0.9% reduction in triglyceride in the patients treated with 850mg of omega-3 fatty acids at 6 months(113). The Study on Prevention of Coronary Atherosclerosis by Intervention with Marine Omega-3 fatty acids (SCIMO) also

showed a significant reduction in TG (10%) at 6 months with significantly less progression of coronary artery disease assessed angiographically(114).
In our study, we observed a 17.5% fall in triglyceride concentration in the patients with nephrotic range proteinuria compared with a 5.9% fall in the control group therefore if we extrapolate from these studies, a significant reduction in CV risk should be evident. However, even although studies have suggested that hypertriglyceridaemia is a risk factor for CHD independent of other risk factors(260), the evidence regarding the benefit of triglyceride lowering on mortality is less robust. Since we commenced this study, there have been further studies examining fibrates, which have also addressed previous concerns that fibrates increase serum creatinine levels. The FIELD (Fenofibrate Intervention and Event Lowering in Diabetes) trial showed a 29% reduction in plasma triglyceride and 12% reduction in LDL-C in patients with diabetes but the reduction in the primary end-point of cardiovascular disease was not significant. They observed a 15% higher increase in serum creatinine in the fibrate group but this was no longer evident following discontinuation suggesting that this was due to a functional rather than a structural change(261). The JELIS (Japan EPA Lipid Intervention Study) randomised 18 645 patients with total cholesterol of 6.5mmol/l or greater to 1800mg of EPA and statin or statin alone with a 5 year follow-up. They showed a 19% relative reduction in coronary events in the EPA treated group with a 9% reduction in triglyceride in this group compared with 4% reduction in the statin alone arm ($p<0.0001$). Both treatments produced only small changes in HDL-C(259).
A recent study examining the effect of Torcetrapib (a potent CETP inhibitor) showed a 72% increase in HDL-C and 25% reduction in LDL-C but treatment

was shown to increase mortality and CV events and so was terminated prematurely(262). The mechanism behind this is unclear. The role of HDL subfractions may play an important role in the protective effect of HDL-C. This may, in part, account for the results observed in this study. Further work examining the effect of Torcetrapib on HDL subfractions may clarify this. The previously mentioned fish oil studies showed a reduction in CV risk with smaller reductions in TG levels. This implies that the action of omega-3 fatty acids on lipids and lipoproteins is not solely responsible for the effect they have on reducing CV risk. As discussed in chapter 2, in contrast to fibrates, omega-3 fatty acids act in a number of different ways. It is likely that the combination of these different mechanisms act to lower CV risk in this population of patients. In addition to triglyceride lowering, we observed significant reductions in remnant lipoprotein, small dense LDL and postprandial lipaemia. However, the effect we observed on LDL is concerning therefore we would suggest that monotherapy with omega-3 fatty acids is unadvisable in this population of patients.

Our study was not designed to examine the effect of omega-3 fatty acids on renal progression and proteinuria. Studies have suggested that omega-3 fatty acids slow renal progression in patients with IgA nephropathy(156;263). Investigators from the Mayo clinic showed that treatment with omega-3 fatty acids reduced renal progression in patients with idiopathic IgA nephropathy and persistent proteinuria over a period of 2 years(156). The same investigators examined these patients over a mean follow-up of 6.4 years. Once again, they demonstrated reduced rates of renal progression in the fish

oil treated patients. However, levels of proteinuria did not differ between the two groups(263) and lipid parameters were not measured in these patients. Treatment with HMG Co reductase inhibitors has also been shown to reduce renal progression. A meta-analysis of 27 randomised controlled trials examined the effects of statins on change in eGFR (264). They showed that patients treated with statins had 1.2 ml/min per year slower decline in GFR than controls and a reduction in proteinuria. Thus, the combination of omega-3 fatty acids and statins may reduce renal progression further particularly in a population with a high risk of progressive renal failure due to the presence of significant proteinuria.

In this thesis, we have demonstrated that omega-3 fatty acids were well tolerated with beneficial effects on triglyceride-rich lipoproteins, postprandial lipaemia and HDL metabolism in patients with nephrotic range proteinuria. However, we have also shown that omega-3 fatty acids increase LDL-C in these patients which may offset these benefits.

9.6 Further Work

Further work is required to clarify certain areas which we have studied. We plan to carry out electron microscopy of the chylomicrons and $VLDL_1$ in order to determine their size and whether they differ between the two populations. Kinetic studies examining chylomicron and $VLDL_1$ metabolism in patients with nephrotic range proteinuria compared with controls may clarify the differences observed between our work and previously published work.

In terms of future treatment options, a study examining the effect of combination therapy with HMG Co A reductase inhibitors together with omega-3 fatty acids looking at their effect on lipids and lipoproteins, in particular, postprandial lipaemia and their effect on LDL-C. Furthermore, their combined effect on renal progression and proteinuria would also be of interest in this population of patients. These further studies would allow us to effectively tailor lipid therapy in this high-risk population of patients. However, such large scale prospective studies examining CV or renal progression end-points may prove difficult to undertake due to the low incidence of this condition and the large numbers that would be required to be recruited.

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