HDL functionality and LDL quality: the influence of obesity, obstructive sleep apnoea and pharmacological intervention

HDL FUNCTIONALITY AND LDL QUALITY: THE INFLUENCE OF OBESITY, OBSTRUCTIVE SLEEP APNOEA AND PHARMACOLOGICAL INTERVENTION

A thesis submitted to The University of Manchester
for the degree of Doctor of Medicine, MD
in the Faculty of Medical and Human Sciences

2013

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School of Medicine
Cardiovascular Medicine
HDL functionality and LDL quality: the influence of obesity, obstructive sleep apnoea and pharmacological intervention

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**ABBREVIATIONS (in alphabetical order)**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABCA-I</td>
<td>ATP-binding cassette transporter A1</td>
</tr>
<tr>
<td>ABCG-I</td>
<td>ATP-binding cassette transporter GI</td>
</tr>
<tr>
<td>ABCG-4</td>
<td>ATP-binding cassette transporter G4</td>
</tr>
<tr>
<td>ADA</td>
<td>American Diabetes Association</td>
</tr>
<tr>
<td>AE</td>
<td>Adverse events</td>
</tr>
<tr>
<td>AGE</td>
<td>Advanced glycation end-products</td>
</tr>
<tr>
<td>AHI</td>
<td>Apnoea hypo-apnoea index</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immuno-deficiency syndrome</td>
</tr>
<tr>
<td>apoAI</td>
<td>Apolipoprotein AI</td>
</tr>
<tr>
<td>apoAII</td>
<td>Apolipoprotein AII</td>
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<tr>
<td>ApoB</td>
<td>Apolipoprotein B</td>
</tr>
<tr>
<td>ApoJ</td>
<td>Apolipoprotein J</td>
</tr>
<tr>
<td>apoM</td>
<td>Apolipoprotein M</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BHT</td>
<td>Butylated hydroxytoluene</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAPD</td>
<td>Continuous ambulatory peritoneal dialysis</td>
</tr>
<tr>
<td>CE</td>
<td>Cholesteryl esters</td>
</tr>
<tr>
<td>CETP</td>
<td>Cholesteryl esters binding protein</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
</tr>
<tr>
<td>CHE</td>
<td>Cholesterol esterase</td>
</tr>
<tr>
<td>CHO</td>
<td>Cholesterol oxidase</td>
</tr>
<tr>
<td>CHOD-PAP</td>
<td>Cholesterol oxidase phenol 4-aminoantipyrine peroxidase</td>
</tr>
<tr>
<td>CIMT</td>
<td>Carotid intima media thickness</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic kidney disease</td>
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<tr>
<td>CPAP</td>
<td>Continuous positive airway pressure</td>
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CRP</td>
<td>C reactive protein</td>
</tr>
<tr>
<td>CV</td>
<td>Cardio vascular</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardio vascular disease</td>
</tr>
<tr>
<td>CVR</td>
<td>Cardio vascular risk</td>
</tr>
<tr>
<td>DALY</td>
<td>Disability Adjusted Life Years</td>
</tr>
<tr>
<td>DGAT-2</td>
<td>Diacylglycerol acyl transferase 2</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DP1</td>
<td>Prostaglandin D2 receptor subtype 1</td>
</tr>
<tr>
<td>DSMB</td>
<td>Data and safety monitoring board</td>
</tr>
<tr>
<td>eGFR</td>
<td>Estimated glomerular filtration rate</td>
</tr>
<tr>
<td>EAS</td>
<td>European Atherosclerosis Society</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra acetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immuno sorbent assay</td>
</tr>
<tr>
<td>ERN</td>
<td>Extended release niacin</td>
</tr>
<tr>
<td>FBG</td>
<td>Fasting blood glucose</td>
</tr>
<tr>
<td>G6PD</td>
<td>Glucose-6-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>Gly apoB</td>
<td>Glycated apoB</td>
</tr>
<tr>
<td>GOD-PAP</td>
<td>Glucose oxidase phenol 4-aminoantipyrine peroxidase</td>
</tr>
<tr>
<td>GPO-PAP</td>
<td>Glycerol phosphate oxidase phenol 4-aminoantipyrine peroxidase</td>
</tr>
<tr>
<td>GPR109a</td>
<td>G-protein coupled receptor 109a</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HbA1c</td>
<td>Glycated hemoglobin A1c</td>
</tr>
<tr>
<td>HD</td>
<td>Hemodialysis</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>HDL-C</td>
<td>High density lipoprotein cholesterol</td>
</tr>
<tr>
<td>HeFH</td>
<td>Heterozygous familial hypercholesterolemia</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immuno-deficiency virus</td>
</tr>
<tr>
<td>HMEC-1</td>
<td>Human micro vascular endothelial cells</td>
</tr>
<tr>
<td>HOMA$\beta$</td>
<td>Homeostasis mode assessment beta cell function</td>
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</tbody>
</table>
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<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>HOMA-IR</td>
<td>Homeostasis mode assessment – insulin resistance</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSDA</td>
<td>N-(2-hydroxy-3-sulphopropyl)-3,5-dimethoxyaniline</td>
</tr>
<tr>
<td>HSL</td>
<td>Hormone sensitive lipase</td>
</tr>
<tr>
<td>Hs-CRP</td>
<td>High sensitivity CRP</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule 1</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin 1</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>K&lt;sub&gt;2&lt;/sub&gt;EDTA</td>
<td>Potassium ethylene diamine tetra acetic acid</td>
</tr>
<tr>
<td>KBr</td>
<td>Potassium bromide</td>
</tr>
<tr>
<td>LCAT</td>
<td>Lecithin:cholesterol acyl transferase</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LDL-C</td>
<td>Low density lipoprotein cholesterol</td>
</tr>
<tr>
<td>LOX-1</td>
<td>Lectin-like oxidized LDL receptor-1</td>
</tr>
<tr>
<td>Lp-PLA&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Lipoprotein phospholipase 2</td>
</tr>
<tr>
<td>LRP</td>
<td>Laropiprant</td>
</tr>
<tr>
<td>Lyso-PC</td>
<td>Lyso phosphatidyl choline</td>
</tr>
<tr>
<td>Lp(a)</td>
<td>Lipoprotein (a)</td>
</tr>
<tr>
<td>LPO</td>
<td>Lipid peroxide</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Macrophage chemoattractant protein 1</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-morpholinopropanesulphonic acid</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>MSD</td>
<td>Merck Sharp &amp; Dohme</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADP</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NCEP ATP III</td>
<td>National cholesterol education program adult treatment panel III</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>NEFA</td>
<td>Non esterified fatty acid</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Nuclear factor kappa b</td>
</tr>
<tr>
<td>NHS</td>
<td>National health service</td>
</tr>
<tr>
<td>NICE</td>
<td>National institute of clinical excellence</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NSAIDS</td>
<td>Non steroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>OSA</td>
<td>Obstructive sleep apnoea</td>
</tr>
<tr>
<td>OxLDL</td>
<td>Oxidized LDL</td>
</tr>
<tr>
<td>OxNEFA</td>
<td>Oxidized NEFA</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet activating factor</td>
</tr>
<tr>
<td>PAF-AH</td>
<td>Platelet activating factor acetylhydrolase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidyl choline</td>
</tr>
<tr>
<td>PD</td>
<td>Peritoneal dialysis</td>
</tr>
<tr>
<td>PEG-CHE</td>
<td>Polyethylene glycol pre-treated cholesterol esterase</td>
</tr>
<tr>
<td>PEG-CHO</td>
<td>Polyethylene glycol pre-treated cholesterol oxidase</td>
</tr>
<tr>
<td>PLTP</td>
<td>Phospholipid Transfer Protein</td>
</tr>
<tr>
<td>PIPES</td>
<td>Piperazine-1,4-bis(2-ethanesulphonic acid</td>
</tr>
<tr>
<td>PLA-2</td>
<td>Phospholipase 2</td>
</tr>
<tr>
<td>POD</td>
<td>Peroxidase</td>
</tr>
<tr>
<td>PON1</td>
<td>Paraoxonase 1</td>
</tr>
<tr>
<td>RCT</td>
<td>Reverse cholesterol transport</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>S1P</td>
<td>Sphingosine-1-phosphate</td>
</tr>
<tr>
<td>SAA</td>
<td>Serum amyloid A</td>
</tr>
<tr>
<td>SAE</td>
<td>Serious adverse effect</td>
</tr>
<tr>
<td>sCAM</td>
<td>Soluble cell adhesion molecule</td>
</tr>
<tr>
<td>SdLDL</td>
<td>Small dense molecule</td>
</tr>
<tr>
<td>sE-SELECTIN</td>
<td>Soluble E-SELECTIN</td>
</tr>
</tbody>
</table>
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>sICAM-1</td>
<td>Soluble ICAM-1</td>
</tr>
<tr>
<td>SPSS</td>
<td>Statistical Package for the Social Sciences</td>
</tr>
<tr>
<td>SR-A</td>
<td>Scavenger receptor A</td>
</tr>
<tr>
<td>SR-B1</td>
<td>Scavenger receptor B1</td>
</tr>
<tr>
<td>TBARS</td>
<td>Thiobarbituric reactive substances</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered solution</td>
</tr>
<tr>
<td>TC</td>
<td>Total cholesterol</td>
</tr>
<tr>
<td>TEM</td>
<td>Trans-endothelial migration</td>
</tr>
<tr>
<td>TG</td>
<td>Triglycerides</td>
</tr>
<tr>
<td>THP1 monocytes</td>
<td>Human acute monocytic leukemia cell line</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethylbenzidine</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>TXA2</td>
<td>Thromboxane A2</td>
</tr>
<tr>
<td>ULN</td>
<td>Upper limit of normal</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule 1</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
</tr>
<tr>
<td>VLDL-C</td>
<td>Very low density lipoprotein cholesterol</td>
</tr>
<tr>
<td>VLDL-TG</td>
<td>Very low density lipoprotein triglycerides</td>
</tr>
<tr>
<td>VAP II</td>
<td>Vertical auto profile II</td>
</tr>
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HDL functionality and LDL quality: the influence of obesity, obstructive sleep apnoea and pharmacological intervention

ABSTRACT
University of Manchester
Rahul Yadav
Doctor of Medicine, MD
HDL functionality and LDL quality: the influence of obesity, obstructive sleep apnoea and pharmacological intervention
Date: 28th August 2013

Aims: LDL oxidation plays an important role in the initiation and progression of atherosclerosis. HDL impedes oxidation, glycation and glycoxidation in vitro and there is evidence to suggest paraoxonase-1 (PON1) plays an important role in this. 1. In patients with dyslipidaemia treated with statins, I assessed the relationship of serum PON1 activity with in vitro HDL antioxidant capacity, susceptibility of LDL to oxidation and the protection offered by HDL. 2. I studied the effect of the presence and severity of obstructive sleep apnoea (OSA) in morbidly obese patients on HDL anti-oxidant and anti-inflammatory functions. 3. I investigated the influence of extended release niacin/ laropiprant (ERN/LRP) versus placebo in patients who had persistent dyslipidaemia despite receiving high doses of potent statins. I assessed the effect of ERN/LRP on mediators of vascular inflammation and HDL’s in vitro anti-oxidant function.

Methods: 1. LDL isolated from dyslipidemic patients was incubated with and without HDL, in the presence of Cu^{2+}. Similarly isolated HDL was incubated alone. Lipid peroxides (LPO) generated over 3 hours were measured. Patients were divided into 2 groups based on median serum PON1 activity. 2. 41 morbidly obese patients were divided into two groups based on the presence or absence of OSA (“OSA” and “no OSA” group) or on severity of OSA (high or low apnoea-hypoapnoea index (AHI) groups). I studied HDL’s ability to protect itself from in vitro oxidation and measured serum PON1 activity, tumor necrosis factor α (TNFα) and intercellular adhesion molecule 1 (ICAM1). 3. This was a randomised double blind cross over trial, where I studied the effect of ERN/LRP compared to placebo in 27 patients who had high LDL-C inspite of maximum tolerated doses of statins. I measured lipid profile, apolipoproteins, cholesteryl ester transport protein (CETP) activity, paraoxonase 1 activity (PON1), oxidised LDL (oxLDL) and related mediators of vascular inflammation. I also examined the capacity of HDL to protect LDL from in vitro oxidation.

Results and conclusion: 1. In statin treated dyslipidemic patients the capacity of HDL to protect itself and LDL from oxidation in vitro is significantly better in individuals with higher serum PON1 activity. 2. The capacity of HDL to protect itself from in vitro oxidation in morbidly obese patients is reduced with onset and severity of OSA. The differences in TNFα and ICAM1 levels may suggest endothelial dysfunction due to OSA. Oxidative damage of PON1 attributable to OSA could be a mechanism for HDL and endothelial dysfunction. 3. Treatment with ERN/LRP resulted in a significant improvement in HDL-C but did not affect HDL’s in vitro anti-oxidant function in patients who had persistent dyslipidaemia despite high doses of potent statins. For the first time I have shown that ERN/LRP reduces mediators of vascular inflammation.
HDL functionality and LDL quality: the influence of obesity, obstructive sleep apnoea and pharmacological intervention

DECLARATION

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Name of candidate: Dr Rahul Yadav

Date: 28th August 2013
HDL functionality and LDL quality: the influence of obesity, obstructive sleep apnoea and pharmacological intervention

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THE AUTHOR

I am a specialist trainee in Diabetes and Endocrinology in the North West Deanery. I took time out of the training program for two years in order to pursue this research. The experiments in this thesis were developed under the supervision of Dr Handrean Soran, Ms Yifen Liu and Dr Valentine Charlton-Menys. I was fully responsible for the recruitment of patients, statistical analysis and the interpretation of all the data from the experimental chapters.
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   Soran H, Hama S, **Yadav R**, Durrington PN.


CHAPTER ONE: INTRODUCTION

1.1 Coronary heart disease

“Misfortunes always come in by a door that has been left open for them.”

Czechoslovakian proverb

The World Health Organization’s Atlas for Heart Disease and Stroke illustrates that globally in 2002 coronary heart disease (CHD) accounted for 6.8% and 5.3% of Disability Adjusted Life Years (DALYs) in men and women respectively. DALYs are the same as years of life lost to disability and death, and therefore suggest a more meaningful way of projecting disease burden as opposed to simply the resulting death. The burden of CHD is expected to rise from 47 million DALYs in 1990 to 82 million DALYs in 2020 [1]. These figures are similar to HIV/AIDS and unipolar depressive disorders. CHD is now the leading cause of death worldwide and knows no borders. 3.8 million men and 3.6 million women die from CHD each year [2].

The basis of CHD is regarded to be atherosclerosis. Hypercholesterolemia is a major contributor to atherosclerosis. The incidence of CHD is much lower in rural China and Japan where average plasma cholesterol concentration is 4.0 mmol/l as compared to the United Kingdom where it is 5.9 mmol/l and CHD is a major cause of death [3].

1.2 Low density lipoprotein (LDL)

1.2.1 LDL metabolism in health (Figure 1.1)

Cholesterol is an important component of cell membranes as it provides stability/rigidity and reduces permeability of the membranes. It is also a precursor of steroid hormones. Cholesterol in the body either originates from the diet or is synthesised in the liver and gut. The liver is the most important site for cholesterol metabolism. After absorption from the gut, cholesterol is packed in chylomicrons and transported to the liver. Cholesterol is then packed in very low density lipoprotein (VLDL) along with triglycerides in the liver and released into the circulation. This VLDL converts into low density lipoprotein (LDL) as it loses its triglycerides by the action of lipoprotein lipase. LDL is rich in cholesterol and is the main source of cholesterol for various tissues. The uptake of LDL is facilitated by receptors present on the surface of cells needing the
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cholesterol. All excess LDL in the circulation is taken up by the liver via LDL receptor, scavenger receptor-BI (SR-BI), LDL receptor-related protein (LRP) and non-receptor medicated uptake. High density lipoprotein (HDL) transports the excess cholesterol from the tissues back to the liver either directly or via LDL [4]. Hypercholesterolemia is essential for the atheromatous process and lipid lowering is an most important step in management of atherosclerosis [5].

1.2.2 Apolipoprotein B 100

There is only one apolipoprotein B 100 (apoB) molecule in each LDL particle, therefore apoB concentration represents LDL particle numbers as opposed to LDL-cholesterol (LDL-C) which simply represents the amount of cholesterol in LDL particles [4]. The Apolipoprotein-related Mortality RISk Study (AMORIS) [6] was designed to compare LDL-C and apoB as markers of risk of fatal MI. (175,553 Swedes followed up for 6 years). Apo B was found to be superior in predicting events at all ages for both men and women compared to LDL-C. A meta-analysis based on epidemiological studies including 233,455 subjects and 22,950 events reported that in United States adult population over a 10-year period, a non-HDL-C strategy would prevent 300,000 more cardio-vascular (CV) events than an LDL-C strategy, whereas an apoB strategy would prevent 500,000 more CV events than a non-HDL-C strategy [7]. However, there is evidence from a recent meta-analysis that among statin-treated patients, on-treatment levels of LDL-C, non-HDL-C, and apoB were each associated with risk of future major cardiovascular events, but the strength of this association was greater for non-HDL-C than for LDL-C and apoB [8]. Statins alone or in combination with ezetimibe are effective in significantly lowering apoB levels [9].

1.2.3 Small dense LDL

The role of small dense LDL (sdLDL) as a risk factor for coronary heart disease has been well established [10]. Cholesteryl ester transfer protein (CETP), as a key enzyme in reverse cholesterol transport, mediates the transfer of cholesteryl esters (CE) from cholesterol-rich LDL to triglyceride (TG) rich VLDL in exchange for TGs. This lipid exchange promotes the generation of smaller LDL particles with higher density (sdLDL) [4] (Figure 1.1). There is a conformational change in the apoB present on sdLDL
leading to a reduced affinity to hepatic LDL receptors [11, 12]. This increases the residence time of sdLDL in the circulation and makes it more susceptible to oxidative modification [4]. SdLDL preferentially undergoes atherogenic modifications like oxidation [13] and glycation [14, 15] \textit{in vivo}. Small dense LDL has also been shown to preferentially undergo oxidation [16, 17] and glycation [15] \textit{in vitro}.

Patients with metabolic syndrome or type 2 diabetes may have marginally higher LDL-C compared to the general population but their CV risk is much higher possibly because of higher sdLDL [4, 18, 19]. The proportionally high sdLDL in these patients is due to insulin resistance, delivery of excess non-esterified fatty acids (NEFA) to the liver, increased output of VLDL from the liver and higher CETP activity [4]. The Quebec cardiovascular study showed that in patients with high ApoB levels but normal size LDL (i.e high LDL-C) there is twofold increase in CV risk compared to a six fold increase in CV risk in patients with high sdLDL levels [20].
Figure 1.1 Lipoprotein metabolism: Left: interaction between HDL and the cell membrane probably mediated by scavenger receptor-BI (SR-BI) promotes the hydrolysis of intracellular cholesteryl ester (CE) to form free cholesterol and its passage to the cell membrane (translocation). Hence it can cross to become incorporated in preb HDL and smaller HDL3 particles (resorption) either directly or via SR-BI and ATP-binding cassette A1 (ABCA1). ATP-binding cassette transporter G1
(ABCG1), ATP-binding cassette transporter G4 (ABCG4) and caveolin may also play a role. Middle: major influences on circulating HDL levels are the efflux of cholesterol from the liver through ABCA1. This cholesterol combining with apolipoprotein AI (apoAI) creates an HDL particle large enough to avoid glomerular filtration, the rate of removal of CE from HDL under the agency of cholesteryl ester transfer protein (CETP) and the rate of removal of CE from HDL by hepatic SR-BI (also known as CLA-1). Right: large triglyceride-rich VLDL secreted by the liver in states of hypertriglyceridaemia is readily converted to small dense low density lipoprotein (SD-LDL) which influences serum apolipoprotein B levels, but not LDL-C. The process involves transfer of CE from buoyant LDL back to VLDL in exchange for triglyceride, which is then removed by hepatic lipase leaving cholesterol-depleted SD-LDL in the circulation. This is not readily removed by the LDL receptors. CETP activity is increased in hypertriglyceridaemia, which is also responsible for decreasing HDL concentration and particle size. SD-LDL is more susceptible to atherogenic modifications. Modified LDL is avidly taken up by macrophages via scavenger receptor AI (SR-AI), scavenger receptor AII (SR-AII) and CD36 of macrophages. 1,2-DAG, 1,2-diacylglycerol; ACAT, acyl-CoA: cholesterol O-acyltransferase; FC, free cholesterol; G protein, guanosine triphosphate-binding protein; inositol P3, inositol 1,4,5 triphosphate; NCEH, neutral cholesteryl ester hydrolase; PIP2, phosphatidylinositol 4,5 biphosphate; TG, triglyceride. Adapted with permission from [4].
1.2.4 Oxidized LDL

1.2.4.1 Mechanism of LDL oxidation

LDL oxidation is an important atherogenic modification [21, 22]. The process of oxidation starts with generation of reactive oxygen species or free radicals (ROS) in the vessel wall or elsewhere. A free radical is an atom or a molecule that can exist independently despite one or more unpaired electrons in its orbit. But it may have a tendency to seek or share electrons in order to gain stability. For example oxygen when it exists as a diatomic molecule has two outer orbitals with only one electron in each, moving parallel to each other. Oxygen therefore needs to find two electrons moving in the opposite direction from its own two electrons, in order to gain stability. This is a two step process, making it less reactive as compared to an atom or molecule needing just one electron in order to gain stability. Highly reactive species (hydrogen dioxy/ hydroxyl) are formed during diatomic oxygen’s endeavour to gain an inert state [4].

\[
\begin{align*}
O_2^- + e & \rightarrow O_2^- + H^+ \rightarrow HO_2^- \quad \text{(Hydrogen dioxy radical (highly reactive))} \\
& \text{Superoxide radical} \\
HO_2^- + e & \rightarrow H_2O_2 \rightarrow 2OH^- \quad \text{(Hydroxyl radical (highly reactive))} \\
& \text{Hydrogen peroxide} \\
2OH^- + 2e & \rightarrow 2H_2O
\end{align*}
\]

These ROS attack the circulating or tissue (sub-endothelial) LDL or the cells which form a part of the vasculature. Polyunsaturated fatty acids (present in the outer layer of LDL, cell membrane) have more double bonds, therefore are more susceptible to attack and damage (peroxidation) by ROS. A good example for this would be phosphatidyl choline which has an unsaturated fatty acyl group in the 2nd position. When a hydroxyl free radical attacks this part and extracts hydrogen, the remaining phospholipid itself becomes a free radical or lipid peroxide, which undergoes rearrangement. It then accepts a diatomic oxygen molecule to form a peroxy radical. This is highly reactive itself and can attack other phospholipids to produce free
radicals and lipid hydroperoxides. Two lipid hydroperoxides react together to form more peroxyl radicals and also alkoxyl radicals (chain reaction) [4]. Transition metal cations like copper can further catalyse these reactions and a similar method has been used for LDL in vitro studies [23]. Peroxyl radicals are capable of damaging cholesterol esters and cholesterol as well [24]. Hydrocarbon chains containing alkoxyl groups break away to form aldehydes or ketones which can damage deoxyribonucleic acid (DNA) and are cytotoxic [4]. There are suggestions that these processes of phospholipid oxidation may lead to conformational changes in the structure of apoB [25, 26].

Observational prospective human cohort studies have been interpreted as supporting a role for antioxidants in the prevention of CHD [27, 28]. However, firm recommendations to take antioxidant supplements to treat or prevent CHD require evidence derived from randomized controlled studies. In primary prevention studies, low dose alpha-tocopherol does not reduce the incidence of coronary events (ATBC study) [29], and beta-carotene either has no effect or increases the incidence of coronary events and cancer death (ATBC, CARET, Physician's Health studies) [30, 31]. Secondary preventions, those with smaller populations and shorter duration of follow up have shown some benefit from alpha-tocopherol (CHAOS, SPACE) [32, 33], but larger randomized studies indicate no benefit from treatment with alpha-tocopherol (HOPE, GISSI, PPP) [34-36]. Another study with antioxidant combinations also showed no benefit (HATS) [37]. On the basis of these data, supplements of alpha-tocopherol and beta-carotene cannot be recommended for the treatment or prevention of CHD. Fundamental and applied research may yet find a role for antioxidant supplements in the treatment of coronary disease.

1.2.4.2 Factors promoting LDL oxidation

ROS are naturally present in the mitochondria or peroxisomes but are tightly regulated. Under hypoxic conditions this regulation may be lost leading to leakage of ROS to the outside. Macrophages as a part of their inherent actions to kill bacteria or disable potentially modifying macromolecular complexes, can also release ROS [4]. Myeloperoxidase (MPO) is an enzyme present in azurophyllic granules of monocytes and catalyzes the formation of ROS. The MPO/hydrogen peroxide ($\text{H}_2\text{O}_2$)/chloride system can generate reactive species that cause lipid peroxidation
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and oxidize LDL in the vicinity of the growing plaque [38]. MPO has been linked to oxidative stress, inflammation and atherosclerosis [38-40]. During inflammation, cyclooxygenases and lipoxygenases also contribute to the generation of free radicals [41, 42]. The metabolic syndrome in obese patients with or without diabetes is associated with increased LDL lipid peroxidation [43]. A similar observation was made with increasing body mass index (BMI) [44]. Certain changes in LDL structure make it more susceptible to oxidation. These include formation of sdLDL [12] or glycation of LDL apoB [45]. This structural transformation of LDL interferes with the uptake of this modified LDL by its normal hepatic receptors. Ultimately this oxidized LDL lodges on to scavenger receptors present on macrophages like scavenger receptor A and B1 (SRA, SRB1), CD36 and lectin-like oxidised LDL receptor-1 (LOX-1) [46, 47] and these receptors are also upregulated by oxidised LDL (oxLDL) (Figure 1.1).

1.2.4.3 Role of oxLDL in promoting atherosclerosis
Uptake of oxLDL by macrophages leads to cholesterol accumulation in the macrophages and foam cell formation [24]. Furthermore, oxidised LDL attracts monocytes and macrophages to the site of atheroma formation, prevents macrophages from leaving the site, stimulates endothelial cells to become proinflammatory and reduces production of nitric oxide (NO) [48-50]. Oxidized LDL propagates an immune response which leads to production of LDL directed antibodies. These complexes are then readily taken up by macrophages to create more foam cells [4, 51].

1.2.5 Lipoprotein phospholipase A2
Lipoprotein phospholipase A2 (Lp-PLA2) is produced by a wide range of inflammatory and non-inflammatory cells [52]. Initially Lp-PLA2 was recognized by its action on platelet activating factor (PAF) and was therefore called PAF acetylhydrolase (PAF-AH) [53]. The extracellular Lp-PLA2 circulates in association primarily with LDL (80-85%) and with HDL (15-20%) [54]. Lp-PLA2 activity strongly correlates with LDL-C, apoB and sdLDL apoB [55, 56]. There is also a strong correlation between Lp-PLA2 mass and activity (r=0.86, P<0.001) [57].
Previously studies have suggested that Lp-PLA2 may have a role to play in protection against atherosclerosis. In presence of oxidized phospholipids, Lp-PLA2 removes these fragments acting as an antioxidant. Matsuzawa et al. [58], suggested
that the over expression of Lp-PLA2 protects the cells from reactive oxygen species (ROS)-induced apoptosis through oxidized phospholipids hydrolysis. Lourida et al. [59] showed that Lp-PLA2 activity is important for reducing the immunogenicity of oxLDL. More recently, Noto et al. [60] showed in animals that Lp-PLA2 (activity) protects lipoproteins from oxidation, producing less pro-atherogenic lipoproteins and preserving HDL functions.

But a systematic review [61] comprising 14 studies and more than 20,000 patients reported that Lp-PLA2 is significantly associated with cardio-vascular disease (CVD) (5 studies measured Lp-PLA2 activity and 9 measured mass). The risk estimate appears to be relatively unaffected by adjustment for conventional CVD risk factors. The authors suggested that measurement of Lp-PLA2 may be useful in CVD risk stratification and that Lp-PLA2 may represent a potential therapeutic target for CVD risk reduction. Another recent and more comprehensive meta-analysis [62] comprising 32 studies and more than 70,000 patients demonstrated that Lp-PLA2 activity and mass each show continuous associations with risk of coronary heart disease, similar in magnitude to that with non-HDL cholesterol or systolic blood pressure in the population studied.

On the same lines, the association of Lp-PLA2 activity with inflammatory reactions represents the majority of the studies in literature in the recent years. Lp-PLA2 activity results in conversion of phosphatidyl choline (PC) to lysophosphatidyl choline (lyso-PC) and oxidized non-esterified fatty acids (ox NEFA). Karabina and co-workers [63] showed that dyslipidemic patients exhibit higher Lp-PLA2 activity than control subjects in all LDL subfractions, resulting in higher lyso-PC production during in vitro oxidation. Another study demonstrated that Lp-PLA2 activity can only convert oxidized PC to lyso-PC and Lp-PLA2 is unable to modify structurally normal PC [64]. This may suggest that oxidation of LDL associated phospholipids is necessary for activation of Lp-PLA2 which then hydrolyzes the oxidized phospholipids. Further support for this was found in another study which demonstrated that Lp-PLA2 activity is mainly associated with electro-negative LDL and that Lp-PLA2 was bound to the electro-negative LDL regardless of LDL size [65]. This study also showed that this electronegative LDL which has 5 fold higher Lp-PLA2 activity is enriched in products of Lp-PLA2 reaction (Lyso-PC and ox NEFA).
Lyso-PC are involved with atherosclerotic process and show a deleterious role of Lp-PLA2 activity [66]. Lyso-PC from apoptotic cells contribute to attract monocytes and primary macrophages [67, 68]. Studying the effects of oxLDL, Kuniyasu et al. [69] demonstrated that oxLDL, and particularly, the lyso-PC present in this particle, enhances the plasminogen activator inhibitor-1 expression.

1.2.6 LDL glycation and its role in atherosclerosis

Glycation of LDL is an increasingly important area in dyslipidaemia and atherosclerosis [70]. Glycation of lipoproteins and their associated apolipoproteins leads to a structural and therefore functional change. These modified particles cannot be taken up by their natural receptors [71, 72]. They are susceptible to further modification like oxidation or are taken up by scavenger receptors on macrophages (Figure 1.1) [70, 73, 74] which will ultimately lead to foam cell formation and atherosclerosis [75]. There is evidence to suggest that LDL glycation is in fact glycoxidation, which takes place even when LDL is incubated with glucose under liquid nitrogen in absence of oxidizing conditions [14, 76].

Glycation involves non-enzymatic condensation of glucose or its α-oxoaldehyde metabolites, with proteins in order to form stable covalent adducts. Exposed lysine residues on LDL apoB are prone to condensation with glucose [77, 78]. This process is non-enzymatic and forms an unstable Schiff’s base which then undergoes further rearrangement to form a ketamine ‘Amadori product’. As this cycle continues, oxidative fragmentation takes place to yield advanced glycation end products (AGE) [79, 80]. \textit{In vivo} glycation of LDL is minimal in healthy subjects [81, 82]. Glycation increases with elevated LDL [15], diabetes [81, 82] and is maximal in a diabetic patient with dyslipidaemia [83]. Small dense LDL is preferentially glycated in non-diabetic patients, patients with type 2 diabetes and metabolic syndrome [15].

1.2.6.1 Mechanisms that promote \textit{in vivo} glycation

\textbf{Hyperglycaemia:} Serum glycated apoB was significantly higher in the diabetic patients when compared with controls. In the same study a positive correlation was established between percentage glycated apo B and glycated haemoglobin (HbA1c) [81]. During \textit{in vitro} glycation studies, up to 80mmol of glucose concentration is needed for a number of days to allow glycation to take place [15]. However, \textit{in vivo} glycation can occur even with physiological glucose concentrations. This may be related to presence of a number of more reactive metabolites of glucose in
circulation which are highly potent glycating agents (for example glucose-6-phosphate, methylglyoxal and glycoaldehyde [73, 84-86]). These α-oxoaldehydes can be derived from glucose, from Schiff’s base during the glycation process or from the polyol pathway [87].

**Small dense LDL:** Higher glycated LDL in patients with diabetes is not exclusively related to glycaemia, but also related to presence of sdLDL (Figure 1.1). SdLDL concentration is high in patients with diabetes [88]. SdLDL is preferentially glycated compared with more buoyant LDL in both diabetic and non-diabetic dyslipidemia states [14, 15, 89]. This is possibly due to the longer residence time of sdLDL in circulation; higher proportion of apoB is exposed on the surface of sdLDL or may be other structural changes which favour glycation [90, 91]. The amount of sdLDL may be more important in determining concentration of glycated apoB rather than the degree of dysglycaemia [14]. Statin treatment is reported to reduce glycated LDL in diabetic dyslipidemia [92]. It is important to mention here that statins [93] reduce CV risk to a greater extent as compared to treatment of dysglycaemia [94] in diabetes and it has been shown that statins are associated with lower levels of both sdLDL and glycated apoB [89].

### 1.2.6.2 Glycoxidation independent of reactive oxygen species or transition metals

The process of glycation also yields free radicals therefore this process could be glycoxidation rather than pure glycation [95]. Buccala and co-workers showed that this process does not require transition metals, enzymes or free radicals and that both glycation and oxidation can be inhibited by aminoguanidine which is an AGE inhibitor [45]. This has subsequently been shown in other studies as well [73]. Generation of lipid peroxides (LPO) correlates with LDL glycation during *in vitro* glycation studies [15] although the extent was much less than glycation and facilitating further oxidation would need other systems as mentioned above.

### 1.2.6.3 Vascular complications from glycated LDL

*In vitro* studies suggest that LDL glycation is sufficient for rapid lipid accumulation in macrophages even in the absence of significant oxidation [96]. Glycated LDL is significantly concentrated in atherosclerotic plaques [97] and its role in propagating the process of atherosclerosis has also been elucidated [98, 99]. Glycated LDL can alter endothelial function and potentiate prothrombotic events [100, 101].
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1.3 High density lipoprotein

In 1975, Miller and Miller [102] reported an inverse association between plasma HDL cholesterol (HDL-C) concentration and coronary heart disease (CHD). This was confirmed by numerous additional studies worldwide and HDL was established as an independent risk factor for CHD [103]. Furthermore, HDL-C remains as an independent predictor of cardiovascular events in statin-treated patients at all levels of LDL-C [104]. The most accepted mechanistic explanation has been that HDL facilitates uptake of peripheral cholesterol and its return to the liver for excretion in the bile and faeces, a concept first introduced by Glomset in 1968 that was termed reverse cholesterol transport (RCT) (figure 1.1) [105]. It has been suggested that RCT could be a protective mechanism against atherosclerosis. More recently, a variety of other functions of HDL have been described, primarily based on in-vitro studies (figure 1.2). These include anti-inflammatory, antioxidant, antiglycation, antithrombotic, nitric oxide – inducing and antimicrobial activities [4, 106-109]. Furthermore, a role of HDL in the immune system [110-112] and as a safe location to release toxic metabolites like lysolecithin and lipid peroxide breakdown products such as aldehydes has been described [109, 113]. These findings have raised the important question of whether a substantial portion of the protective effect of HDL may be due to functions beyond RCT. Some 100 different proteins have been identified in HDL [114]. Variations in its composition, particularly in paraoxonase-1 (PON1), apolipoprotein AI (apoAI), lecithin:cholesterol acyl transferase (LCAT), Lp-PLA2, serum amyloid A (SAA) and apolipoprotein J (apoJ) have been linked with HDL’s functions [109, 114, 115].
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1.3.1 HDL: Reverse cholesterol transport (figure 1.1)
In peripheral tissues excess cholesterol is esterified and packaged as cytoplasmic droplets of the intensely hydrophobic cholesteryl ester. The interaction of HDL, particularly preβ HDL, with the outer cell membrane, can stimulate the hydrolysis of intracellular cholesteryl esters and the passage of the free cholesterol across the cell
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membrane wherein it can then be incorporated in the surface of HDL molecules. The movement of free cholesterol out of storage droplets of cholesteryl ester is termed translocation [4]. ATP-binding cassette transporter G4 (ABCG4) [116], ATP-binding cassette transporter G1 (ABCG1) [116] and caveolin [117] are also involved in shifting the balance from storage to cholesterol efflux.

Three major mechanisms account for the efflux of cholesterol across the outer cell membrane. One is a nonspecific efflux from the cell surface by physicochemical cholesterol exchange between the cell membrane and lipoproteins in the tissue fluid. HDL is the most efficient lipoprotein acceptor, because it possesses the enzyme LCAT which can esterify any unesterified cholesterol entering its outer surface after which it will move into the intensely hydrophobic central core, leaving the surface of the HDL able to accept more unesterified cholesterol. This process converts preβ HDL to larger HDL3 particles. LCAT activity is greater in smaller HDL particles, particularly preβ HDL and HDL3 [4]. LCAT is important in the process of RCT by generating a gradient of free cholesterol from cells to HDL [118]. The second major mechanism for cellular efflux is via the ABCA1 receptor [119]. This mechanism involves the binding of preβ HDL to the ABCA1 receptor wherein it receives unesterified cholesterol. The third mechanism is the SR-BI receptor. This receptor is clearly important in the hepatic uptake of cholesterol from larger HDL particles (HDL2) and perhaps from other lipoproteins such as LDL during their passage through the hepatic sinusoids, but in peripheral cells it can also function to facilitate the movement of cholesterol out of the cell [4].

The smaller HDL particles, particularly preβ HDL and HDL3, and their regeneration from larger particles are thus particularly important for RCT. Preβ HDL has a molecular weight in the range of 45 000–80 000 Da. It is lipid deficient and comprises principally two molecules of apoAI and phospholipids. It is small enough to enter the tissue fluid readily. After the acquisition by HDL of free cholesterol by the action of LCAT from the tissues and its esterification and packaging into its core the next stage of RCT is transfer of the cholesteryl ester to the liver. This will also have the effect of reducing the size of the HDL particle, from HDL2 dimensions, back to HDL3 (remodelling) [4].

Apo AI is catabolized by liver and kidney. The rate of catabolism of apoAI by the liver is too low to account for the return of even a small fraction of the cholesterol
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that the liver exports into the circulation, therefore, the clearance mechanism is unlikely to involve the catabolism of the whole HDL particle as it does in the case of LDL. Hence, there must be routes for cholesteryl ester to leave HDL and enter the liver without involving catabolism of the whole particle. The most important of these involves the SR-I receptor (also known as CLA-I in humans) [4].

1.3.2 HDL: Anti-atherogenic/anti-inflammatory function and NO production

Potential anti-inflammatory effects of HDL have been elucidated through cell culture and in-vivo studies. These effects could be due to the PON1 [120] and phospholipase A2 [121] associated with HDL. Moreover, HDL has been shown to reduce reactive oxygen species (ROS) generation and neutrophils infiltration in a collared carotid artery animal model [122], tumor necrosis factor mediated induction of intracellular adhesion molecule 1 (ICAM1) and vascular cell adhesion protein 1 (VCAM1) in cultured aortic endothelial cells [123] and can reduce the area of myocardial infarction in an ischemia-reperfusion model [124]. In patients with hyperlipidemia, low HDL-C levels were associated with elevated plasma concentrations of soluble ICAM1 (sICAM1) and soluble E-selectin (sE-selectin), but patients with high HDL-C had plasma soluble cellular adhesion molecules (sCAM) concentration similar to healthy controls. Also HDL-C was inversely and significantly associated with sICAM1 and sE-selectin in individuals with low plasma HDL-C but not in those with normal or elevated HDL-C [125]. In the same study, fenofibrate-induced increase in HDL-C in patients with low HDL-C was associated with a significant reduction of plasma sICAM1 and sE-selectin concentrations. Therefore, HDL may be a therapeutic target in patients with acute coronary syndromes or other types of acute tissue injury. Furthermore, HDL is also known to improve vascular reactivity by increasing nitric oxide bioavailability [126].

Patients with high levels of apoAI and HDL-C have a decreased risk of recurrent venous thromboembolism [127]. Potential antithrombotic properties of HDL include enhancing the capacity of activated protein C or S to cleave factor Va, HDL’s stimulatory effect on endothelial cell prostacyclin synthesis by inducing cyclooxygenase-2 expression and providing its substrate, arachidonic acid, and impeding apoptosis of endothelial cells, in particular larger HDL particles, because of its capability to stimulate nitric oxide synthesis [107]. All these observations are based on in-vitro experiments and there is a lack of in-vivo evidence.
HDL functionality and LDL quality: the influence of obesity, obstructive sleep apnoea and pharmacological intervention

In a clinical study, apoAI Milano and phospholipid complexes were infused into patients with acute coronary syndromes. There was a significant regression of coronary atherosclerosis compared with baseline as measured by intravascular ultrasound studies [128]. It is important to mention that antiatherogenic properties of apoAI Milano may not be superior to wild type apoAI [129] and some researchers have also questioned whether individuals with apoAI Milano mutation have protection against atherosclerosis [130]. Nevertheless, this is a strong evidence that infusion of apoAI leads to regression of atheroma [128].

There is new evidence from in-vitro [130] and animal [131] studies in support of the ability of platelets to alter the properties of endothelial cells in order to assist penetration of monocytes and lymphocytes into the arterial wall, which might indicate a role of platelets in the initiation of atheromatous plaque formation. HDL has been shown to inhibit platelet activation through various mechanisms, which have been at least partially elucidated. In-vitro studies showed reduced platelet aggregation in response to collagen, thrombin and ADP in the presence of HDL [132]. It has been hypothesized that inhibition of platelet activation by HDL, is via HDL receptor SR-B1 present on the platelets [133]. Thrombosis may be promoted by apoptotic and dysfunctional endothelial cells by producing membrane micro-particles and enhancing the adhesive reaction between platelets and leucocytes [134]. HDL may prevent this by maintaining the integrity of the endothelial cell monolayer [135, 136]. Therefore, there is some credible data suggesting that suppression of platelet activity might be one of the many mechanisms through which HDL protects against atherogenesis, but practical applications of most of these preliminary observations are as yet unknown.

1.3.3 HDL: Anti-oxidant effect and safe location for LPS

When HDL was incubated with LDL under oxidizing conditions the accumulation of lipid peroxides on LDL was decreased, but the concentration of lipid peroxides on HDL remained similar to that observed when HDL alone underwent oxidation [108, 137, 138]. The lipid peroxides accumulating on HDL, regardless of whether LDL was present, rapidly reached a plateau. This capacity of HDL to decrease lipid peroxide accumulation proved to be a result of a hydrolytic enzymatic activity for which PON1 emerged as the most credible candidate [139].
PON1 is a protein of 354 amino acids with a molecular mass of 43000 Da. It is synthesized in the liver [4] and located on a subspecies of HDL that also contains apo J (also called clusterin) and apoAI with or without apolipoprotein AII (apoAII) [4]. PON1 is strongly lipophilic and requires a lipid environment to maintain its activity. Based on kinetic parameters of paraoxonases towards different substrates, it is assumed that lactones are the likely physiological substrates [140]. Hydrolysis of homocysteine thiolactone by PON1 is considered to be protective against coronary artery disease [141]. The location of PON1 on HDL probably serves two purposes. Firstly, HDL is the most widely distributed of the lipoproteins in tissue fluid. Therefore, its location on HDL would allow it to have the widest distribution for its possible biological role to protect the outer cell membranes from damage by the products of lipid peroxidation and perhaps also by homocysteine [142]. Secondly, PON1 accelerates hydrolysis of oxidized glycerophospholipids, such as phosphatidyl choline, which typically have fatty acyl hydroperoxides groups derived from linoleate in its Sn2 position and would lead to the production of lysoPC. This is potentially damaging as discussed earlier. However, we know that it can safely be produced on HDL because substantial quantities are produced there by the activities of LCAT in esterifying free cholesterol [4]. Additionally, HDL is associated with lower levels of conjugated dienes in plasma from healthy people and those with CHD and human HDL infused into hypercholesterolaemic rabbits also exhibits the capacity to lower the plasma levels of conjugated dienes [143, 144]. There is evidence that HDL from patients with coronary artery disease is defective both in its ability to prevent LDL oxidation by artery wall cells and in its ability to inhibit the biologic activity of oxidized 1-palmitoyl-2-arachidonoylsn- glycerol-3-phosphocholine [145]. A small study showed that patients with high HDL but low PON1 were more susceptible to CHD than patients with low HDL but high PON1 [146].

However, the role of PON1 as a credible candidate in HDL’s antioxidant function has been controversial, largely because the most highly purified preparations of PON1 isolated from HDL and recombinant water soluble variants of PON1 do not hydrolyze lipid peroxides [147, 148]. Yet, it is exceptionally difficult to separate PON1 from other HDL components, such as apoAI and PLA2, without subjecting it to conditions in which it becomes dissociated from the lipid environment essential
for its catalytic activity in reactions involving lipid substrates. Furthermore, the creation of mutations making PON1 more polar [149] is similarly going to compromise its activity in hydrolyzing hydrophobic substrates. The biochemical approach to determine which components of HDL contribute to its anti-oxidative activity thus has its limitations and evidence from other sources must be considered. The mechanism of transfer of substrates for PON1 from LDL to HDL are not clear. Phospholipid transfer protein may play a role here. Various studies have revealed that PLTP facilitates the transfer of a spectrum of different lipid molecules, including diacylglycerol, phosphatidic acid, sphingomyelin, phosphatidyl choline, phosphatidylglycerol, cerebrosides and phosphatidyl ethanolamine [150, 151]. The key role of PLTP in lipoprotein metabolism undoubtedly centers on its lipid transport function of transferring phospholipids between HDL particles and of lipids between apoB-containing lipoproteins and HDL. In this latter capacity, during lipolysis of chylomicrons and very low density lipoproteins (VLDL) by lipoprotein lipase, PLTP is essential for the transfer of excess surface lipids from triglyceride-rich lipoproteins to HDL [152, 153] and thereby, facilitates the formation of smaller lipoprotein remnants, contributes to the formation of LDL, and assists in the maturation of HDL particles. Furthermore, PLTP-mediated phospholipid transport among HDL particles is another mechanism whereby PLTP modulates HDL size and composition. This so-called HDL conversion into larger and smaller particles was first described by Rye and Barter [154] and subsequently shown to be mediated by PLTP [155, 156]. This process involves the transfer of surface lipids, the formation of enlarged HDL through particle fusion, and the formation of small lipid-poor apoA-1/phospholipid complexes with preβ-mobility [157]. ApoA1 and LCAT may also assist transfer of PON1 substrates from LDL to HDL. It might be possible that the transfer of these substrates is due to direct physical contact as both LDL and HDL are found in blood in abundance.

Most patients with dyslipidemia are on statin therapy. It is imperative to study HDL’s antioxidant and anti-glycation function in these patients and the role played by PON1 in this.

1.3.4 Role of apolipoprotein M in HDL anti-oxidant function

Apolipoprotein M (ApoM) is predominantly associated with HDL and to a minor degree to LDL, VLDL and chylomicrons [158]. Elsoe and co-workers incubated
HDL isolated from wild type mice, apoM-deficient mice, and two lines of apoM transgenic mice with 2-fold and 10-fold increased plasma apoM, respectively either alone or with LDL in the presence of copper. The results suggested that apoM may increase HDL’s antioxidant effect by binding oxidized phospholipids. Also apoM enrichment did affect apoA1 or PON1 in this study suggesting that apoM has a distinct and unique role in promoting anti-oxidant function of HDL [159]. It has been suggested that the lipocalin structure of apoM may enable it to bind oxidized phospholipids allowing apoM to increase the ability of HDL to act as a sink for oxidation products. This would indicate that the antioxidant mechanism of apoM is different from that of PON1 thus providing HDL with diverse means of preventing oxidation. Another study demonstrated that apoM, by delivering sphingosine-1-phosphate (S1P) to the S1P receptor on endothelial cells, promotes vascular stability [160].

1.3.5 HDL: antiglycation property
Oxidation is known to accompany glycation and it has been argued that glycation should more properly be described as glycoxidation [75]. In keeping with this, it has previously been reported that when LDL is glycated in vitro, small quantities of LPO are also generated even under the conditions we impose there is no external source of oxygen free radicals [161]. It has recently been suggested that HDL is able to impede in vitro LDL apoB glycation and PON1 may be important for this function of HDL [76].

1.3.6 HDL: Anti-apoptotic function
HDL protects endothelial cells against apoptosis induced by oxLDL. The specific component of HDL implicated in such cyto-protection was revealed in a study where human micro vascular endothelial cells (HMEC-1) were incubated with mildly oxLDL in the presence or absence of the physicochemically distinct HDL subpopulations fractionated from normolipidemic human plasma (n=7) by isopycnic density gradient ultracentrifugation [162]. The authors concluded that small, dense, lipid-poor HDL 3 potently protects endothelial cells from primary apoptosis and intracellular ROS generation induced by mildly oxLDL, and that apoA1 was pivotal to such protection. Plasma membrane receptors and transporters involved in cellular cholesterol efflux, including SR-BI and ABCA1 and ABCG1,
may potentially mediate the cyto-protective effects of HDL via their interaction with apoAI and ensuing cellular efflux of toxic oxidized lipids [163].

S1P circulates at high nanomolar concentrations primarily associated with small, dense HDL 3 [164], and is likely derived from red blood cells and/or platelets [165]. Equally, S1P can function as a ligand for G protein-coupled S1P receptors on endothelial and smooth muscle cells, thereby enhancing cell growth and survival [166]. Consistent with these findings, intracellular signal transduction induced by HDL associated lysosphingolipids, primarily S1P, may in part account for the cyto-protective effects of HDL [167].

1.3.7 Serum amyloid A

Epidemiological studies previously have suggested a link between serum SAA and CVD [168, 169]. It is not clear as yet whether elevated SAA just reflects the risk factors for atherosclerosis or if it has a causal relationship with the disease process [170]. SAA is a family of acute phase proteins synthesized in the liver when stimulated by TNFα, interleukin 1 (IL-1) or interleukin 6 (IL-6) and it has been suggested that SAA may alter HDL function especially reverse cholesterol transport [170, 171]. SAA is the main apolipoprotein associated with HDL during the acute phase response as it displaces apoAI [172].

1.4 Obstructive sleep apnoea

1.4.1 Definition and gradation

Obstructive sleep apnoea (OSA) refers to pauses in breathing during sleep due to narrowing of the upper airway despite continuing respiratory effort. Deposition of excessive adipose tissue around the upper airways in obese patients causes this narrowing of airway when they are in a supine position. When the subject is asleep and the neck muscles are relaxed the excess adipose tissue collapses the airway under gravitational pull.

Studies suggest that up to 60% of the population may snore during sleep but not all snorers develop apnoea [173, 174]. The Wisconsin sleep study demonstrated that 9% of females and 24% of males in the study population had at least mild sleep apnoea. Also 2% females and 4% males had symptoms with this [175]. In the UK 5% of the population may have at least mild sleep apnoea and 0.03% have the severe form [176]. In the context of OSA, an episode of apnoea is defined as complete cessation
of breathing for 10 or more seconds. Hypo-apnoea is at least 50% reduction in air flow associated with 2-4 % desaturation. The diagnosis and severity of OSA are established by calculating the number of apnoea episodes per hour (apnoea/hypo apnoea index or AHI)

- No OSA (<5 episodes/hours)
- Mild OSA (5-15 episodes/hour)
- Moderate OSA (15-30 episodes/hour)
- Severe OSA (>30 episodes/hour)

1.4.2 OSA promotes insulin resistance and cardiovascular risk

Many epidemiological, population based and clinic based studies reported an independent correlation between presence and severity of sleep apnoea and

- Degree of insulin resistance, fasting glucose, postprandial glucose [177-180]
- Prevalence of diabetes [181]
- Cardiovascular risk factors or CVD [182] (like hypertension [183], coronary artery disease [184, 185], stroke [186], heart failure [187] and cardiac arrhythmias [188])

This correlation persists even after correction for BMI, age, gender and other risk factors. The chronic state of intermittent hypoxia can lead to increased oxygen free radical stress [189] (hypoxia and reoxygenation cycles), systemic inflammation [190], dyslipidaemia [182, 191-193], reduced nitric oxide (NO) and endothelial dysfunction [191]. It has also been suggested that the degree of insulin resistance may increase with increasing AHI [194]. OSA is a state of inflammation as evidenced by elevated levels of C-reactive protein (CRP), IL-6, nuclear factor-κB (NF-κB), TNF-α, ICAM-1, VCAM-1 and E-selectin. This may suggest that OSA is a predisposing factor for endothelial dysfunction, increased inflammation and atherogenesis [195].

1.4.3 Effect of obstructive sleep apnoea on lipoproteins

Adverse effects of OSA on lipid profile are well documented. The sleep heart study established a significant increase in the levels of total cholesterol (TC) and TG and reduction in the levels of HDL-C with increasing severity of sleep apnoea there was [182, 192]. Lavie et al showed that thiobarbituric reactive substances (TBARS) and peroxides (PD) were elevated in patients with OSA with or without CVD as compared to controls. These were directly related to the severity of OSA [196].
Barcelo *et al* showed higher lipid peroxidation and LDL susceptibility to further oxidation in OSA patients as compared to controls [197]. Kizawa *et al* [191] demonstrated that there is an important role of angiotensin II and oxLDL-mediated LOX-1 (oxLDL receptor) upregulation in endothelial cell injury in patients with OSA. Similar results were reported by other investigators [198]. This is all thought to be due to intermittent hypoxia and reoxidation cycles that produce ROS, which then start a chain reaction. Some improvement with continuous positive airway pressure (CPAP) has been observed [199, 200]. According to Vatansever *et al* [201], OSA causes increments in oxidative damage and decreases adiponectin levels. This group also demonstrated endothelial dysfunction by cell incubation techniques and measuring markers. They postulated that recurrent hypoxia-reoxygenation attacks in OSA patients may activate oxidative stress, elevating sympathetic activity and leading to low levels of adiponectin.

### 1.4.4 Dysfunctional HDL in obstructive sleep apnoea

Tan and co-workers [193] showed that lipoproteins and apolipoproteins in patients with OSA were similar to controls. But the HDL was dysfunctional in OSA patients and lead to higher oxidised LDL levels in vivo and lower protection to further mild LDL oxidation on exposure to atmospheric oxygen. The degree of dysfunctionality of HDL and levels of oxidised LDL were directly related to AHI/ severity of OSA [193].

Lavie [196] studied PON1 activity in controls and OSA patients with and without CVD, but did not match the subjects for age and sex. This group found that PON1 activity was significantly different between controls and OSA patients with CVD, although there was also a trend showing difference in PON1 activity between controls and OSA patients without CVD. Contrary to these findings Kotani *et al* [202] showed that in age and sex matched OSA patients/ controls (no CVD) although paraoxonase activity was similar, arylesterase (lactonase) activity was significantly lower in the OSA group. Both paraoxonase and arylesterase activities improved with CPAP treatment for 6 months and both correlated well with improvement in HDL-C levels.

### 1.4.5 Regulation of endothelial function by HDL associated PON1

#### 1.4.5.1 Adhesion molecules in atherosclerosis
HDL functionality and LDL quality: the influence of obesity, obstructive sleep apnoea and pharmacological intervention

An early event in atherogenesis is the adhesion of monocytes to the endothelium [203, 204] via adhesion molecules such as ICAM-1 and VCAM-1 [205, 206]. ICAM-1 (CD54) is expressed on many cell types and is involved in both monocyte and lymphocyte adhesion to activated endothelium [206, 207]. VCAM-1 (CD106) is a member of the immunoglobulin-like super-family and is primarily involved in the adhesion of mononuclear leukocytes to the endothelium [205]. Both ICAM-1 and VCAM-1 are rapidly induced by the inflammatory cytokines IL-1 and TNF-α [208].

1.4.5.2 HDL and adhesion molecules

In patients with hyperlipidemia, low HDL-C levels were associated with elevated plasma concentrations of soluble ICAM-1 (sICAM-1) and soluble E-selectin (sE-selectin), but patients with high HDL-C had plasma soluble cellular adhesion molecules (sCAM) concentration similar to healthy controls. Also HDL-C was inversely and significantly associated with sICAM-1 and sE-selectin in individuals with low plasma HDL-C but not in those with normal or elevated HDL-C [125]. In the same study, fenofibrate-induced increase in HDL-C in patients with low HDL-C was associated with a significant reduction of plasma sICAM-1 and sE-selectin concentrations.

1.4.5.3 Protective role of HDL associated PON1

HDL-associated PON1 has been shown to have anti-oxidant and anti-inflammatory potential mainly by protecting lipids of HDL [113] and LDL from oxidative modifications [209, 210]. Most likely, these protective properties depend on the peroxidase and esterase activity of PON1 allowing the detoxification of oxidized molecules such as phospholipids and lipid hydroperoxides [211, 212]. Serum PON1 is suggested to contribute to the established anti-atherogenic function of HDLs which is, at least partly, attributable to their anti-oxidative properties [209, 210, 213]. Based on these observations it is vital to study the effect of the presence and severity of OSA in morbidly obese patients on HDL’s anti-oxidant and anti-inflammatory functions. This would further augment our understanding of the relationship between OSA and atherosclerosis.

1.5 Extended release niacin with laropiprant: Efficacy, clinical effectiveness and safety

1.5.1 Introduction
HDL functionality and LDL quality: the influence of obesity, obstructive sleep apnoea and pharmacological intervention

Statins effectively reduce LDL-C and have revolutionized the treatment of dyslipidemia and CVD [5, 214-217]. However, there is a residual CV risk of 60-70% despite optimal statin treatment [215, 217-220]. This may reflect the failure of TC and LDL-C measurement to represent the atherogenic potential of certain factors and dyslipidemias like LDL heterogeneity, atherogenic apo B containing particles beyond LDL and also low levels of HDL-C [219]. The risk of developing CVD is inversely related to HDL-C [221-223]. Therefore there is a need for drugs with an HDL-C raising effect. Interest in niacin has re-emerged because of its apo-B containing lipoprotein lowering and HDL-C raising effects. [224] [225-227]. There is intriguing evidence, although only from small scale studies that the use of niacin is associated with a reduction in CV morbidity and mortality rates [228, 229]. The discovery of a receptor for niacin that explains some of its effects has also led to greater interest in this drug. The clinical use of niacin has been limited by the side effect of cutaneous flushing.

1.5.2 Chemistry

1.5.2.1 Niacin (Nicotinic acid) (figure 1.3)

Niacin (3-pyridinecarboxylic acid) (figure 1.3) is a water-soluble B complex vitamin (vitamin B3). Its amide, nicotinamide, requires conversion to nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP) to function as a vitamin. However, only niacin affects lipid levels and the therapeutic hypolipidemic dose of niacin is 20-40 times higher than the effective vitamin dose.

1.5.2.2 Laropiprant (figure 1.3)

Laropiprant (LRP), (3R)-4-(4-chlorobenzyl)-7-fluoro-5-(methylsulfonyl)-1,2,3,4-tetrahydrocyclopenta[b] indol-3-yl]acetic acid (figure 1.3) inhibits prostaglandin D2 release, which is the main mediator of niacin induced cutaneous flushing. LRP has been combined with ERN (ERN/LRP) in order to reduce flushing and is being marketed as Tredaptive® by Merck, Sharp and Dohme (MSD).
HDL functionality and LDL quality: the influence of obesity, obstructive sleep apnoea and pharmacological intervention

<table>
<thead>
<tr>
<th>Drug name</th>
<th>Tredaptive (Extended release niacin/ laropiprant)</th>
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<tbody>
<tr>
<td>Phase</td>
<td>Launched</td>
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<tr>
<td>Indication</td>
<td>Dyslipidemia</td>
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<tr>
<td>Pharmacology description</td>
<td>Niacin: Inhibits hepatic diacylglycerol acyl transferase 2, stimulates macrophage ABCA1 transporter, reduces vascular inflammation Laropiprant: Blocks DP1 receptors</td>
</tr>
<tr>
<td>Route of administration</td>
<td>Alimentary, by mouth</td>
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<td>Chemical structure</td>
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![Chemical structure](image)

Niacin

Laropiprant

Pivotal trial(s)

HPS2 THRIVE, AIM HIGH

Figure 1.3. Drug summary

1.5.3 Pharmacodynamics of niacin

1.5.3.1 Mechanism of action (figure 1.4)

Niacin’s mode of action is only partially understood on the basis of *in vitro* studies. Niacin reduces hepatic production of apo-B 100 associated lipoproteins, decreases HDL catabolism, promotes reverse cholesterol transport and lowers vascular inflammation [219]. The G-protein coupled receptor 109a (GPR109a) is a Gαi coupled transmembrane receptor expressed in adipose tissue [230]. In mice, niacin has been shown to inhibit hormone sensitive lipase (HSL) in adipocytes through GPR109a receptor, preventing adipocyte lipolysis and release of NEFA into the circulation [230]. Reduced delivery of NEFA to the liver decreases TG production and lipidation of VLDL in the liver. In addition, the final pathway in hepatic TG
assembly catalyzed by diacylglycerol acyl transferase 2 (DGAT2) may be inhibited by niacin [231]. Niacin treatment in humans may reduce liver fat content by its ability to inhibit DGAT2 [232].

Niacin may also improve HDL-C levels and HDL’s antiatherogenic properties through three potential pathways. Firstly, niacin reduces CETP mediated lipid exchange of TG in VLDL with CE in HDL by reducing hepatic VLDL output and by reducing CETP gene expression [233-235]. This shifts HDL towards the more buoyant cholesterol rich HDL2 form and elevates HDL-C levels [235]. Secondly, niacin down-regulates the expression of ATP synthase beta chain, a cell surface receptor in the liver that facilitates HDL/Apo A1 holoparticle endocytosis thereby reducing hepatic removal of HDL [236, 237]. Lastly, niacin promotes cholesterol efflux from monocytes/macrophages to nascent HDL by stimulating ATP binding cassette transporter 1 (ABCA1) [238, 239], which is the first step in reverse cholesterol transport (figure 1.4).

The various effects of niacin on lipoproteins and insulin resistance may be due to its ability to modulate gene expression [240].

**1.5.3.2 Pleiotropic and anti-inflammatory effects (figure 1.4)**

Pleiotropic roles for niacin, including protection of endothelial function, anti-oxidative and anti-inflammatory effects have been proposed. Glucose-6-phosphate dehydrogenase (G6PD) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which are upregulated by niacin, may acquire anti-oxidant properties by virtue of their ability to generate nicotinamide adenine dinucleotide phosphate (NADPH) and nicotinamide adenine dinucleotide (NADH) from NAD+ [241, 242]. NADPH decreases cellular ROS [243]. Niacin is a precursor of NAD+ and has been shown to increase cellular concentrations of NAD+ and NADPH [244-246].

Niacin has been reported to reduce glutathione levels, inhibit LDL oxidation and angiotensin II induced ROS production, decrease TNFα induced VCAM-1 and monocyte chemoattractant protein-1 (MCP-1) secretion and lower TNFα induced monocyte adhesion [245, 247] *in vitro*. It suppresses pro-atherogenic chemokines and elevates adiponectin through G-protein coupled receptors in adipose tissues [248]. The results from *in vitro* studies on THP1 monocytes (human acute monocytic leukemia cell line) have suggested that niacin’s anti-inflammatory activities could also be mediated through the GPR109a receptor present on monocytes and
HDL functionality and LDL quality: the influence of obesity, obstructive sleep apnoea and pharmacological intervention

macrophages [249]. Niacin reduces inflammatory markers like CRP and Lp-PLA2 [250]. Niacin treated endothelial cells do not promote platelet activation [251]. Therefore, niacin may have a role in inhibiting vascular inflammation and other key events in early atherogenesis, which is separate from its conventional role as a lipid-regulating agent.
HDL functionality and LDL quality: the influence of obesity, obstructive sleep apnoea and pharmacological intervention

Figure 1.4. Proposed mechanisms of action of niacin. A. Niacin prevents adipocyte lipolysis and release of FFA by inhibiting HSL through GPR109a receptor. B. Niacin promotes DNA repair and reduces ROS by increasing G6PD and GADPH gene expression. C. Effects of niacin on cultured HAEC suggest its role in inhibiting key events in early atherogenesis. D. Niacin reduces hepatic TG synthesis and VLDL output by inhibiting DGAT2. E. Niacin prevents hepatic HDL holoparticle endocytosis by down regulating expression of ASBC. F. Niacin decrease CETP gene expression, mass and activity leading to a decrease in exchange of HDL CE for VLDL TG. This increases HDL-C (in particular HDL2). G. Niacin promotes efflux of FC from atheroma site macrophages to nascent HDL by stimulating ABCA1
HDL functionality and LDL quality: the influence of obesity, obstructive sleep apnoea and pharmacological intervention


1.5.3.3 Niacin-induced flushing and role of laropiprant (figure 1.5)

Niacin causes cutaneous flushing mainly of the face and chest and a feeling of warmth or burning. Although mild flushing has been reported at niacin doses as low as 250mg, at therapeutic doses of 1500 mg to 2000 mg, it can become intolerable [252, 253] resulting in drug non-compliance [254, 255]. The incidence of flushing has been reduced by the use of slow-release preparations and also by combining it with LRP [256]. Flushing is caused by prostaglandin D2 (PGD2). Niacin interacts with the receptor GPR109a on the Langerhans’ cells in the skin leading to the release of PGD2 [257-259]. This, in turn, causes dilatation of dermal capillaries via prostaglandin D2 receptor subtype 1 (DP1) present on the vascular smooth muscles in the skin vasculature. LRP competitively and selectively blocks PGD2 (DP1) receptor (figure 1.5) [260, 261] thereby antagonizing the vasodilatory effect of niacin. This action reduces the incidence and severity of niacin-induced flushing. Although LRP also has a weak affinity to the thromboxane A2 (TXA2) receptor, therapeutic doses of LRP have no clinically relevant effect on bleeding time and collagen-induced platelet aggregation [262, 263].
HDL functionality and LDL quality: the influence of obesity, obstructive sleep apnoea and pharmacological intervention

Figure 1.5. Mechanism of action of laropiprant, NSAIDS and aspirin in abolition of niacin induced flushing. NSAIDS and aspirin prevent production of both categories of prostaglandins (housekeeping and inflammatory) by inhibiting both COX-1 and COX-2 enzymes. Laropiprant is specifically targeted towards blocking PGD2 mediated stimulation of DP1 receptors on dermal blood vessels. (Key: COX-1 – cyclooxygenases 1, COX-2 – cyclooxygenases 2, DP1 - prostaglandin D2 receptor subtype 1, LRP – laropiprant, NSAIDS – non steroidal anti-inflammatory drugs, PGD2 – prostaglandin D2, PGE2 – prostaglandin E2, PGF2α – prostaglandin F2α, PGI2 – prostaglandin I2, PL-A2 – phospholipase A2, TXA2 – thromboxane A2 (reproduced after permission from Expert Opinion, Informa)
1.5.4 Pharmacokinetics of niacin
The predominant pathway with low doses of niacin or lower rates of absorption (slow-release preparation) results in the formation of nicotinamide and NAD, which are potentially hepatotoxic. High doses of niacin or immediate release preparations quickly saturate this pathway. In the second pathway nicotinuric acid is formed by the conjugation of glycine with niacin. With increasing doses of niacin, a dose-proportional level of nicotinuric acid is detectable in the circulation as the second pathway exhibits first order kinetics across the dose range. Immediate-release preparations of niacin divert the drug towards this pathway leading to minimal hepatotoxicity but excessive flushing [219]. The excessive hepatotoxicity from slow-release niacin and excessive flushing from immediate-release niacin have been demonstrated in a head-to-head trial of the two formulations [264]. Modern extended-release preparations available commercially are intermediate-release and, therefore, minimize both flushing and liver toxicity without affecting efficacy [265, 266].

1.5.5 Efficacy of niacin
Three ERN monotherapy trials [267-269], which included subjects with HDL-C levels of 1.1-1.2 mmol/l at baseline, showed a dose-dependent increment in HDL-C (30%) up to a maximum ERN dose of 2.5 gm. Similarly there were ERN dose-dependent reductions in levels of LDL-C (22%), TG (39%) and lipoprotein (a) (Lp(a)) (30%). Adding ERN to baseline statin therapy led to significant improvements in atherogenic lipid profiles [270, 271]. Use of ERN monotherapy or ERN-statin combination over 98 weeks, confirmed that niacin had a sustained favorable effect on lipoproteins [272]. In patients with hyperlipidemia and combined dyslipidemia a combination of ERN and simvastatin (2000mg/40mg) resulted in a less atherogenic lipid profile compared to atorvastatin monotherapy (40 mg) [273].

The effect of niacin on diabetic dyslipidemia, characterized by high TG, high atherogenic sdLDL and low HDL-C was studied in a 16 week double-blind placebo-controlled trial with 148 diabetic patients (47% statin treated) [274]. Significant dose-dependent increase in HDL-C and reduction in TG and LDL-C were observed in the ERN arm compared to the placebo arm. Small dense LDL is more susceptible to oxidation and glycation compared to more buoyant LDL; hence it is assumed to be more atherogenic [92, 275, 276]. A study
assessing lipoprotein particle size by nuclear magnetic resonance spectroscopy (NMR) in patients with metabolic syndrome or type-2 diabetes showed that ERN preferentially increased buoyant HDL and reduced the sdLDL subtype [277]. Similar results have been obtained in studies using either ERN monotherapy [269] or a ERN-statin combination [278].

In recent years it has become increasingly evident that lipoprotein(a) (Lp(a)) is modestly associated with CVD risk in a continuous and independent manner [279]. A European Atherosclerosis Society Consensus Panel on Lp(a) concluded that it probably has a causal role in CVD [280]. The structure of Lp(a) is similar to a LDL particle but for an additional molecule of apolipoprotein(a) [apo(a)] [281]. The apoB100 and apo(a) associated with Lp(a) are bound by a disulphide bond [282]. The apo(a) molecule is structurally similar to plasminogen and plasmin [282]. Higher Lp(a) levels in an individual may predispose to increased risk of CVD via prothrombotic or anti-fibrinolytic effects. Lp(a) is also a source of cholesterol for the expanding atheroma in the subintimal space [280]. In patients at high CV risk the recommended target for Lp(a) is ~1.29 mmol/l (50 mg/dl) [280]. Niacin, anacetrapib and estrogen have been shown to lower Lp(a) [283-287].

1.5.6 Clinical effectiveness of niacin (table 1.1)

The Coronary Drug Project (CDP), a double-blind placebo-controlled study with total mortality as the primary endpoint, compared various lipoprotein-altering medications in 8341 statin naive men with history of electrocardiogram (ECG) documented myocardial infarction (MI). Other outcomes included CV events and effect on lipoproteins over a period of 5.0-8.5 years. Niacin treatment did not lower total mortality within this period but reduced non fatal MI by 26% [228]. However, a 15-year follow-up showed that niacin reduced all cause mortality by 11% compared to placebo [288].

The ARBITER trials and Oxford Niaspan study compared combination ERN and statin treatment with statin alone on carotid intima-media thickness (CIMT) and carotid artery wall area (Table 1.1). In ARBITER-2, significant slowing of CIMT progression was found in both groups [289]. There was no difference between the ERN and statin arms though there was a trend towards significance ($p=0.08$) in favor of combination treatment. Subjects who completed ARBITER-2 were enrolled in ARBITER-3 in order to clarify whether treatment with ERN beyond 12 months
would have had a greater impact on CIMT [290]. All participants in ARBITER-3 were also receiving statins. This study showed that further treatment with ERN when added to statin treatment for 12-24 months significantly reduced progression of CIMT compared to patients on placebo (p≤0.001). Moreover, when changes in LDL-C and TG were controlled, only changes in HDL-C were independently associated with regression of CIMT (p=0.001).

ARBITER 6-HALTS (Table 1.1) was designed to elucidate whether in statin treated high CV-risk patients it would be more beneficial to add ERN to elevate HDL-C or add ezetimibe in order to lower the LDL-C further [291]. The study was stopped prematurely after 14 months by the independent steering committee on the basis of efficacy. This study demonstrated that adding ERN causes a significant regression of CIMT when combined with a statin and that niacin is superior to ezetimibe (p=0.003). This outcome was possibly due to ERN induced elevation in HDL-C levels and reduction in TG. ARBITER 6-HALTS also showed that incidence of major CV events was lower in the ERN group than in the Ezetimibe group (1% vs. 5%, p=0.04), although the study was not designed for this outcome. The Oxford Niaspan study was a randomized placebo-controlled trial that compared effects of ERN versus placebo in patients with high CV risk and receiving statins. The outcome of this study suggested that ERN reduced carotid artery wall area (p=0.03) and there was an inverse relationship between this outcome and HDL-C [292].

The HDL Atherosclerosis treatment study (HATS) [37] and the 10-year follow up data from the Familial Atherosclerosis Treatment Study (FATS) [293] suggest that treatment with niacin may reduce CV events and death. The HATS study showed that in coronary heart disease patients on statin treatment, with LDL-C at target but low HDL-C, the addition of niacin can further reduce CV events by 90% compared to placebo. Seventy five patients who completed the FATS trial (Table 1.1) were started on triple therapy of niacin 2.5g/day, Colestipol 20 g/day and Lovastatin 40 mg/day after the unblinding process and were followed up for 10 years. These patients had a 19.8% reduction in all cause mortality compared to 1.3 % in patients who returned to usual care (p<0.001).

AIM-HIGH (Atherothrombosis Intervention in Metabolic Syndrome with Low HDL/High Triglycerides: Impact on Global Health) was the first randomized controlled secondary prevention trial designed to confirm the findings of previous
studies that elevating HDL-C using high dose ERN would lead to reduction in CV morbidity and mortality independent of LDL-C reduction achieved using statins [294]. The trial included 3414 statin-treated patients with metabolic syndrome and previous history of cardiovascular disease with low HDL-C (<1.03 for men and 1.3 mmol/l for women) and high TG (1.7-4.5 mmol/l). Five hundred and fifteen participants also received ezetimibe in order to maintain their LDL-C between 1.03-2.06 mmol/l. This trial had a 99% power to detect a 25% reduction in the primary endpoints: CHD death, non-fatal MI, ischemic stroke, hospitalization for acute coronary syndrome or symptom driven coronary or cerebral revascularization [295]. After a mean follow up of 3 years, HDL-C increased by 25%, LDL-C and TG decreased by 12% and 28.6 % respectively. However, the data and safety monitoring board (DSMB) noted that high-dose ERN offered no benefits beyond statin therapy alone in reducing CV events despite a significant improvement in HDL-C, LDL-C and TG. The DSMB also noted an unexplained albeit not significant increase in rate of ischemic stroke in the ERN group (28 strokes, 1.6 %) compared to the control group (12 strokes, 0.7%, p=0.11) and consequently AIM-HIGH was terminated prematurely [296]. An association between niacin and stroke has not been seen in any other trial. A large proportion of the excess strokes seen with niacin treatment in AIM-HIGH occurred after stopping treatment. The lack of added benefit of ERN in AIM-HIGH may mean that it simply does not work but this conclusion may be questioned. The population studied had a low average baseline LDL-C of 1.8mmol/l possibly because a high proportion of patients entering the study had been treated with lipid lowering agents including statins for a number of years: 40% for more than 5 years. This limits the potential for further benefit but the results should not be extended to patients who did not achieve such optimal LDL-C levels. Separation of the effects of elevating HDL-C from lowering LDL-C is intrinsically difficult and the trial design is open to criticism. Simvastatin and ezetimibe in both the control and ERN combination treatment groups were titrated to an LDL-C target. This resulted in a higher proportion of patients on 80mg simvastatin and ezetimibe in the placebo arm. The overall reduction in LDL-C in the placebo group of 7.5 mg/dl (0.2 mmol/l, 9.8 %) was similar to that in the treatment group of 11.0 mg/dl (0.3 mmol/l, 14.8%) but the goal of equivalence of LDL-C was not achieved. The placebo group received 150-200 mg of immediate-release niacin
to produce flushing as an aid to blind treatment. A rise in HDL of 3.8 mg/dl (0.1 mmol/l, 9.7%) was noted in the control group compared with 9.3 mg/dl (0.24 mmol/l, 21%) in the treatment group. The conclusions from AIM-HIGH remain controversial.

In early 2013 Merck withdrew the drug Tredaptive (ERN/LRP) from the existing markets and also announced its decision to not apply for a license with FDA. This was based on the lack of benefit on mortality or morbidity seen in large scale trial HPS2-THRIVE (Treatment of HDL to reduce the incidence of vascular events: A randomized trial of the long-term clinical effects of rising HDL cholesterol with niacin and MK-0524) [297]. This study aimed to assess the clinical effects of combined daily ERN/LRP (2g/40mg) versus placebo in 20,000 patients with pre-existing atherosclerotic vascular disease who are all receiving simvastatin 40mg. This study like AIM-HIGH also aimed to lower LDL-C to below 2mmol/l by using simvastatin ± ezetimibe and then assess the effect of ERN/LRP on cardiovascular mortality and morbidity by its ability to elevate HDL-C. 25,673 patients were recruited in this study and received treatment for a median 3.9 years. Again elevation of HDL-C in this manner did not show any further benefit on mortality.
HDL functionality and LDL quality: the influence of obesity, obstructive sleep apnoea and pharmacological intervention
### Table: Clinical Trials

<table>
<thead>
<tr>
<th>Trial (n)</th>
<th>Subjects and follow up period</th>
<th>DB</th>
<th>PC</th>
<th>R</th>
<th>Treatment</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDP (8341) [228, 288]</td>
<td>Post MI men, 5-15 years</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>Placebo or Niacin or clofibrate or oestrogen or dextrothyroxine</td>
<td>26% ↓ non-fatal MI at 5 years, Niacin vs placebo (p&lt;0.005); 11%↓ in mortality at 15 years, Niacin vs placebo (p=0.0004)</td>
</tr>
<tr>
<td>CLAS (162) [298]</td>
<td>Post CABG men, Progressive atherosclerosis, 2 years</td>
<td>×</td>
<td>✔</td>
<td>✔</td>
<td>Placebo vs Niacin + colestipol</td>
<td>↓ coronary global change score, Niacin vs placebo (p&lt;0.0001); ↓ % of subjects with new lesions (p&lt;0.04) in bypass grafts; ↓ average number of lesions per subject that progressed (p&lt;0.03)</td>
</tr>
<tr>
<td>FATS (120) [229]</td>
<td>Men with CAD, high apoB &amp; positive family history of CVD, 2.5 years</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>Placebo (+/- colestipol) vs simvastatin + colestipol; (colestipol in placebo group only if high LDL-C)</td>
<td>Less lesion progression and more regression in 9 proximal coronary segments (p&lt;0.005) in simvastatin vs placebo group; fewer deaths, MI or revascularization in simvastatin or Niacin group vs placebo</td>
</tr>
<tr>
<td>UCSF-SCOR (72) [299]</td>
<td>HeFH, 26 months</td>
<td>×</td>
<td>×</td>
<td>✔</td>
<td>Diet +/- colestipol vs Niacin + colestipol +/- lovastatin</td>
<td>CA stenosis in controls progressed, in Niacin group regressed (p=0.03)</td>
</tr>
<tr>
<td>HATS (160) [37]</td>
<td>CHD, Low HDL-C, normal LDL-C, 3 years</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>Placebo + simvastatin vs Niacin + simvastatin</td>
<td>Coronary stenosis progression in placebo group and regression in Niacin + simvastatin group (p&lt;0.001); 24 % first CV events in placebo but 3% in Niacin + simvastatin group (p=0.03)</td>
</tr>
<tr>
<td>ARBITER-2 (167) [289]</td>
<td>CHD, low HDL-C, 1 year</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>All patients on background statins, Placebo vs ERN</td>
<td>No significant difference in CIMT progression between the 2 groups (0.08)</td>
</tr>
<tr>
<td>ARBITER-3</td>
<td>Patients completing ARBITER-2, 2</td>
<td>×</td>
<td>✔</td>
<td>×</td>
<td>All patients on</td>
<td>Net regression of CIMT at 1year (p&lt;0.001) and 2 years</td>
</tr>
<tr>
<td>Study</td>
<td>Duration</td>
<td>Eligibility Criteria</td>
<td>Comparator</td>
<td>Intervention</td>
<td>Results</td>
<td></td>
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<tr>
<td>ARBITRE 6-HALTS (208) [291]</td>
<td>14 months</td>
<td>CHD or CHD risk equivalent, LDL-C &lt; 2.6 mmol/l and HDL-C &lt; 1.3-1.4 mmol/l</td>
<td>Placebo vs ERN</td>
<td>All patients on background statins. Started on Niacin or ezetimibe study drugs</td>
<td>ERN (vs. ezetimibe) produced greater change from baseline in mean CIMT (p=0.003). Lower major CV events in ERN group vs. ezetimibe group (p=0.04).</td>
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<tr>
<td>Oxford Niaspan study (71) [292]</td>
<td>1 year</td>
<td>Low HDL-C and either type 2 diabetes and CHD or carotid/peripheral atherosclerosis</td>
<td>Placebo vs ERN</td>
<td>All patients on background statins, Placebo vs ERN</td>
<td>ERN reduced carotid artery wall area (vs placebo, p=0.03)</td>
<td></td>
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<tr>
<td>AIM-HIGH (3414) [296]</td>
<td>32 months</td>
<td>Metabolic syndrome, CHD</td>
<td>Placebo vs ERN, all patients on background statins, 515 patients on ezetimibe to reduce LDL-C to &lt; 2.06 mmol/l</td>
<td>No significant difference in CV events, increase in ischaemic strokes in ERN group vs placebo (1.6% vs 0.7%)</td>
<td></td>
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</tr>
<tr>
<td>HPS2-THRIVE (25673) [297]</td>
<td>3.9 years</td>
<td>Cardiovascular disease</td>
<td>Placebo vs ERN/LRP, all patients on background statins ± ezetimibe to reduce TC to &lt;3.5 mmol/l</td>
<td>Major vascular event 13.2% vs 13.9%, p=0.29. Mortality data not published as yet.</td>
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</table>

1.5.7 Current Guidelines on HDL targets and treatment

The American Diabetes Association (ADA) concurs that a low level of HDL-C often associated with elevated TG, is the most prevalent pattern of diabetic dyslipidemia. The ADA recommends target levels of TG <150 mg/dl (1.7 mmol/l) and HDL-C >40 mg/dl (1.0 mmol/l) in men and >50 mg/dl (1.3 mmol/l) in women [300]. However, there is less evidence for modifying these lipid fractions with niacin than that for statin therapy [301]. The ADA recommends that if the HDL cholesterol is <40 mg/dl (1.0 mmol/l) and the LDL cholesterol is 100–129 mg/dl (2.6–3.3 mmol/l), gemfibrozil or niacin might be used, if a patient is intolerant to statins but recognizes that niacin is the most effective drug for raising HDL-C [300].

The European Atherosclerosis Society (EAS) recognizes the importance of HDL-C in CV risk but does not make any target recommendations due to lack of trial evidence. The EAS also recognizes that elevating HDL and lowering TG are important strategies in high-risk patients with metabolic syndrome or type-2 diabetes, and recommends combining a statin with niacin or a fibrate for such patients. The EAS suggests that ERN/LRP may be useful in view of its improved tolerability due to reduced flushing compared with niacin alone [302].

In the UK, The National Institute of Clinical Excellence (NICE) in its guidance for diabetic dyslipidemia accepts low HDL-C as a risk factor for CVD and advises raising HDL-C as ‘Safe Target’. However, NICE does not recommend any specific target levels for HDL-C in type-2 diabetes but instead recommends managing the overall CV risk rather than individual lipid levels [303]. Also NICE is unable to give any general recommendation on the use of niacin in diabetic dyslipidaemia due to lack of outcome trials in type-2 diabetics and flushing side effects despite use of prophylactic aspirin, dose titration and extended release formulations of niacin. NICE has also issued guidance on modification of lipids for the primary and secondary prevention of CVD. Niacin has been included in a group of drugs along with fibrates, ion exchange resins and ezetimibe to be used if statins are not tolerated in secondary prevention only [304].

The effects of different statins on HDL-C levels, relationships between changes in HDL-C and changes in LDL-C, and predictors of statin-induced increases in HDL-C were investigated in a meta-analysis of 32,258 dyslipidemic patients included in 37 randomized studies using rosuvastatin, atorvastatin, and simvastatin.
[305]. Rosuvastatin, atorvastatin, and simvastatin all raised the concentrations of both HDL-C and apoAI across the range of doses used. The percentage increase in HDL-C was paralleled by a comparable increase in apoA-I for each of the statins at most doses studied. The magnitude of the increases in HDL-C, however, varied with statin doses and, at any given dose, also between different statins. In the case of rosuvastatin, there was a dose-dependent increase in HDL-C (from 5.5% to 7.9%) over the dose range of 5–40 mg/day. The apoAI increase with rosuvastatin, while of a similar magnitude to that of HDL-C, appeared, however, not to be dose dependent, being similar (between 6% and 6.5%) across the dose range. In the case of atorvastatin, the increase in HDL-C was inversely related to dose, being 4.5% at the 10 mg dose and falling to 2.3% at a dose of 80 mg/day. A similar inverse relationship with atorvastatin dose was also observed for apoA1 (4.7% increase at 10 mg down to 0.1% increase at 80 mg). For simvastatin, the increase in HDL-C (as was observed with rosuvastatin) was dose dependent, ranging from 4.2% at 10 mg to 5.3% at 80 mg. The effect of simvastatin on apoA-I was similar to that on HDL-C, ranging from an increase of 5.2% at 10 mg to 5.9% at the 80 mg dose. Therefore increases in HDL-C were positively related to statin dose with rosuvastatin and simvastatin but inversely related to dose with atorvastatin. There was no apparent relationship between reduction in LDL-C and increase in HDL-C, whether analyzed overall for all statins (correlation coefficient = 0.005) or for each statin individually. Percentage increase in apolipoprotein A-I was virtually identical to that of HDL-C at all doses of the three statins. Baseline concentrations of HDL-C and TG and presence of diabetes were strong, independent predictors of statin-induced elevations of HDL-C.

1.5.8 Efficacy of niacin and laropiprant in trials (table 1.2)

1.5.8.1 Phase III studies

Three phase III studies that were designed to assess the efficacy and safety of ERN/LRP have been published.

In a comparison of the efficacy of ERN/LRP vs. ERN vs. placebo in patients with various degrees of CV risk (Table 1.2) [283], both ERN/LRP and ERN had a significantly superior effect ($p<0.001$) on all lipid parameters including ApoB, Apo AI and Lp(a) compared to placebo. The lipid modifying effect of ERN/LRP and ERN were similarly consistent with no effect of LRP on lipids. The dose escalation
HDL functionality and LDL quality: the influence of obesity, obstructive sleep apnoea and pharmacological intervention

for ERN/LRP and ERN in this study was rapid, starting with 1gm and progressing to 2gm in 4 weeks). In a post hoc subgroup analysis in patients with and without metabolic syndrome [306], ERN/LRP and ERN had similar favorable lipid modifying effects on LDL-C, HDL-C and TG compared to placebo. The effect of ERN/LRP and ERN on increasing plasma glucose levels was similar in both groups. ERN/LRP and ERN have been compared in a second study with a slower rate of increase in dose of ERN (by 500mg every 4 weeks). Patients in the ERN group were allowed to take aspirin or non-steroidal anti-inflammatory drugs (NSAIDS) prior to taking the ERN in order to reduce niacin induced flushing (Table 1.2) [256]. Despite these measures patients on ERN had higher rates of discontinuation, which could account for the superior LDL-C lowering and HDL-C raising effect of ERN/LRP compared with ERN.

A third phase III study has assessed the efficacy and safety of ERN/LRP + Simvastatin vs. ERN/LRP vs. Simvastatin [307]. There was a 47.9% reduction in LDL-C from baseline in the ERN/LRP + Simvastatin group as compared to 17% in ERN/LRP group and 37% in Simvastatin group at the end of 12 weeks (p<0.05). A similar pattern was observed for TG and apoB (p<0.05). Also there was a 27.5% elevation in HDL-C from baseline in the ERN/LRP + Simvastatin group as compared to 23.4% in ERN/LRP group and 6% in Simvastatin group at the end of 12 weeks (p<0.05). The ERN/LRP + Simvastatin group showed a significant increase in apoA1 (8.6%) and decrease in Lp(a) (19.6%) as opposed to the Simvastatin monotherapy group (2.3% and 0% respectively, p<0.05). These findings suggest that a combination of ERN/LRP and Simvastatin improves pro-atherogenic lipid profiles better than either drug on its own. This study did not show any additional benefit of ERN/LRP on high sensitivity C-reactive protein (hsCRP) when added to Simvastatin.

1.5.8.2 Post marketing studies

Adding ERN/LRP to statin has been shown to have a greater effect than doubling statin dose in improving lipid profile (LDL-C, -10% vs. -5.5%; HDL-C, 15.8% vs. 0.2%; TG, -17.6% vs. -4%; apoB, -10.7% vs. -2.4%; apoAl, 6.9% vs. 1% and Lp(a), -18.2% vs. 0%; p<0.001 for all) [308]. In the same study, 58% participants in the ERN/LRP + statin group were able to achieve national cholesterol education program adult treatment panel III (NCEP ATP III) LDL-C targets as opposed to
44.9% in the higher statin dose group ($p=0.003$). All these studies have concentrated on primary hypercholesterolemia or combined dyslipidemia patients with varying prevalence of diabetes. MacLean evaluated the efficacy and safety of ERN/LRP vs. placebo in 796 patients with type 2 diabetes randomized 4:3 to ERN/LRPT or placebo [309]. Approximately 80% of the participants were on statins with or without other lipid modifying drugs. The remaining participants were naive of any lipid modifying drugs. Compared with placebo, ERN/LRP reduced LDL-C by 17.9%, TG by 23.1%, apoB by 17.1%, Lp(a) by 25% and increased HDL-C and apoAI by 23.2% and 8.2% respectively, hsCRP decreased by 16.9%. In the ERN/LRP group, 17.6% patients needed intensification of anti-hyperglycemic regimen compared to 8.2% patients in the placebo arm over 9 months. This study demonstrated that in diabetic patients with persistent dyslipidemia despite existing lipid altering regimen, addition of ERN/LRP produced further positive lipid modification and the glucose elevating effects of ERN/LRP was manageable in the majority of patients.
HDL functionality and LDL quality: the influence of obesity, obstructive sleep apnoea and pharmacological intervention
<table>
<thead>
<tr>
<th>Study (1st author, year, DB, R, PC) (n=reference)</th>
<th>Target patient population. Drugs/ drug combinations compared.</th>
<th>Inclusion criteria</th>
<th>Study design</th>
<th>Follow up period</th>
<th>Number (%) of patients on statins</th>
<th>Percentage change from baseline for ERN/LRP group</th>
</tr>
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<tbody>
<tr>
<td><strong>Phase III studies</strong></td>
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<tr>
<td>Maccubbin, 2008, DB, R, PC (n=1613) [283]</td>
<td>Primary hypercholesterolemia &amp; mixed dyslipidemia. PB vs ERN vs ERN/LRP.</td>
<td>All with ↑CHD risk/T2DM, LDL-C ≤2.59 or CVR ≥2, LDL-C &lt;3.37 or CVR 0-1, LDL-C 3.37 – 4.92</td>
<td>3 groups. PB or ERN 1gm or ERN/LRP 1gm for 4 weeks; then dose doubled in all groups for 20 weeks</td>
<td>24 weeks</td>
<td>1045 (67%)</td>
<td>-18 *** 20 *** -22 ***</td>
</tr>
<tr>
<td>Maccubbin, 2009, DB, R, PC (n=1452) [256]</td>
<td>Primary hypercholesterolemia &amp; mixed dyslipidemia. ERN vs ERN/LRP.</td>
<td>All with ↑CHD risk/T2DM, LDL-C &lt;3.36 or CVR ≥2, LDL-C ≤4.13 or CVR 0-1, LDL-C no restriction</td>
<td>2 groups. ERN/LRP 1gm for 4 weeks, then 2 gm for 12 weeks. ERN 500mg for 4 weeks, then dose increased by 500mg every 4 weeks till reached 2gm</td>
<td>16 weeks</td>
<td>677 (47%)</td>
<td>-11 ** 20 *** -18</td>
</tr>
<tr>
<td>Gleim, 2009, DB, R, PC (n=1398) [307]</td>
<td>Primary hypercholesterolemia &amp; mixed dyslipidemia. ERN/LRP + simva vs ERN/LRP vs simva</td>
<td>&gt; 1 CVR, LDL-C 3.37-4.14 or 0-1 CVR, LDL-C 3.37-4.92. CHD or ↑ CVR or T2DM excluded due to statin washout period</td>
<td>7 groups. ERN/LRP 1gm + simva (10/20/40mg) or ERN/LRP 1gm or simva (10/20/40mg) for 4 weeks. Doses of ERN/LRP and simva doubled in both groups for next 8 weeks (simva maximum 40mg)</td>
<td>12 weeks</td>
<td>1203 (86%)</td>
<td>Pooled data from all ERN/LRP + simva group -47.9 * 27.5 * -33.3 *</td>
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<td>Pooled data from ERN/LRP group -17 23.4 -21.6</td>
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<td><strong>Post marketing studies</strong></td>
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<td>Shah, 2010,</td>
<td>Primary hyper</td>
<td>All with ↑CHD</td>
<td>2 groups. ERN/LRP 1gm</td>
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<tr>
<td>Study</td>
<td>Design</td>
<td>Intervention</td>
<td>Comparator</td>
<td>Endpoints</td>
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<tr>
<td>DB, R, PC (n=1216) [308]</td>
<td>Cholesterol &amp; mixed dyslipidemia. ERN/LRP 2gm + statin (simva 10/20 mg or atrova 10 mg) vs doubled statin dose</td>
<td>risk/T2DM. LDL-C - 2.59-4.14 or CVR ≥2, LDL-C - 3.37 – 4.91 or CVR 0-1, LDL-C 4.14 – 5.69</td>
<td>+ statin for 4 weeks, then ERN/LRP 2gm + statin for 8 weeks. Statin (simva 10/20 mg or atrova 10 mg) for 4 weeks, then dose doubled for 8 weeks</td>
<td>2 years</td>
<td>1216 (100%)</td>
<td>-10 **</td>
</tr>
<tr>
<td>Maclean, 2011, DB, R, PC (n=796) [309]</td>
<td>Type 2 diabetes. ERN/LRP vs PB. All patients on background lipid altering drugs</td>
<td>All T2DM. LDL-C 1.55 - 2.97. 4 weeks lipid modifying period to bring LDL-C to target range</td>
<td>2 groups. ERN/LRP 1gm for 4 weeks, then 2 gm for 32 weeks. PB for 36 weeks.</td>
<td>3 years</td>
<td>637 (80%)</td>
<td>-19 ***</td>
</tr>
<tr>
<td>Armitage, 2013, DB, R, PC (n=25673) [297]</td>
<td>High CVR patients, ERN/LRP vs PB. All patients on simvastatin ± ezetimibe to lower TC&lt;3.5</td>
<td>MI or CVA or PVD (or DM with any of the above or angina/ACS or revascularization). LDL-C standardisation phase leading to ERN/LRP trial phase followed by randomisation</td>
<td>ERN/LRP vs PB</td>
<td>3 years</td>
<td>100%</td>
<td>-20</td>
</tr>
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</table>


* P<0.05; ** P<0.01; *** P<0.001. Data for significance not published as yet for HPS2-THRIVE, results shown are after 8 weeks treatment with ERN/LRP.
1.5.9 Safety of niacin and Laropiprant in various settings

1.5.9.1 Niacin

Niacin infrequently causes nausea, vomiting and diarrhea, mainly at doses >2000 mg/day [310]. The extended-release preparation occasionally causes significant elevation of hepatic transaminases or uric acid levels but these are rarely clinically relevant [272]. Clinical studies have produced little evidence that niacin potentiates myopathy or rhabdomyolysis associated with statins [278, 311]. However, monitoring for any evidence of muscle damage when niacin and statin are used together in the presence of factors predisposing to rhabdomyolysis such as age >70 years, chronic kidney disease stage (CKD) ≥4, personal or family history of hereditary muscular disorders or previous history of muscle damage from statins or fibrates has been advised [312]. In HPS2-THRIVE there was an increased incidence of myopathy especially in Chinese patients taking simvastatin 40mg and ERN/LRP 2000mg/40 mg [297]. Approximately 25% of patients stopped the active drug as opposed to 17% in the placebo at the end of 3.9 years. The main reasons cited for this were side effects related to skin, gastro-intestinal tract, muscles and blood glucose.

Niacin suppresses adipose tissue lipolysis and release of NEFA into the circulation [312, 313], thereby reducing insulin resistance [314]. However, this effect is short lasting with immediate-release niacin, leading to rebound elevation in serum NEFA [4] and significant hyperglycemia [315]. This may explain hyperglycemia seen in animals with artificially reduced β cell function and niacin-induced insulin resistance [316]. ERN preparations are able to overcome this effect to a significant extent. In practice, this may account for the marginal increase in glucose and HbA1c levels with ERN doses ≤2gm/day in diabetic patients [274]. Niacin treatment has also been associated with elevated fasting serum glucose in non-diabetic patients and patients with new-onset diabetes [283, 307]. Results from a 64 week trial where niacin was added to a combination of statin and ezetimibe in dyslipidemic patients suggest that increased fasting glucose and new onset diabetes occurred only during initial up-titration of niacin and often improved or remitted without the need for any additional treatment [317]. However, with appropriate monitoring, ERN ≤2gm/day can be safely used in patients
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with type-2 diabetes [310] with benefits outweighing risks [318]. Niacin may cause approximately 10% reduction in phosphate levels or platelet count but this needs neither regular blood tests nor dose adjustment [310].

Flushing has been reported by most individuals who were started on therapeutic doses of crystalline immediate-release niacin, and it is sometimes accompanied by a transient rash. A clinical practice based study showed that ERN had the second highest discontinuation rates among all drugs used to treat hyperlipidemia with 85% of patients newly started on ERN not renewing their prescriptions in the first year [254]. Approximately 40% of patients were on a sub-therapeutic dose of 500mg ERN months after initiation. Up titration of the drug was difficult and at 1-year only 5.8% were on 1gm and only 2.2% were on doses greater than 1.5gm.

1.5.9.2 Laropiprant

LRP has no effects on lipoproteins and does not interfere with the lipid modifying actions of niacin [283, 319]. Studies of LRP in hyperlipidemic patients have not demonstrated any clinically significant side effects [319]. Concerns about the possible effects of LRP on platelet aggregation have not been substantiated [262, 263, 320, 321].

1.5.9.3 Safety evaluation of ERN/LRP in clinical trials

Approximately 6000 patients have participated in many studies focusing on safety of ERN/LRP combination compared to ERN, statins or placebo [256, 283, 307, 308, 320]. The outcomes suggest that the combination of ERN and LRP is as safe as ERN alone [261] but there is no long-term data on the safety of LRP. LRP added to ERN significantly reduced the rates of flushing and drug discontinuation when compared to ERN alone, thus enabling ERN dose to be titrated to effective doses within 4 weeks [256]. In the same study the incidence of discontinuation due to flushing was significantly lower in patients on ERN/LRP as compared to ERN alone (7.4% vs. 12.4%, \( p=0.002 \)) [256]. Combination of ERN/LRP with simvastatin is well tolerated without significant liver or muscle related side effects. As with niacin alone, ERN/LRP is associated with a slight increase in fasting blood glucose and HbA1c; however, benefits from favorable lipoprotein changes are likely to outweigh any possible detrimental effect associated with this mild increase in glycemic parameters.
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The effect of ERN/LRP on mediators of vascular inflammation and HDL’s anti-oxidant function in humans has not been studied as yet. Therefore I investigated the influence of extended release niacin/laropiprant (ERN/LRP) versus placebo in patients who had persistent dyslipidaemia despite receiving high doses of potent statins as the latter more accurately reflected actual clinical practice. I also assessed the effect of ERN/LRP on mediators of vascular inflammation and HDL’s in vitro anti-oxidant function.
CHAPTER TWO: AIMS OF THESIS

Chapter 2.1 Aims of thesis
A. To assess the relationship of serum PON1 activity with in vitro HDL antioxidant capacity, susceptibility of LDL to oxidation and the protection offered by HDL, in patients with dyslipidaemia treated with statins. To study the effect of PON1 activity on in vivo apoB glycation.
B. To study the effect of the presence and severity of OSA in morbidly obese patients on HDL’s anti-oxidant and anti-inflammatory functions.
C. To investigate the influence of ERN/LRP versus placebo in patients with persistent dyslipidaemia despite receiving high doses of potent statins as the latter more accurately reflected actual clinical practice. To assess the effect of ERN/LRP on mediators of vascular inflammation and HDL’s in vitro anti-oxidant function. To study the effect of adding LRP on patient compliance.
CHAPTER THREE: MATERIALS AND METHODS

3.1 Subjects

3.1.1 High Paraoxonase 1 activity reinforces HDL’s in vitro anti-oxidant function in statin treated dyslipidemic patients.

Fifty-three men and women aged 20-75 years not achieving NCEP ATP III [224] target of LDL-C of less than 1.8 mmol/l (70 mg/l) despite at least 12 months statin treatment were recruited from Central Manchester University Hospital NHS Foundation Trust and Salford Royal Hospital NHS Foundation Trust. They were divided into 2 groups above and below the median PON1 activity (111 nmol/min/ml). The median PON1 activity was 66 nmol/min/ml for the low PON1 group and 176 nmol/min/ml for the high PON1 group. Sixteen patients (30%) had heterozygous familial hypercholesterolemia (HeFH) and the remaining thirty seven patients (70%) had polygenic hypercholesterolemia. 20 patients were taking Rosuvastatin (10 – 40mg, mean dose 30 ± 10mg), 24 were on Atorvastatin (20-80mg, mean dose 60 ± 20mg) and 9 on simvastatin (40mg). Fifteen (30%) patients had type 2 diabetes. The Local Research Ethics Committee approved the project. All participants gave informed consent to the study. All the study patients were seen in the Cardiovascular Trials Unit at Manchester Royal Infirmary or Wellcome Trust Clinical Research Facility (Grafton Street, Manchester).

3.1.2 High Density Lipoprotein Antioxidant Function is Impaired in obese patients with Obstructive Sleep Apnoea

Forty one patients with BMI > 40 kg/m² were recruited from Salford Royal Hospital NHS Foundation Trust obesity management clinic. All patients underwent overnight respiratory variable polysomnography to determine the number of apnoeic or hypopnoeic episodes per hour (AHI). OSA was diagnosed if AHI was ≥ 5/ hour. Patients were divided into two groups based on the presence or absence of OSA (“OSA” and “no OSA” group) or on severity of OSA (high or low AHI groups). The Local Research Ethics Committee approved the project. All participants gave informed consent to the study. All the study patients were seen in Wellcome Trust Clinical Research Facility (Grafton Street, Manchester).
3.1.3 Extended release niacin lowers mediators of vascular inflammation but does not improve *in vitro* HDL antioxidant function in statin treated dyslipidemic patients

Patients were recruited from Central Manchester University Hospitals NHS Foundation Trust and University Hospital of South Manchester. Recruitment began in October 2010 and ended in May 2011. The study was completed in February 2012. Informed consent was obtained from all patients. The study was approved by local ethics committee. All the study patients were seen in the Cardiovascular Trials Unit at Manchester Royal Infirmary. 38 patients were recruited and 27 patients completed the study. 11 patients were lost to follow up.

**Inclusion & exclusion criteria:** Men and women aged 20-75 years with hyperlipidemia on lipid lowering treatment (maximum tolerated statins and/or ezetimibe) but not achieving NCEP ATP III target of LDL-C < 1.8 mmol/l (70 mg/l) were recruited. Ezetimibe was stopped 4 weeks before entry to the trial. Patients were excluded if they were pregnant and/or breast-feeding, if they had significant renal impairment (chronic kidney disease stage 3 or more advanced: eGFR ≤ 59), alanine aminotransferase > 1.5 upper limit of normal (ULN), if they were receiving fibrates and/or Omacor, if they were allergic to niacin and if they had active peptic ulcer disease.

**Randomization and Intervention:** The trial medication Tredaptive® or ERN/LRP was supplied by Merck, Sharp & Dohme Ltd. LRP is an agent that reduces the flushing side effect of ERN. Image-matched placebo for the study was also supplied by Merck, Sharp & Dohme Ltd.

Study patients were asked to attend for 5 visits (figure 3.1). Patients who fulfilled inclusion and exclusion criteria were invited for screening visit when informed consent was obtained. Equivalent rosuvastatin doses were calculated by equating 40 mg simvastatin and 10 mg atorvastatin to 5 mg rosuvastatin [322, 323]. At the first visit, patients taking both statin and ezetimibe were asked to stop ezetimibe. All patients had 4 weeks (placebo period) of receiving placebo. At the end of the placebo period, patients attended for study visit and were randomised to either placebo or ERN/LRP for 12 weeks (treatment period). During the treatment period, patients randomised to study medication received ERN/LRP 1g/20mg for 4 weeks followed by increased dose of
ERN/LRP 2g/40mg for 8 weeks. After the first treatment period all patients repeated the placebo period (4 weeks) before the second treatment period (12 weeks). As before they attended for visits at the end of the placebo period, and at the end of the treatment period. Patients who were randomised to placebo in the first treatment period received ERN/LRP in the second treatment period and vice versa. Fasting blood samples were taken at all the study visits except the screening visit. Concordance with medication was assessed by pill count at each visit.

The Clinical Trials Pharmacist was responsible for the randomisation procedure. Block randomisation was carried out with equal numbers of participants per group using a computer generated list for this cross over trial. Patients and investigators were blinded to individual assignments and to the randomisation procedure.
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<table>
<thead>
<tr>
<th>Visit</th>
<th>Details</th>
</tr>
</thead>
</table>
| 1st visit (n=38) | • 38 patients consented  
• All patients received placebo for 4 weeks (first placebo period) |
| 2nd visit (n=36)  | • Post-placebo blood samples obtained  
• 36 patients randomised to ERN/LRP or placebo for 12 weeks (first treatment period)  
• 2 dropouts during first placebo period (reason unknown) |
| 3rd visit (n=31)  | • Post-treatment blood samples obtained  
• All patients received placebo for 4 weeks (second placebo period)  
• Dropouts during 1st treatment period: (receiving ERN/LRP: 1 due to flushing, 2 reason unknown; receiving placebo: 2 reasons unknown) |
| 4th visit (n=31)  | • Post-placebo blood samples obtained  
• Patients received placebo or ERN/LRP for 12 weeks (second treatment period)  
• No dropouts during 2nd placebo period |
| 5th visit (n=27)  | • Post-treatment blood samples obtained  
• 27 patients completed all study visits  
• Dropouts during 2nd treatment period: (receiving ERN/LRP: 1 due to flushing, 1 SAE abdominal pain; receiving placebo: 1 diarrhoea, 1 SAE abdominal pain) |

Figure 3.1 Study overview. Patients randomised to placebo during the first period received ERN/LRP in the second period and vice versa. In each period the ERN/LRP dose was increased from 1g/20mg to 2g/40mg after 4 weeks.

**3.2 Sample processing**

Blood was withdrawn, after an overnight fast, into BD-vacutainer (plain, potassium ethylene diamine tetra acetic acid (K<sub>2</sub>EDTA) and oxalate/sodium fluoride) tubes. Blood was processed for serum and plasma separation by centrifugation at 2000xg at 4°C for 15 minutes using a Heraeus Labofuge 400R centrifuge (DJB Labcare, Buckinghamshire, UK). Fasting blood glucose was measured in fluoride-plasma; TC, TG, HDL-C, very low density lipoprotein cholesterol (VLDL-C), very low density lipoprotein triglycerides (VLDL-TG), apoB and apoAI were measured in serum using a Cobas Mira autoanalyzer with Horiba-ABX and Roche standards, control and reagents (Horiba ABX-UK, Northampton, UK and Roche Diagnostics, Burgess Hill, UK). The assays were
calibrated before each test run, as recommended by the manufacturer. PON-1 activity was measured in serum samples using paraoxon as substrate [324]. The K₂EDTA-plasma was processed to isolate different density lipoprotein fractions by ultracentrifugation.

All laboratory chemicals were purchased from Sigma Chemical Co., Poole, UK unless otherwise indicated.

3.3 Laboratory processes

3.3.1 Blood glucose

Blood glucose was assayed according to [325] using the Roche glucose oxidase phenol 4-aminoantipyrine peroxidase (GOD-PAP) method and a Roche auto-analyser (Roche Diagnostics Ltd, Burgess Hill, UK) in the Clinical Biochemistry Laboratory of the Manchester Royal Infirmary which participates in a national quality control scheme.

Principle

Glucose oxidase (GOD) converts glucose into gluconate generating H₂O₂. The latter reacts with phenol and 4-aminoantipyrine in the presence of peroxidase (POD) giving a red-coloured quinone which is measurable at 505 nm. The increase in absorbance correlates with the glucose concentration of the sample.

Glucose + O₂ \( \xrightarrow{\text{GOD}} \) Gluconic acid + H₂O₂

H₂O₂ + Phenol + 4-aminoantipyrine \( \xrightarrow{\text{POD}} \) Red quinone + 4H₂O

Reagents

Reagent 1

- Phosphate buffer, pH: 7.4
- Phenol
- 4-Aminoantipyrine
- Glucose oxidase
- Peroxidase

Reagent 2 (Standard)

- Glucose

3.3.2 Lipids and lipoproteins
HDL functionality and LDL quality: the influence of obesity, obstructive sleep apnoea and pharmacological intervention

### 3.3.2.1 Total cholesterol

**Principle**

Cholesterol is measured by an enzymatic photometric cholesterol oxidase phenol 4-aminoantipyrine peroxidase (CHOD-PAP) method according to the method described by Deeg and Ziegenhorn, 1983 [326]. After an enzymatic hydrolysis by cholesterol esterase (CHE), cholesterol is oxidized by cholesterol oxidase (CHO). The $\text{H}_2\text{O}_2$ released reacts with 4-aminoantipyrine and phenol under the catalytic action of POD (Trinder’s reaction) to form the coloured indicator, quinoneimine, which has maximum absorption at 500 nm.

\[
\begin{align*}
\text{CHE} & : \text{Cholesterol ester} + \text{H}_2\text{O} \rightarrow \text{Cholesterol} + \text{Fatty acids} \\
\text{CHO} & : \text{Cholesterol} + \text{O}_2 \rightarrow \text{Cholesterol-3-one} + \text{H}_2\text{O}_2 \\
2\text{H}_2\text{O}_2 + 4\text{-Aminoantipyrine} + \text{Phenol} & \rightarrow \text{POD} \rightarrow \text{Quinoneimine} + 4\text{H}_2\text{O}
\end{align*}
\]

**Reagents**

**i. ABX cholesterol CP**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Good’s buffer pH 6.7</td>
<td>50 mmol/l</td>
</tr>
<tr>
<td>Phenol</td>
<td>5 mmol/l</td>
</tr>
<tr>
<td>4-Aminoantipyrine</td>
<td>0.3 mmol/l</td>
</tr>
<tr>
<td>CHE</td>
<td>≥ 200 units/l</td>
</tr>
<tr>
<td>CHO</td>
<td>≥ 50 units/l</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>≥ 3 K unit/l</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>0.95 g/l</td>
</tr>
</tbody>
</table>

**ii. Diluent: H$_2$O**

**iii. Calibrator: ABX Pentra multicalibrator** (lyophilisate for 3ml) containing 4.16 mmol/l cholesterol isolated from bovine plasma.

The Cobas Mira auto-analyzer (Horiba ABX-UK, Northampton, UK) was programmed as recommended by the kit manufacturer (3µl of sample, 20µl of diluent, 250 µl of reagent; measurements at 500 nm).
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3.3.2.2 Triglyceride (TG)

Principle
Triglyceride was measured by glycerol phosphate oxidase phenol 4-aminoantipyrine peroxidase (GPO-PAP) method after an enzymatic hydrolysis by lipoprotein lipase. Oxidation by glycerol-3-phosphate oxidase released H$_2$O$_2$ which generates the colorimetric indicator, quinoneimine, from 4-aminoantipyrine and phenol under the catalytic action of POD (Trinder’s reaction) that is measured at 500 nm [327].

\[
\text{Triglycerides} + \text{H}_2\text{O} \xrightarrow{\text{Lipoprotein lipase}} \text{Glycerol + fatty acids} \\
\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerokinase}} \text{Glycerol-3-phosphate} + \text{ADP} \\
\text{Glycerol-3-phosphate} \xrightarrow{\text{Glycerol-3-phosphate Oxidase}} \text{H}_2\text{O}_2 + \text{DHAP} \\
2 \text{H}_2\text{O}_2 + \text{4-Aminoantipyrine} + \text{p-Chlorophenol} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + 4 \text{H}_2\text{O}
\]

Reagents
i. ABX triglycerides CP

- PIPES free acid 50 mmol/l
- Sodium hydroxide 3.36g/l
- Triton X-100 1ml/l
- Magnesium salt 14.8 mmol/l
- \textit{p}-Chlorophenol 2.69 mmol/l
- ATP 3.14 mmol/l
- Sodium azide 7.99 mmol/l
- Potassium ferrocyanide 9.94 µmol/l
- 4-aminoantipyrine 0.31 mmol/l
- Lipoprotein lipase 1.9 units/l
- Glycerokinase 0.5050 K unit/l
- Glycerol phosphate Oxidase 4.15 K unit/l
- Peroxidase 0.495 K unit/l
- Distilled water qs 1l/l
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ii. Diluent: H₂O

iii. Calibrator: ABX Pentra multicalibrator (lyophilisate for 3ml) containing 1.64 mmol/l triglycerides isolated from chicken egg yolk.

The Cobas Mira auto-analyzer (Horiba ABX-UK, Northampton, UK) was programmed as recommended by the kit manufacturer (3 µl of sample, 10 µl of diluent, 290 µl of reagent; measurements at 500 nm).

3.3.2.3 High density lipoprotein-cholesterol

Principle

HDL-C was measured by direct second generation homogenous method (Roche Diagnostics, Burgess Hill, UK) using alpha-cyclodextrin sulphate, magnesium ions, and polyethylene glycol pre-treated cholesterol esterase (PEG-CHE) and oxidase (PEG-CHO) to measure HDL-C specifically in the presence of apoB-containing lipoproteins. PEG modified cholesterol esterase and cholesterol oxidase enzymes show selective catalytic activities towards lipoprotein fractions in the order, LDL<VLDL<chylomicron<HDL. In the presence of magnesium ions, α-cyclodextrin sulphate reduced the reactivity of cholesterol, especially in chylomicrons and very-low-density lipoprotein. As a result of combining PEG-modified enzymes and α-cyclodextrin sulphate, selective determination of HDL-C in serum in the presence of a small amount of dextran sulphate can be achieved without the need for precipitation of other apoB-containing lipoproteins [328].

HDL-cholesterol esters are broken down quantitatively into free cholesterol and fatty acids by PEG-CHE. In the presence of oxygen, cholesterol is oxidized by cholesterol oxidase into Δ⁴-cholestenone and H₂O₂ that reacts with 4-aminoantipyrine and HSDA (N-(2-hydroxy-3-sulphopropyl)-3,5-dimethoxyaniline) to form a purple-blue dye. The colour intensity of this dye is proportional to the cholesterol concentration and can be measured at 600nm.
HDL functionality and LDL quality: the influence of obesity, obstructive sleep apnoea and pharmacological intervention

\[
\text{HDL-cholesterol esters + } H_2O \xrightarrow{\text{PEG-CHE}} \text{HDL-cholesterol + RCOOH}
\]

\[
\text{HDL-cholesterol + O}_2 \xrightarrow{\text{PEG-CHO}} \Delta \text{-cholestenone + H}_2O_2
\]

\[
2H_2O_2 + 4\text{-Aminoantipyrine + HSDA + H}^+H_2O \xrightarrow{\text{POD}} \text{Quinoneime +5H}_2O
\]

**Reagents**

**i. Reagent 1**

- 3-morpholinopropanesulphonic acid (MOPS) 19.1 mmol/l, pH 7
- Dextran sulphate 0.5 g/l
- MgSO$_4$.7H$_2$O $\geq$8.11 mmol/l
- HSDA 0.96 mmol/l
- Ascorbate oxidase (microorganism, recombinant) $\geq$ 3 K unit/l
- POD (horseradish) $\geq$ 10 K unit/l

**ii. Reagent 2**

- Piperazine-1,4-bis(2-ethanesulphonic acid (PIPES) 9.9 mmol/l pH7
- PEG-cholesterol esterase (microorganism) $\geq$0.2 K unit/l
- PEG-cholesterol oxidase (microorganism, recombinant) $\geq$7.6 K unit/l
- POD (horseradish) $\geq$20 K unit/l
- 4-Aminoantipyrine 2.46 mmol/l

**iii. Diluent: H$_2$O**

**iv. Calibrator:** 1.42 mmol/l lyophilised HDL-cholesterol isolated from human serum containing 0.4% (w/w) sodium azide.

The Cobas Mira auto-analyzer (Horiba ABX-UK, Northampton, UK) was programmed as recommended by the kit manufacturer (3 µl of sample, 50 µl of diluent, 250 µl of reagent1, 83 µl of reagent2, 12 µl of diluent; measurements at 600 nm).

**3.3.2.4 LDL-cholesterol**

LDL-C levels were calculated from the Friedewald formula [329].
HDL functionality and LDL quality: the influence of obesity, obstructive sleep apnoea and pharmacological intervention

\[
\text{LDL-C} = \text{total cholesterol} - \text{HDL-C} - \frac{\text{TG}}{2.19} \text{ (for mmol/l)}
\]

This formula is only accurate when serum TG concentration does not exceed 4.5

3.3.2.5 Total apoB and apo AI

Principle

ApoB and apoAI were measured using immunoturbidimetric assays adapted for the Cobas-Mira auto-analyzer according to method described by Eugui et al. [330] and Brustolin et al. [331]. The immune complexes formed are measured by turbidimetry with the signal generated correlated directly with the concentration of apoB or apoAI in the sample.

Reagents


ii. ABX Pentra apoAI (apoAI start R1): a fraction of purified immunoglobulins from rabbit antiserum containing 15 mmol/l NaN₃ as stabiliser (Immunogen: ApoAI isolated from human HDL).

iii. ABX Pentra apo calibrator: a lyophilisate for 0.5ml containing 1.41g/l apoAI and 1.05g/l apoB.

iv. Diluent 1: H₂O

v. ABX Pentra sample diluent CP: Saline phosphate buffer containing <0.1% NaN₃.

vi. ABX Pentra accelerator I CP: Polymer solution in saline phosphate buffer containing <0.1% NaN₃.

The Cobas Mira auto-analyzer (Horiba ABX-UK, Northampton, UK) was programmed as recommended by the kit manufacturer (13µl of sample, 30 µl of diluent1, 200µl of accelerator; 16.7 µl of apoB start R1 and 53.3 µl of reagent diluent; measurements at 340 nm) and (7µl of sample, 60 µl of H₂O, 200µl of accelerator; 23.3 µl of apoAI start R1 and 46.7 µl of diluent; measurements at 340 nm after 10 and 15 min) for apoB and apoAI respectively.

3.3.2.6 Serum paroxonase-1 activity (PON1)

Principle
Serum PON-1 activity was determined by a semi-automated micro-titre plate method using paraoxon (O, O-Diethyl O-(4-nitrophenyl) phosphate) as a substrate. The rate of generation of p-nitrophenol was determined at 25°C, with the use of a continuously recording spectrophotometer at 405 nm [324].

**Reagents**

i. Paraoxon (O, O-diethyl p-nitro phenyl phosphate) purchased from Sigma Chemical Co., Poole, UK.

ii. Paraoxonase assay buffer: 100 mmol/l Tris–HCl buffer, pH 8.0 containing 2 mmol/l CaCl$_2$

iii. Decontaminating solution: 2mol/l NaOH.

**Procedures and calculations**

Paraoxon stock solution was prepared freshly as 5.5 mmol/l paraoxon in assay buffer. Mixing was performed at room temperature for at least 1 hour till paraoxon completely dissolve.

In a 96 well micro-titre plate, 10 µl samples were mixed with 200 µl paraoxon stock solution.

The change in OD at 405 nm at 23 ± 0.5°C was recorded using a Labsystems multiskan multisoft plate reader (Labsystems, Hampshire, UK) which was programmed to measure the change in the optical density following a 0.42 min lag phase and directly calculate the PON-1 activity (OD/min x 2057 = nmol ml$^{-1}$min$^{-1}$).

Excess paraoxon solution and all glass and plastic wares were decontaminated by immersion in decontaminating solution (2mol/l NaOH) overnight in fume cupboard.

**3.3.2.7 Lipoprotein isolation for measuring sdLDL apoB, VLDL-C, VLDL-TG and VLDL-apoB**

**Principle**

Lipoproteins were isolated from fresh EDTA-plasma by a simplified ultracentrifugation method according to the method described by Charlton-Menys et al. [332]. The density of plasma was raised as described by Havel et al. [333] by the addition of a concentrated solution of KBr (potassium bromide heavy density solution containing EDTA; typically D=1.33g/ml). This was checked by repeatedly weighing 5 ml aliquots. Solutions of
lower density were prepared by dilution of the stock heavy density solution with 0.15 mmol/l sodium chloride solution (D = 1.006 g/ml) according to the formula:

\[ V = \frac{(D - D1/D2 - D)}{V1} \times ml \]

Where V is the required volume of heavy density solution (Density=1.33 g/ml), D is the density of heavy density solution (=1.33g/ml), D1 is density of the original solution (=1.006 g/ml), D2 is the desired density of the mixture and V1 is the volume of the original solution.

The density of plasma was raised to the desired density by the addition of salt solution according to similar formula taking plasma density of 1.006 g/ml. The density-adjusted plasma aliquots were ultracentrifuged at 435,680x g for 5h at 4°C according to Ordovas et al. [334].

**Reagents**

- **i.** Heavy density solution containing EDTA (D=1.33g/ml): 76.5g sodium chloride NaCl, 177g potassium bromide KBr and 0.05g disodium ethylenediamine tetra-acetic acid dihydrate (Na₂EDTA2H₂O) were dissolved in distilled water and made up to 500 ml. The density of the solution was determined before being used.

- **ii.** 0.15 mmol/l sodium chloride solution (D=1.006 g/ml).

- **iii.** Phosphate-buffered saline (PBS): 8 g/l sodium chloride NaCl, 0.2 g/l potassium chloride KCl, 1.44 g/l disodium hydrogen phosphate Na₂HPO₄ and 0.25 g/l potassium dihydrogen phosphate KH₂PO₄; pH 7.4

**Procedures**

SdLDL was isolated from 1ml aliquot of plasma adjusted to density of 1.044g/ml.

VLDL was isolated from 1ml aliquot of plasma at its background density of 1.006g/ml.

The density-adjusted plasma aliquots were ultracentrifuged in 11 x 34 mm polycarbonate tubes (Beckman Coulter UK, High Wycombe, UK) at 100,000 rpm (435,680 x g) for 5h at 4°C using a Beckman Optima TLX bench top ultracentrifuge fitted with TLA 120.2 fixed angle rotor (Beckman Coulter UK, High Wycombe, UK).

Supernatants and infranatants were removed by aspiration and readjusted to 1ml in volumetric flasks with PBS to restore the original concentrations.

The isolated fractions were as follow:

- D<1.006g/ml Supernatant; VLDL.
D>1.044g/ml Infranatant; sdLDL.

ApoB was measured in all isolated fractions by immunoturbidimetric assay as previously described in section 3.3.2.5. The distribution of apoB was expressed as concentration (mg/dl).

Cholesterol and triglycerides were also measured in the VLDL subfraction in order to yield VLDL-C and VLDL-TG as previously described in sections 3.3.2.1 and 3.3.2.2 respectively.

3.3.2.8 Non HDL-C, LDL apoB and buoyant LDL apoB

Non HDL-C was calculated by the following formula:

\[
\text{Non HDL-C} = \text{TC} - \text{HDL-C}
\]

LDL apoB was calculated by the following formula:

\[
\text{LDL apoB} = \text{Total apoB} - \text{VLDL apoB}
\]

Buoyant LDL apoB was calculated by the following formula:

\[
\text{Buoyant LDL apoB} = \text{LDL apoB} - \text{sdLDL apoB}
\]

3.3.2.9 Glycated LDL apoB

Glycated apoB was measured directly by non-radioactive competitive Enzyme Linked-Immuno-Sorbent Assay (ELISA) method [335, 336] utilizing specific mouse monoclonal antibodies against glycated apoB, ES12 using Glycacor kit (Glycor, Exocell Inc, Pa, USA). The assay has intra- and inter-assay precision with a CV<10% according to the manufacturer. Glycacor kits were purchased from BioGnosis Ltd, Hailsham, UK.

Principle

The mouse monoclonal antibody, ES12, recognizes a specific epitope on glycated apoB in the LDL complex. It is specific for glycated LDL, and does not cross-react with other human plasma proteins including non-glycated LDL [335]. During the primary incubation, ES12 binds to either the glycated LDL immobilized to the microtitre plate well solid phase or to the soluble glycated LDL in standard or experimental samples. Subsequent washing removes reactants in the soluble phase, and the antibody bound to the glycated LDL in the solid phase is detected with HRP-conjugated goat anti-mouse antibody. Because it is a competitive assay, colour intensity, upon development with tetramethylbenzidine (TMB) is inversely proportional to the concentration of glycated
LDL added to the soluble phase. The amount of glycated LDL in the experimental sample is determined from a simultaneously run standard curve using known amounts of glycated apoB in the primary incubation system.

**Test components**
The following components were provided with the kit

- Glycacos assay plate: a multi-strip ELISA microplate pre-coated with a standardized preparation of glycated LDL, and blocked/stored in blocking solution for shipping.
- Glycacos wash buffer: a concentrated wash buffer that is diluted 1:10 before use.
- LDL diluent: a proprietary buffer for the dilution of glycated LDL standard and samples.
- Glycated LDL standard: a lyophilized preparation of human glycated LDL that is reconstituted with 1 ml of distilled water to give targeted concentrations between 0.01-0.1 mg/dl when serially diluted.
- Positive control: a lyophilized glycated LDL that is reconstituted with 0.5 ml of distilled water to give targeted concentrations greater than 0.2 mg/dl (absorbance value less than 0.2)
- Assay control: a lyophilized human plasma that is reconstituted with 0.5 ml of distilled water to give targeted concentrations within the expected range of the assay.
- ES12 anti-glycated LDL: a mouse monoclonal antibody that specifically binds the glycated apoB site within the LDL complex.
- HRP-conjugate: anti-mouse IgG-HRP conjugate of goat origin.
- Colour developer: 3, 3’, 5, 5’-Tetramethylbenzidine (TMB) in buffer salts.
- Colour stopper: 1mol/l sulphuric acid.

**Procedures**

**Preliminary preparations include**
Dilution of wash buffer and reconstitution of glycated LDL standard, positive control and assay control.
Glycated LDL standard preparation by serial dilution using LDL diluent.
Sample dilution by LDL diluent: 1:41 dilution for plasma samples and 1:21 dilution for isolated fractions.
HDL functionality and LDL quality: the influence of obesity, obstructive sleep apnoea and pharmacological intervention

**GlycCOR test plate**

The wells were drained and washed five times with the wash buffer filling the wells each time. Residual fluids have to be removed by inversion on absorbent paper and gentle tapping.

Duplicates of the control and standards wells were prepared as follows

<table>
<thead>
<tr>
<th>Sample</th>
<th>Volume µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL diluent (Blank)</td>
<td>100</td>
</tr>
<tr>
<td>LDL diluent (No antigen control for assay performance)</td>
<td>50</td>
</tr>
<tr>
<td>1:1, 1:2, 1:4, 1:8 and 1:16 standard dilutions</td>
<td>50</td>
</tr>
<tr>
<td>Assay control</td>
<td>50</td>
</tr>
<tr>
<td>Positive control</td>
<td>50</td>
</tr>
</tbody>
</table>

Diluted samples were applied to the microtitre plate in a volume of 50 µl.

The ES12 anti-glycated LDL was reconstituted by 4.4 ml of LDL diluent. Fifty microliters of diluted ES12 anti-glycated LDL were added to each well leaving blank well without any antibody.

The plate was covered and incubated for one hour at room temperature.

The plate was then drained and washed ten times as described previously with blotting after the final wash.

One hundred microliters of HRP-conjugate was added to each well and the plate was covered and incubated for one hour at room temperature.

Plate washing was repeated ten times as described previously followed by adding 100 µl of colour developer to each well and ten minutes incubation at room temperature.
The colour development was stopped by adding 100 µl of colour stopper and absorbance was measured at 453nm using Labsystems multiskan mutisof plate reader (Labsystems, Hampshire, UK).

**Calculation**

Standard curve was prepared by plotting standard concentration against (1-absorption) values to get a logarithmic curve (figure 3.2). The concentration of glycated apoB was determined by graphic analysis from standard curve using Microsoft excel followed by correction for sample dilution.

![Figure 3.2: Standard curve for glycated apoB (Glycacor kit)](image)

**3.3.3 The susceptibility of LDL and HDL to oxidation and the ability of HDL to protect LDL against oxidation (in vitro)**

**3.3.3.1 Isolation of LDL and HDL**

**Principle**

Fresh serum was used to isolate LDL (D= 1.019-1.063 g/ml) and HDL (D= 1.063-1.21 g/ml) by sequential density gradient ultracentrifugation using Beckman L7 ultracentrifuge fitted with 50.4 Ti fixed angle rotor (Beckman Instruments Ltd, Palo Alto, USA) [337].
HDL functionality and LDL quality: the influence of obesity, obstructive sleep apnoea and pharmacological intervention

The density of serum was raised according to Havel et al. method [333] with the addition of concentrated salt solution (heavy density solution containing EDTA; D=1.33g/ml) as previously described.

**Procedures**

Serum density was first adjusted to D=1.019 g/ml using KBr heavy density solution (D=1.33 g/ml) in order to remove VLDL and IDL taking the plasma density as 1.006g/ml.

Serum was mixed with the heavy density solution and then was transferred to 13 x 64 mm polyallomer tubes (Beckman Coulter UK, High Wycombe, UK) ensuring that each tube was completely filled. The tubes were secured with metal caps and centrifuged at 34,000 rpm (144,000 x g), 4°C for 22 hours and 17 minutes. The floating layer consisting of VLDL and IDL was discarded and the infranatant was pooled.

The volume of the pooled infranatants was measured again and the density was increased to D=1.063g/ml before repeating centrifugation as before and the distinct floating bands were carefully separated by means of aspiration as small volumes to represent LDL (D= 1.019-1.063 g/ml).

The process was repeated to the infranatant by increasing density to D=1.21 g/ml in order to isolate HDL (D= 1.063-1.21 g/ml).

Tris buffered saline was prepared for dialysis Tris buffered solution (TBS) {Tris 20 mmol/l (15.8 g/5 litres), normal saline (45 g/5 litres), CaCl2 2 mmol/l (1.47g/ 5 litres), and 0.01% chloramphenicol (50 ml), pH 7.4, 4°C}. LDL and HDL were dialyzed overnight against this TBS to remove density-adjusting salts and then stored at 4°C under N2 for not more than 16 hours. The protein content of LDL and HDL was then measured before the *in vitro* oxidation studies.

**3.3.3.2 Determination of protein content**

The total protein content of the isolated lipoproteins was first determined by the bicinchoninic acid method (Pierce® BCA protein assay kit; Fisher Scientific UK Limited, Loughborough, UK) using bovine serum albumin (BSA) as standard [338].

**Principle**
This method depends on the reducing properties of proteins. In alkaline medium, proteins can reduce cupric to cuprous ions (Biuret reaction) detected with a highly sensitive and selective colourimetric reagent containing bicinechinonic acid (BCA). Chelation takes place between two molecules of BCA and one cuprous ion to give a purple coloured reaction complex measured at 540 nm.

**Reagents**

BCA™ protein assay reagent A: reagent contains sodium carbonate, sodium bicarbonate, BCA™ detection reagent and sodium tartrate in 0.1 mol/l sodium hydroxide.

BCA™ protein assay reagent B: reagent contains 4% cupric sulphate pentahydrate.

BCA™ standard: 2 mg/ml bovine serum albumin in 0.9% sodium chloride solution containing sodium azide.

**Procedures**

Working reagent was freshly prepared by mixing 1 part of reagent B with 50 parts of reagent A. BSA standard (2 mg/ml) was diluted as stated by manufacturer to give concentration range 0-2 mg/ml.

Using a 96 wells microplate, 10 µl of standard and samples were applied as duplicates and 200 µl of the working reagent was added to each well and the plate was mixed thoroughly for 30 seconds. The plate was covered and incubated at 37°C for 30 minutes. The developed colour intensity was measured at 540 nm after the plate being cooled to room temperature using Labsystems multiskan multisoft plate reader (Labsystems, Hampshire, UK).

**Calculation**

The total protein content was determined directly from standard curve (figure 3.3).
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Figure 3.3: Total protein standard curve using BSA as standard (BCA method; r=0.999).

3.3.3.3 In vitro oxidation:
Ability of the patient’s HDL to limit lipid peroxide formation in patient’s LDL in the presence of Cu$^{2+}$ was tested by El-Saadani method [23]. Susceptibility of LDL and HDL to in vitro oxidation was also tested using the same method. The oxidation of iodide (I$^-$) by lipid hydroperoxides (LCOOH) proceeds according to the following reaction:

$$\text{LCOOH} + 2\text{H}^+ + 3\text{I}^- \rightarrow \text{LOH} + \text{H}_2\text{O} + \text{I}_3^-$$

The quantity of triiodide anion (I$_3^-$) released in the oxidation reaction is directly proportional to the lipid peroxide accumulated in the sample. The molar absorptivity value ($\varepsilon$) of I$_3^-$ is $2.46 \times 10^4 \text{ l.mol}^{-1}.\text{cm}^{-1}$ at 365 nm [339].

Reagents:

i. PBS: 8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na$_2$HPO$_4$ and 0.25 g/L KH$_2$PO$_4$; pH 7.4.

ii. 0.5 mM CuSO$_4$ (FW 159.6) was prepared as 50mM stock solution (0.798 g/100ml water). 1ml was then diluted to 100ml to give 0.5 mM solution.

iii. 2 mM EDTA Disodium Salt (FW 372.24) was prepared as 20mM stock solution: (0.74 g/100ml water). 1ml was diluted to 10ml to give 2 mM solution.

iv. Cholesterol-Iodide reagent (CHOD-Iodide reagent) as, 0.2 M potassium dihydrogen phosphate (FW 136.09); 27.22 g/l, 0.12 M potassium Iodide (FW 166.01); 19.92 g/l, 0.15 mM sodium azide (FW 65.01); 0.0098 g/l, 0.2% Triton X-100, 0.1g/l benzalkonium chloride (FW 471.5), 10 µM ammonium molybdate (FW 1235.9); 0.0124 g/l. The solution is stored in dark at 4°C.
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**v.** 4 mM butylated hydroxytoluene (BHT) (FW 220.36) was prepared as 40mM stock solution (88.1 mg/10ml ethanol). 1ml was diluted to 10ml in ethanol to give 4 mM solution.

**Procedure:**
The volumes of LDL and HDL equivalent to 0.25 mg protein were calculated.
PBS was oxygenated using 99.9% oxygen.
The required amount of Cholesterol-Iodine working reagent was prepared as follows *(prepared fresh each time)*:

- 2 mM EDTA= 5µl X (Number of samples +5) X 5 (e.g. 250µl for 5 samples)
- 4 mM BHT= 2.5µl X (Number of samples +5) X 5 (e.g. 125µl)
- Cholesterol-Iodine reagent= 0.5 ml X (Number of samples +5) X 5 (e.g. 25ml)

Incubation mixtures (table 3.1) with LDL (0.25 mg protein) or HDL (0.25 mg protein) or both LDL & HDL (0.25 mg protein each), oxygenated PBS and copper were prepared. Copper was added to the mixture at the end and immediately 50 µl of this mixture was added to 0.5 ml of the working reagent (mixture-WR) in order to stop the oxidation process.

This mixture-WR was stored for 30 minutes in dark and absorbance was measured against CHOD at 365 nm using UVi light XTD2 spectrophotometer equipped with eight-position automatic sample changer.

<table>
<thead>
<tr>
<th>Prepare a total of 500 µl</th>
<th>LDL (volume in µl = 0.25 mg LDL)</th>
<th>HDL (volume in µl = 0.25 mg LDL)</th>
<th>PBS (volume in µl)</th>
<th>Cu+2 (volume in µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation mixtures</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL</td>
<td>LDL</td>
<td>-</td>
<td>495 - LDL</td>
<td>5</td>
</tr>
<tr>
<td>LDL+HDL</td>
<td>LDL</td>
<td>HDL</td>
<td>495-(LDL+HDL)</td>
<td>5</td>
</tr>
<tr>
<td>HDL</td>
<td>-</td>
<td>HDL</td>
<td>495-HDL</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 3.1 Mixture components for *in vitro* oxidation

The remaining mixture was then incubated for 3 hours at 37°C. At the end of 3 hours oxidation was again halted as above and absorbance measured.
LPO was calculated from absorbance after correction against blank using the equation

\[
\text{LPO \text{ nmol/l}} = (\text{Abs 365}/2.46) \times 1100.
\]

Net LPO generated at 3 hours was calculated by subtracting 0 hr LPO from 3 hr LPO.

3.3.4 Biomarkers: principles of methods

**TNFα, adiponectin, MCP-1 and ICAM-I** were all measured using DuoSet ELISA development kits from R&D Systems (Abingdon, UK). Plasma was used for the determination of adhesion molecules, while serum was used for the others. First, the capture antibody (mouse anti-human antibody) is bound to a microtitre plate to create a solid phase. A blocking buffer containing BSA is then added. Following a wash, standards and samples are then incubated with the solid phase antibody (that captures the antigen). After further washing, detection antibody (biotinylated mouse anti-human antibody) is added to each well thus completing the sandwich. Following a wash, each well is incubated with streptavidin-HRP. After further washing, the substrate TMB solution (Sigma-Aldrich, Poole, Dorset) is added and colour develops in proportion to the amount of bound HRP. Colour development is stopped by addition of strong acid and the intensity then measured at \( \lambda = 450\text{nm} \).

**Oxidised LDL (ox-LDL)** was measured in plasma PG using kits from Mercodia AB (Upsala, Sweden). Mercodia Oxidized LDL ELISA is a solid phase two-site enzyme immunoassay. It is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the oxidized apolipoprotein B molecule. During incubation oxidized LDL in the sample reacts with anti-oxidized LDL antibodies bound to microtiteration well. After washing, which removes non-reactive plasma components, a peroxidase conjugated anti-human. Apolipoprotein B antibody recognizes the oxidized LDL bound to the solid phase. After a second incubation and a simple washing step that removes unbound enzyme labeled antibody, the bound conjugate is detected by reaction with 3,3', 5,5'-TMB. The reaction is stopped by adding acid to give a colorimetric endpoint, then read spectrophotometrically

**CETP activity** was measured using a fluorometric method using assay kits (ab65383) from Abcam, UK. This kit uses a donor molecule containing a fluorescent self-quenched neutral lipid that is transferred to an acceptor molecule in the presence of CETP. CETP-
mediated transfer of the fluorescent neutral lipid to the acceptor molecule results in an increase in fluorescence.

**Lyso-PC** was measured in serum using Azwell lyso-PC enzymatic assay kits from Cosmo Bio, Japan. Lyso-PC is hydrolyzed with lysophospholipase, followed by glycerophosphorylcholine phosphodiesterase and choline oxidase. Subsequently, generated hydrogen peroxides are colorimetrically measured in the presence of peroxidase.

**Insulin** was determined in plasma using Mercodia ELISA kits from Diagenics Ltd. This ELISA is a solid phase two-site enzyme immunoassay. It is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation insulin in the sample reacts with peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound to microplate wells. A simple washing step removes unbound enzyme labelled antibody. The bound conjugate is detected by reaction with 3,3’,5,5’-TMB. The reaction is stopped by adding acid to give a colorimetric endpoint that is read spectrophotometrically.

**Homeostasis mode assessment beta cell function** (**HOMA β**) was calculated using the formula HOMA β = (20 X insulin mU/l)/(glucose – 3.5 mmol/l) [340]. **Homeostasis mode assessment – insulin resistance** (**HOMA-IR**) was calculated using the formula HOMA IR = (insulin X glucose)/22.5 [340].

**Lp-PLA2** mass was determined in plasma using USCN Lifescience ELISA kits from Hölzel Diagnostika Handels GmbH. This assay employs the quantitative sandwich enzyme immunoassay technique. Antibody specific for Lp-PLA2 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Lp-PLA2 present is bound by the immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific for Lp-PLA2 is added to the wells. After washing, avidin conjugated horseradish peroxidase (HRP) is added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution is added to the wells and colour develops in proportion to the amount of Lp-PLA2 bound in the initial step. The colour development is stopped and the intensity of the colour is measured.
Caslake and co-workers conducted a study to ascertain the relation between Lp-PLA2 mass and activity (PAF-hydrolysing capability) in post MI patients or patients with stable coronary artery disease or healthy controls (n=148) [57]. They showed a strong correlation ($r=0.86, P<0.001$) between Lp-PLA2 mass and activity in plasma thus suggesting that Lp-PLA2 contributes the majority of the PAF-hydrolysing capability of plasma. For measuring Lp-PLA2 mass, they developed an immunoassay that offered a simple, sensitive, and reliable method for the detection of Lp-PLA2 in plasma compared to activity measurements.

**Apo M** was assayed in serum using Bluegene E01A0522 kits from Hölzel Diagnostika Handels GmbH, Köln, Germany. This ELISA kit uses Sandwich-ELISA as the method. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to antigen. Standards or samples are added to the appropriate micro ELISA plate wells and combined to the specific antibody. Then a biotinylated detection antibody specific for antigen and Avidin-HRP conjugate is added to each micro plate well successively and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain antigen, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in colour. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the colour turn yellow.

**MPO** was determined in plasma by ELISA using kits from R&D Systems Ltd. This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for MPO has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any MPO present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for MPO is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and colour develops in proportion to the amount of MPO bound in the initial step. The colour development is stopped and the intensity of the colour is measured. While MPO expression or protein level measurements can provide some information regarding the abundance of the MPO molecule, the enzymatic activity of MPO can vary considerably between individuals even if the amount of MPO present is similar [341].
Besides effects such as age and gender, multiple polymorphisms have been identified both with decreased [342] and increased [341] MPO activity. Furthermore, as MPO can be inhibited by endogenous inhibitors, MPO activity does not always correspond to MPO protein or expression levels [343, 344].

Evaluating MPO activity is crucial to understanding its effects in inflammation, and it is not surprising that MPO activity assays are widely used in the literature for this purpose. However, no consensus has been reached on which of the many available assays to use. This is further complicated by the fact that most available probes (e.g. TMB, o-dianisidine, guaiacol) are general peroxidase substrates, lacking specificity towards MPO. Moreover, tissue inhibitors of MPO can interfere with assays [345], and peroxidase activity of myoglobin and hemoglobin can also alter assay results [346]. More importantly, different assays have not yet been compared to each other, and validation and standardization are much needed to verify results from different studies.

**SAA** was assayed by ELISA using kits from Invitrogen Ltd, Paisley, UK. This kit is a solid phase sandwich ELISA. A highly purified monoclonal antibody against Hu SAA has been coated onto the wells of the microtiter strips provided. During the first incubation, standards of known Hu SAA content, controls, and unknown samples are pipetted into the coated wells, followed by the addition of biotinylated second monoclonal antibody. After washing, Streptavidin-Peroxidase (enzyme) is added. This binds to the biotinylated antibody to complete the four-member sandwich. After a second incubation and washing to remove the entire unbound enzyme, a substrate solution is added, which is acted upon by the bound enzyme to produce colour. The intensity of this coloured product is directly proportional to the concentration of Hu SAA present in the original specimen.

**Lp(a)** was determined using ELISA kits from Diagenics Ltd. This assay employs a quantitative sandwich enzyme immunoassay technique that measures Human Lipoprotein(a) in less than 4 hours. A polyclonal antibody specific for Human Lp(a), has been pre-coated onto a 96-well microplate with removable strips. Lp(a) in standards and samples is sandwiched by the immobilized antibody and biotinylated polyclonal antibody specific for Lp(a), which is recognized by a streptavidin-peroxidase conjugate.
All unbound material is then washed away and a peroxidase enzyme substrate is added. The colour development is stopped and the intensity of the colour is measured.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Intra-assay coefficient of variance (%)</th>
<th>Inter-assay coefficient of variance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CV provided by kit manufacturer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPO</td>
<td>&lt;8</td>
<td>&lt;8</td>
</tr>
<tr>
<td>ICAM1</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Insulin</td>
<td>&lt;5</td>
<td>&lt;6</td>
</tr>
<tr>
<td>ApoM</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Lp-PLA2</td>
<td>&lt;10</td>
<td>&lt;12</td>
</tr>
<tr>
<td>Lp(a)</td>
<td>&lt;4</td>
<td>&lt;6</td>
</tr>
<tr>
<td>Glycated apoB</td>
<td>3.5</td>
<td>14.9</td>
</tr>
<tr>
<td>LysoPC</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>CV calculated in lab</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAA</td>
<td>6.1</td>
<td>7.4</td>
</tr>
<tr>
<td>TNFα</td>
<td>5.9</td>
<td>13.1</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>3.2</td>
<td>9.3</td>
</tr>
<tr>
<td>MCP1</td>
<td>7.2</td>
<td>7.3</td>
</tr>
<tr>
<td>oxLDL</td>
<td>5.8</td>
<td>4.6</td>
</tr>
</tbody>
</table>

Table 3.2. This table displays the intra-assay and inter-assay coefficient of variance (CV) for various biomarkers measured.

3.4 Statistical analyses

The distribution or normality of data was determined using Kolmogorov-Smirnov test, D’Agastino and Pearson omnibus normality test and Shapiro-Wilk normality test on GraphPad Prism (GraphPad Software Inc., California, USA). Results are expressed as mean ± standard deviation (SD) for parametric data and median (interquartile range) for
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non-parametric data. SPSS statistical software (SPSS, Illinois, USA) was used for all comparisons and correlations. Statistical significance of differences between groups was determined using the Student’s $t$-test or Mann Whitney test, taking $p<0.05$ as statistically significant. Statistical significance of correlations was determined using Spearman’s test, taking $p<0.05$ as statistically significant.
CHAPTER FOUR: High Paraoxonase 1 activity reinforces HDL’s *in vitro* anti-oxidant function in statin treated dyslipidemic patients.

4.1 Introduction

LDL oxidation is an important atherogenic modification [21, 22]. Statins effectively reduce LDL-C and have revolutionized the treatment of dyslipidemia and CVD [216, 217]. However, there is a residual CV risk of 60-70% despite optimal statin treatment [218-220]. The risk of developing CVD is inversely related to HDL-C [221-223]. The anti-atherogenic role of HDL could be mediated by a number of mechanisms or functions that it possesses. The most accepted mechanistic explanation has been that HDL facilitates uptake of peripheral cholesterol and its return to the liver for excretion in the bile and faeces, a concept first introduced by Glomset in 1968 that was termed RCT [105]. It has been suggested that RCT could be a protective mechanism against atherosclerosis. More recently, a variety of other functions of HDL have been described, primarily based on *in vitro* studies. These include anti-inflammatory, antioxidant, antiglycation, antithrombotic, nitric oxide – inducing and antimicrobial activities [4, 106-109]. Furthermore, a role of HDL in the immune system [110-112] and as a safe location to release toxic metabolites like lysolecithin and lipid peroxide breakdown products such as aldehydes has been described [109, 113]. Foam cells are critical for all stages of atherosclerosis. However unmodified LDL is taken up slowly by macrophages *in vitro*. It must undergo modifications like oxidation and glycation before it can trigger foam cell formation [347]. There is convincing evidence to support that HDL protects LDL from oxidation. Previous studies have tried to elucidate the pathways through which HDL impedes LDL lipid peroxidation. Lipid peroxides could be transferred from LDL to HDL [348] wherein it has been reported that HDL metabolizes lipid peroxides safely [137]. When HDL was incubated with LDL under oxidizing conditions the accumulation of lipid peroxides on LDL was decreased, but the concentration of lipid peroxides on HDL remained similar to that observed when HDL alone underwent oxidation [108, 137, 138]. The lipid peroxides accumulating on
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HDL, regardless of whether LDL was present, rapidly reached a plateau. This capacity of HDL to decrease lipid peroxide accumulation proved to be a result of a hydrolytic enzymatic activity for which PON1 emerged as the most credible candidate [139]. By the virtue of this characteristic PON1 may have an anti-atherogenic role particularly in high risk patients [213, 349].

Statins, which act primarily by lowering LDL-C may also possess antioxidant activity [350] attributable to their capacity to scavenge hydroxyl and peroxyl radicals [351] and their ability to prevent oxidative damage to DNA [352]. Statin treatment of dyslipidaemic patients has been shown to reduce oxidizability of lipoproteins [353]. In animal studies an atherogenic diet reduces PON1 whereas statins increase PON1 and reduce lipoprotein oxidation [354]. In man there is evidence that statin treatment increases PON1 activity [355-357]. Other studies show that treatment of statin naïve dyslipidaemic patients with statins leads to reduced LDL oxidizability both in vivo and in vitro and increases PON1 activity [355, 356, 358]. These human in vitro studies included incubation of LDL alone and not LDL with or without HDL in order to test the effect of statins on LDL oxidizability in vitro. Therefore it is not well understood whether serum PON1 activity influences HDL’s antioxidant capacity and its capacity to protect LDL from oxidation in vitro in statin treated patients.

This study looked at:
- in vitro oxidizability of LDL alone or with HDL and of HDL alone, in statin treated dyslipidaemic patients.
- relation of intrinsically high and low PON1 on the oxidizability of HDL and LDL in these patients.
- effect of PON1 on in vivo LDL glycation as it has recently been suggested that HDL is able to impede in vitro LDL apoB glycation and PON1 may be important for this function of HDL [76].
- factors effecting PON1 activity in this cohort

4.2. Methods

4.2.1. Subjects
Fifty-three men and women aged 20-75 years, not achieving NCEP ATP III target of LDL cholesterol of less than 1.8 mmol/l (70 mg/l) despite at least 12 months statin treatment, were recruited from Central Manchester University Hospital NHS Foundation Trust. They were divided into 2 groups above and below the median PON1 activity (111 nmol/min/ml). The median PON1 activity was 66 nmol/min/ml for the low PON1 group and 176 nmol/min/ml for the high PON1 group. Sixteen patients (30%) had HeFH, either definite or possible according to Simon Broome’s criteria and the remaining thirty seven patients (70%) had polygenic hypercholesterolemia. 20 patients were taking Rosuvastatin (10 – 40mg, mean dose 30 ± 10mg), 24 were on Atorvastatin (20-80mg, mean dose 60 ± 20mg) and 9 on simvastatin (40mg). Fifteen (30%) patients had type 2 diabetes. The Local Research Ethics Committee approved the project. All participants gave informed consent to the study.

4.2.2. Isolation of lipoproteins
Blood samples were collected between 9 and 11 a.m. after participants had fasted from 10 p.m. the previous day. Serum and EDTA-plasma were isolated by centrifugation at 2000 × g for 15 min at 4°C within 2 h of collection and were maintained at that temperature until further use. Serum was used for cholesterol, triglyceride and HDL-C assays and EDTA-plasma for apolipoprotein B100 (apoB). Lipoprotein fractions were prepared by sequential density gradient ultracentrifugation without the addition of EDTA in a Beckman preparative M8-55M ultracentrifuge with a 50.4Ti fixed angle rotor using 13 × 64 mm polyallomer tubes at speeds of 34,000 rpm (144,361 × g) for 22 h [333, 359]. Supernatant LDL, density range 1.019-1.063g/ml and supernatant HDL density range 1.063-1.21g/ml were isolated and the fractions dialyzed against TBS overnight at 4 °C. LDL and HDL protein concentration were determined using BCA [338].

4.2.3. In vitro studies
Susceptibility of lipoprotein fractions to oxidation by Cu^{+2} in vitro was assessed by incubating 0.25 mg protein/ml of lipoprotein fractions with 5 µl copper sulphate (concentration) for 3 hours at 37°C in a Gallenkamp Economy Size 1 Incubator (Gallenkamp, Leicester, UK). LPO production was measured by spectrophotometry at
365 nmol at baseline and 3 hours [23]. LDL and HDL were incubated separately and together in this system.

4.2.4. Other laboratory analyses

Cholesterol and TG were determined using CHOD-PAP and GPO-PAP methods respectively, with reagents from ABX Horiba-UK, Northampton, UK. HDL-C was measured by a direct second generation homogeneous method (Roche Diagnostics, Burgess Hill, UK). LDL-C was estimated using Friedewald Formula. ApoB and apo A1 were assayed immunoturbidimetrically. A Cobas Mira auto-analyzer (ABX Horiba-UK, Northampton, UK) was employed for all these assays. Serum paraoxonase 1 activity (PON1) was determined by a semi-automated micro-titre plate method using paraoxon (O,O-Diethyl O-(4-nitrophenyl) phosphate) as a substrate and read by spectrophotometer at 405 nm [324]. Small dense LDL apoB concentration (d = 1.044 – 1.063g/ml) was determined as previously described. HbA1c and fasting blood glucose were measured using the routine automated methods in the Department of Clinical Biochemistry, Central Manchester University Hospitals NHS Foundation Trust. Ox-LDL was measured in plasma by a direct ELISA sandwich technique with a kit from Mercodia (Upsala, Sweden). Glycated LDL was assayed in plasma using Glyacor ELISA kits from BioGnosis Ltd, Hailsham, UK. Insulin was determined in plasma using Mercodia ELISA kits from Diagenics Ltd. Lp-PLA2 mass was determined in plasma using USCN Lifescience ELISA kits from Hölzel Diagnostika Handels GmbH. Insulin sensitivity or HOMA β was calculated using the formula HOMA β = (20 X insulin mU/l)/(glucose mmol/l – 3.5). HOMA-IR was calculated using the formula HOMA IR = (insulin mU/l X glucose mmol/l)/22.5.

4.2.5. Statistical analyses

Results are expressed as mean ± SD for parametric data and median (interquartile range) for non-parametric data. Statistical significance of differences between groups was determined using the Student’s t-test (for parametric data) or Mann Whitney test (for non parametric data), taking p< 0.05 as statistically significant. Statistical significance of correlations was determined using Spearman’s test, taking p< 0.05 as statistically significant. The distribution or normality of data was determined using Kolmogorov-Smirnov test, D’Agastino and Pearson omnibus normality test and Shapiro-Wilk
normality test on GraphPad Prism (GraphPad Software Inc., California, USA). SPSS statistical software (SPSS, Illinois, USA) was used for all comparisons and correlations.

4.3. Results All patients grouped together:
LPO generated at 3 hours when HDL was incubated in vitro alone and together with LDL, were significantly lower compared with LDL incubated alone {15 (5 – 30) vs 44 (23 – 55) vs 108 (50 – 121) nmol/ml respectively} (p<0.0001) (Figure 4.1).

![Graph showing LPO (nmol/ml) for 3h LDL LPO, 3h LDL+HDL LPO, and 3h HDL LPO](image)

**Figure 4.1** Lipid peroxides (LPO) generated in 3 hours on incubating LDL or LDL+HDL or HDL in all 53 patients put together. Note that on incubating HDL alone or with LDL, LPO generated were significantly lower compared with incubating LDL alone. * p<0.0001. LPO – lipid peroxides.

PON1 activity inversely correlated with LPO generated *in vitro* when HDL was incubated alone and together with LDL (n=53), $r = -0.3$, $P < 0.03$ and $r = -0.3$, $P < 0.04$ respectively. However, PON1 did not correlate with LPO generated when LDL alone was incubated ($r= -0.04$, $p= 0.8$) (Table 4.1). I noted that the PON1 activity showed no significant correlation with apolipoprotein A1 (apoA1) ($p=0.5$) and there was no correlation between serum apoA1 and LPO generated in any of the incubation mixtures.
HDL functionality and LDL quality: the influence of obesity, obstructive sleep apnoea and pharmacological intervention

<table>
<thead>
<tr>
<th>Independent variable</th>
<th>Dependent variable</th>
<th>Spearman’s correlation coefficient (r)</th>
<th>p value (Spearman’s)</th>
<th>Kendall’s tau correlation coefficient (r)</th>
<th>p value (Kendall’s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PON1</td>
<td>3 hr LDL LPO</td>
<td>+0.04</td>
<td>0.8</td>
<td>-0.039</td>
<td>0.7</td>
</tr>
<tr>
<td>PON1</td>
<td>3 hr LDL+HDL LPO</td>
<td>-0.3</td>
<td>0.04</td>
<td>-0.20</td>
<td>0.04</td>
</tr>
<tr>
<td>PON1</td>
<td>3 hr HDL LPO</td>
<td>-0.3</td>
<td>0.03</td>
<td>-0.22</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Table 4.1 Significant correlations between PON1 and other variables (all patients, n = 53). gly ApoB – glycated apolipoprotein B, ApoM – apolipoprotein M, LPO – lipid peroxides.

**Low and high PON1 group comparisons:**

The subjects in the low and high PON1 groups were similar in age, gender, smoking status, body mass index (BMI), blood pressure, statin type and dose used and prevalence of type 2 diabetes, CVD and hypertension (Table 4.2).

<table>
<thead>
<tr>
<th>Basic characteristics</th>
<th>Low PON1 activity (n=27)</th>
<th>High PON1 activity (n=26)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>53 ± 9</td>
<td>54 ± 12</td>
<td>0.8</td>
</tr>
<tr>
<td>Gender (M, F)</td>
<td>13, 14</td>
<td>12, 14</td>
<td>0.9</td>
</tr>
<tr>
<td>Type 2 diabetes n (%)</td>
<td>8 (30)</td>
<td>7 (26)</td>
<td>0.7</td>
</tr>
<tr>
<td>Hypertension n (%)</td>
<td>16 (59)</td>
<td>16 (62)</td>
<td>0.9</td>
</tr>
<tr>
<td>Known CVD n (%)</td>
<td>11 (41)</td>
<td>8 (31)</td>
<td>0.45</td>
</tr>
<tr>
<td>HeFH n (%)</td>
<td>8 (30%)</td>
<td>7 (26%)</td>
<td>0.9</td>
</tr>
<tr>
<td>BMI (kg/m²) mean</td>
<td>31 (28 – 45)</td>
<td>33 (29 – 43)</td>
<td>0.7</td>
</tr>
<tr>
<td>Systolic BP mean (mmHg)</td>
<td>139 ± 21</td>
<td>131 ± 13</td>
<td>0.1</td>
</tr>
<tr>
<td>Diastolic BP mean (mmHg)</td>
<td>79 ± 11</td>
<td>74 ± 10</td>
<td>0.08</td>
</tr>
<tr>
<td>Smoking status n (%)</td>
<td>5 (19)</td>
<td>7 (27)</td>
<td>0.5</td>
</tr>
<tr>
<td>Statin type (R, A, S)</td>
<td>12, 10, 5</td>
<td>8, 14, 4</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Table 4.2 Basic characteristics of low and high PON1 groups. Values are in mean ± SD or median (interquartile range). M – male, F – female, CVD – cardiovascular disease, BMI – body mass index, BP – blood pressure, R – rosuvastatin, A – atorvastatin, S – simvastatin, PON1 – paraoxonase 1, HeFH – heterozygous familial hypercholesterolemia. Statistical tests used – T test for parametric data and Mann-Whitney for non-parametric data.
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There was no significant difference between the two groups in TC (5.9 vs 5.6 mmol/l, p = 0.9), TG (1.7 vs 1.7 mmol/l, p = 0.6), HDL-C (1.4 vs 1.4 mmol/l, p = 0.8), LDL-C (3.4 vs 3.3 mmol/l, p = 0.9), apoB (1.2 vs 1.2 g/l, p = 0.9) and apo1 (1.4 vs 1.3 g/l, p = 0.6) (Table 4.3).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Low PON1 activity (n=27)</th>
<th>High PON1 activity (n=26)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Cholesterol (mmol/l)</td>
<td>5.9 (4.6 – 6.5)</td>
<td>5.6 (5.2 – 6.4)</td>
<td>0.9</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.7 (1.3 – 2.8)</td>
<td>1.7 (1.2 – 2.1)</td>
<td>0.6</td>
</tr>
<tr>
<td>HDL-C (mmol/l)</td>
<td>1.4 ± 0.33</td>
<td>1.4 ± 0.34</td>
<td>0.8</td>
</tr>
<tr>
<td>LDL-C (mmol/l)</td>
<td>3.4 (2.5 – 4.3)</td>
<td>3.3 (2.7 – 4.2)</td>
<td>0.9</td>
</tr>
<tr>
<td>ApoB100 (g/l)</td>
<td>1.2 ± 0.4</td>
<td>1.2 ± 0.4</td>
<td>0.9</td>
</tr>
<tr>
<td>ApoAI (g/l)</td>
<td>1.4 (1.3 – 1.5)</td>
<td>1.3 (1.2 – 1.5)</td>
<td>0.6</td>
</tr>
<tr>
<td>Glycated apoB (mg/dl)</td>
<td>5.1 (3.4 – 5.9)</td>
<td>4.2 (3.0 – 4.9)</td>
<td>0.026</td>
</tr>
</tbody>
</table>

Table 4.3 Basic lipoproteins and apolipoproteins in low and high PON1 groups. Values are in mean ± SD or median (interquartile range). HDL-C – high density cholesterol, LDL-C – low density lipoprotein cholesterol, ApoB100 – apolipoprotein B100, ApoAI – apolipoprotein AI. Statistical tests used – T test for parametric data and Mann-Whitney for non-parametric data.

There was no significant difference between the LPO present in vitro at 0 hours in the mixtures containing LDL alone or LDL added to HDL, in the 2 groups (Figure 4.2). This means that HDL did not contribute any LPO to the incubation mixture at 0 hours.
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Figure 4.2 LPO at 0 hours in LDL or LDL+HDL mixtures in low PON1 activity and high PON1 activity groups. There is no significant difference in within group LPO formation at 0 hours. LPO – lipid peroxides, PON1 – paraoxonase 1.

LPO generated when HDL was incubated alone and together with LDL were significantly lower in the high PON1 group compared to low PON1 group {HDL = 14 ± 13 vs 25 ± 20 nmol/ml, P <0.03 and 34 ± 21 vs 50 ± 27 nmol/ml, P <0.02 respectively} (table 4.4).

<table>
<thead>
<tr>
<th>Mixture</th>
<th>Low PON1 activity lipid-peroxides (nmol/ml) (n=27)</th>
<th>High PON1 activity lipid-peroxides (nmol/ml) (n=26)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL</td>
<td>108 ± 48</td>
<td>92 ± 54</td>
<td>0.25</td>
</tr>
<tr>
<td>LDL+HDL</td>
<td>50 ± 27</td>
<td>34 ± 21</td>
<td>0.02</td>
</tr>
<tr>
<td>HDL</td>
<td>25 ± 20</td>
<td>14 ± 13</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Table 4.4 Lipid peroxides (LPO) generated at 3-0 hours on incubating LDL or LDL+HDL or HDL in low and high PON1 groups (in vitro). Values are in mean ± SD as data was parametric. Statistical test used – T test.
HDL functionality and LDL quality: the influence of obesity, obstructive sleep apnoea and pharmacological intervention

Total serum glycated apoB was significantly lower in the high PON1 group as compared to the low PON1 group (4.2 (3.0 – 4.9) vs 5.1 (3.4 – 5.9) mg/dl, p <0.03) and PON1 activity showed a negative correlation with total serum glycated apoB (n=53), Spearman’s r = -0.32, p<0.04. There was no significant difference in other factors that may affect serum glycated apoB concentration between low and high PON1 groups, including LDL-C, sdLDL apoB, HbA1c, FBG, insulin, HOMA β and HOMA-IR (table 4.5).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Low PON1 activity (n=27)</th>
<th>High PON1 activity (n=26)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PON1 activity (nmol/ml/min)</td>
<td>66 (54 – 80)</td>
<td>176 (161 – 214)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LDL-C (mmol/l)</td>
<td>3.4 (2.5 – 4.3)</td>
<td>3.3 (2.7 – 4.2)</td>
<td>0.9</td>
</tr>
<tr>
<td>sd LDL apoB (g/l)</td>
<td>0.27 (0.17 – 0.31)</td>
<td>0.26 (0.11 – 0.35)</td>
<td>0.7</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>6.1 (5.7 – 6.3)</td>
<td>6.0 (5.5 – 6.4)</td>
<td>0.8</td>
</tr>
<tr>
<td>FBG (mmol/l)</td>
<td>5.4 (4.9 – 6.1)</td>
<td>5.4 (4.9 – 6.0)</td>
<td>0.8</td>
</tr>
<tr>
<td>Insulin</td>
<td>12.1 (8.1 – 17.9)</td>
<td>11 (7.2 – 18.0)</td>
<td>0.7</td>
</tr>
<tr>
<td>HOMAβ</td>
<td>121.7 (77.7 – 177.0)</td>
<td>113.7 (64.4 – 171.0)</td>
<td>0.5</td>
</tr>
<tr>
<td>HOMA - IR</td>
<td>2.8 (2.2 – 5.6)</td>
<td>2.9 (1.7 – 4.6)</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Table 4.5 Comparing factors affecting glycation, in low and high PON1 groups. sdLDLapoB – small dense LDL apolipoprotein B, FBG – fasting blood glucose, HOMAβ – homeostatic model assessment of β cell function, HOMA IR - homeostatic model assessment of insulin resistance. Values are in median (interquartile range) as data was non-parametric. Statistical test used – Mann-Whitney.

There was no significant difference in factors that might affect PON1 activity between low and high PON1 groups, including age, gender, BMI, alcohol consumption, hormone replacement therapy in post-menopausal women, smoking, diabetes, menopause, thyroid disease, chronic renal or liver failure, apoA1, oxLDL, Lp-PLA2, use of aspirin or omega 3 fatty acids (table 4.6).
HDL functionality and LDL quality: the influence of obesity, obstructive sleep apnoea and pharmacological intervention

<table>
<thead>
<tr>
<th>Variable</th>
<th>Low PON1 activity (n=27)</th>
<th>High PON1 activity (n=26)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Factors that may increase PON1 activity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low alcohol consumption n (%)</td>
<td>18 (67)</td>
<td>14 (54)</td>
<td>0.4</td>
</tr>
<tr>
<td>HRT (n)</td>
<td>0/9</td>
<td>0/9</td>
<td></td>
</tr>
<tr>
<td>Aspirin use n (%)</td>
<td>11 (40)</td>
<td>11 (42)</td>
<td>0.9</td>
</tr>
<tr>
<td>Omega 3 fatty acids (n)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>ApoAI (g/l) (median)</td>
<td>1.4 (1.3 – 1.5)</td>
<td>1.3 (1.2 – 1.5)</td>
<td>0.6</td>
</tr>
<tr>
<td><strong>Factors that may decrease PON1 activity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking status n (%)</td>
<td>5 (19)</td>
<td>7 (27)</td>
<td>0.5</td>
</tr>
<tr>
<td>Type 2 diabetes n (%)</td>
<td>8 (30)</td>
<td>7 (26)</td>
<td>0.7</td>
</tr>
<tr>
<td>Type 1 diabetes (n)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>High alcohol consumption (n)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Menopause in females n (%)</td>
<td>9/14 (65)</td>
<td>9/14 (65)</td>
<td>1</td>
</tr>
<tr>
<td>Hypo or hyperthyroidism</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Chronic renal failure</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Chronic liver disease</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>oxLDL (mg/l)</td>
<td>14.5 (11.2 – 20.3)</td>
<td>14.8 (12.3 – 18.1)</td>
<td>0.9</td>
</tr>
<tr>
<td>LP-PLA2 (ng/ml)</td>
<td>721 ± 232</td>
<td>669 ± 245</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Factors that may increase or decrease PON1 activity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>53 ± 9</td>
<td>54 ± 12</td>
<td>0.8</td>
</tr>
<tr>
<td>Gender (M, F)</td>
<td>13, 14</td>
<td>12, 14</td>
<td>0.9</td>
</tr>
<tr>
<td>BMI (kg/m²) mean</td>
<td>31 (28 – 45)</td>
<td>33 (29 – 43)</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Table 4.6 Comparing factors known to affect PON1 activity, in low and high PON1 groups. Values are in mean ± SD or median (interquartile range). HRT – hormone replacement therapy, apoA1 – apolipoprotein A1, oxLDL – oxidised LDL, LP-PLA2 – lipoprotein phospholipase2. Statistical tests used – T test for parametric data and Mann-Whitney for non-parametric data.
4.4. Conclusion:

In statin treated dyslipidemic patients, HDL protects LDL against oxidation in vitro. In these patients the capacity of HDL to protect itself and LDL from oxidation in vitro is significantly better in individuals with higher serum PON1 activity. These results provide evidence that PON1 associated with HDL plays an important role in HDL’s in vitro anti-oxidant function. In the same cohort PON1 activity may also protect ApoB from glycation in vivo. The reasons for variation in PON1 activity amongst this group of well matched statin treated dyslipidemic patients is not evident and needs further research and exploration. PON1 activity may play a role in modifying residual risk in high risk populations through protecting LDL from glycation and oxidation. This needs further investigations.
CHAPTER FIVE: High Density Lipoprotein Antioxidant Function is Impaired in obese patients with Obstructive Sleep Apnoea

5.1. Introduction

LDL oxidation is an important atherogenic modification [21, 22]. OxLDL attracts monocytes to the site of atheroma formation, prevents macrophages from leaving the site and stimulates endothelial cells to become proinflammatory [48-50]. An early event in atherogenesis is the adhesion of monocytes to the endothelium [203, 204] via adhesion molecules such as (intercellular adhesion molecule 1) ICAM1 [205, 206]. Mast cells, neutrophils and macrophages release pro-inflammatory cytokines such as TNFα that induce expression of adhesion molecules in endothelium and recruitment of leukocytes, which is essential to the pathogenesis of vascular inflammatory diseases [360]. Another study through an in vitro model of trans-endothelial migration (TEM) showed that TNFα stimulated endothelium interacts with monocytes and this interaction plays an important role in the regulation of foam cell formation and oxidation of LDL in situ [361]. It has been suggested that the products of phospholipid peroxidation originating from oxLDL may propagate vascular inflammation by promoting increased production of TNFα and ICAM1 [362].

HDL-associated PON1 has been shown to have anti-oxidant and anti-inflammatory potential mainly by protecting phospholipids on the surface of HDL [113] and LDL from peroxidation [209, 210]. Most likely, these protective properties depend on the peroxidase and esterase activity of PON1 allowing the detoxification of oxidized molecules such as phospholipids and lipid hydroperoxides [211, 212]. PON1 accelerates hydrolysis of oxidized glycerophospholipids, such as phosphatidyl choline, which typically have fatty acyl hydroperoxide groups derived from linoleate in its Sn2 position and would lead to the production of lysophosphatidyl choline. This is potentially damaging to cell membranes. However, we know that it can safely be produced on HDL because substantial quantities are produced there by the activities of LCAT in esterifying-free cholesterol [4].
HDL-C is inversely associated with ICAM1 concentration in individuals with low plasma HDL-C [125]. Cockerill and co-workers incubated human umbilical vein endothelial cells (HUVEC) with HDL before and after stimulation of the HUVEC’s with TNFα. They showed that HDL is able to inhibit TNFα induced expression of ICAM1 [123]. Another study examined the ability of HDL from haemodialysis (HD) and continuous ambulatory peritoneal dialysis (CAPD) patients to suppress the expression of adhesion molecules in endothelial cells and in monocytes and to inhibit the uptake of oxidized LDL by macrophages. Based on the results the researchers proposed that decreased ability of HDL to suppress expression of adhesion molecules in endothelial cells and the uptake of oxidized LDL by macrophages can be one of the risk factors for atherosclerosis development. They suggested that this could be due to oxidative modification of HDL and the significantly reduced PON1 activity in these patients [363]. Loued and co-workers through the medium of in vitro studies demonstrated that purified PON1 (12.5 to 50 μg/mL) had a pro-inflammatory effect in the presence of oxLDL, increasing ICAM1 levels in Ea.hy926 cells by 33.0% and 40.6% (p<0.001) respectively, and had an anti-inflammatory effect in the presence of oxHDL, causing a 3-fold reduction in ICAM1 levels. PON1 also caused a significant decrease in TNFα and oxidized phospholipid induced ICAM1 expression [364]. This indicates PON1 associated with HDL is vital for its function. Aviram and colleagues have shown that oxidized LDL inactivates human PON1 in a time-dependent manner [365]. ApoM has also been proposed to assist HDL in its anti-oxidant and vasculo-protective functions [159, 160].

OSA refers to pauses in breathing during sleep due to narrowing of the upper airway despite continuing respiratory effort. Deposition of excessive adipose tissue around the upper airways in obese patients causes this narrowing of airway when they are in a supine position. Many studies have reported an independent correlation between presence and severity of sleep apnoea and cardiovascular disease or risk factors [182] like hypertension [183], coronary artery disease [184, 185] and stroke [186]. The chronic state of intermittent hypoxia due to sleep apnoea can lead to increased oxygen free radical stress [189] (hypoxia and reoxygenation cycles) and endothelial dysfunction [191]. This process may propagate atherogenesis.
Previous studies have shown increased lipid peroxidation in patients with sleep apnoea [197]. There is evidence that in patients with OSA, the HDL is dysfunctional [193] and the PON1 activity is reduced [196, 202].

**In summary** there are suggestions that oxidatively modified HDL has low PON1 activity and is unable to remove lipid peroxides originating from oxidized LDL, from the site of atheroma formation. This could lead to activation of monocytes and macrophages which release TNFα. TNFα promotes recruitment of monocytes from the circulation by inducing expression of adhesion molecules on the endothelial cell surface. It is these monocytes that convert to macrophages in the sub-endothelial space and engulf oxidized LDL to become foam cells. This may initiate the process of atheroma formation.

Obstructive Sleep apnoea is an independent risk factor for cardio-vascular disease when other factors have been accounted for. There is increased oxidative stress in patients with sleep apnoea and high apnoea-hypoapnoea index, which could modify HDL. **But the mechanisms that link oxidatively modified HDL with increased atherogenesis in these patients have not been elucidated so far.**

**Hypothesis:**

Patients with obstructive sleep apnoea may have dysfunctional HDL due to low PON1 activity and ApoM component of HDL. Dysfunctional HDL is less able to protect itself from *in vitro* oxidation and has reduced ability to protect the endothelium.

5.2. Methods

5.2.1. Subjects

Forty one patients with body mass index (BMI) > 40 kg/m² were recruited from Salford Royal Hospital NHS Foundation Trust obesity management clinic. All patients underwent overnight respiratory variable polysomnography to determine the AHI. OSA was diagnosed if AHI was ≥ 5/ hour. These patients were divided into two groups based on the presence or absence of OSA ("OSA" and "no OSA" group) in order to assess the effect of presence of OSA. These patients were also divided into two groups around the median AHI (high or low AHI groups) in order to assess the effect of severity of OSA.

5.2.2. Isolation of lipoproteins
Fasting blood samples were collected after participants had fasted from 10 p.m. the previous day. Serum and EDTA-plasma were isolated by centrifugation at 2000 × g for 15 min at 4°C within 2 hours of collection and were maintained at that temperature until further use. Serum was used for cholesterol, triglyceride and HDL-C assays and EDTA-plasma for apolipoprotein B100 (apoB). HDL was prepared by sequential density gradient ultracentrifugation without the addition of EDTA in a Beckman preparative M8-55M ultracentrifuge with a 50.4Ti fixed angle rotor using 13 × 64 mm polyallomer tubes at speeds of 34,000 rpm (144,361 × g) for 22 h [333, 359]. HDL density range 1.063-1.21 g/ml was isolated and the fraction dialyzed against TBS overnight at 4 °C. HDL protein concentration was determined using Bicinchoninic acid [338].

5.2.3. In vitro studies
Susceptibility of HDL to oxidation by copper in vitro was assessed immediately after isolation by incubating 0.25 mg protein/ml of the lipoprotein fraction with 5 µl copper sulphate (concentration) for 3 hours at 37°C in a Gallenkamp Economy Size 1 Incubator (Gallenkamp, Leicester, UK). LPO production was measured by spectrophotometry at 365 nmol at baseline and 3 hours [23]. Group comparisons were made between OSA and obese non-OSA controls and between OSA patients with AHI above and below the median.

5.2.4. Other laboratory analyses
Cholesterol and TG were determined using CHOD-PAP and GPO-PAP methods respectively, using reagents from ABX Horiba-UK, Northampton, UK. HDL-C was measured by a direct second generation homogeneous method (Roche Diagnostics, Burgess Hill, UK). LDL-C was estimated using Friedewald Formula. ApoB and apo A1 were assayed immunoturbidimetrically. A Cobas Mira auto-analyzer (ABX Horiba-UK, Northampton, UK) was employed for all these assays. Serum PON1 was determined by a semi-automated micro-titre plate method using paraoxon (O,O-Diethyl O-(4-nitrophenyl) phosphate) as a substrate and read by spectrophotometer at 405 nm [324]. Small dense LDL apoB concentration (d = 1.044 – 1.063 g/ml) was measured as previously described. HbA1c and fasting blood glucose were measured using the standard laboratory methods of the Department of Clinical Biochemistry, Central
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Manchester University Hospitals NHS Foundation Trust. Insulin was determined in plasma using Mercodia ELISA kits from Diagenics Ltd. Insulin sensitivity or HOMA $\beta$ was calculated using the formula $\text{HOMA } \beta = (20 \times \text{insulin})/(\text{glucose} – 3.5)$ [340]. HOMA-IR was calculated using the formula $\text{HOMA IR} = (\text{insulin} \times \text{glucose})/22.5$ [340]. TNF$\alpha$ and adiponectin were measured in serum, and ICAM1 in plasma all using DuoSet® ELISA development kits from R&D Systems (Abingdon, UK). Apolipoprotein M (Apo M) was assayed in serum using Bluegene E01A0522 kits from Hölzel Diagnostika Handels GmbH, Köln, Germany.

5.2.5. Statistical analyses
Results are expressed as mean ± SD for parametric data and median (interquartile range) for non-parametric data. Statistical significance of differences between groups was determined using the Student’s $t$-test (for parametric data) or Mann Whitney test (for non parametric data), taking $p< 0.05$ as statistically significant. Statistical significance of correlations was determined using Spearman’s test, taking $p< 0.05$ as statistically significant. The distribution or normality of data was determined using Kolmogorov-Smirnov test, D’Agastino and Pearson omnibus normality test and Shapiro-Wilk normality test on GraphPad Prism (GraphPad Software Inc., California, USA). SPSS statistical software version 16.0 (SPSS, Illinois, USA) was used for all comparisons and correlations. We did not use the multiple regression models to study the strength of relationships between various parameters in this study due to the relatively small number of patients.

5.3. Results:
5.3.1. Obese OSA and “no OSA” groups:
The two groups were matched for age, gender, smoking history, BMI, waist circumference, blood pressure, equivalent rosuvastatin dose and prevalence of diabetes, hypertension and cardiovascular disease (Table 5.1).
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<table>
<thead>
<tr>
<th>Clinical characteristic</th>
<th>Obese OSA group (n=29)</th>
<th>Obese no OSA group (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>48 ± 9</td>
<td>44 ± 10</td>
</tr>
<tr>
<td>Gender Male/Female (%)</td>
<td>15/85</td>
<td>30/70</td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>30</td>
<td>20</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>Cardiovascular disease (%)</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>Type 2 diabetes (%)</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Body Mass Index (kg/m²)</td>
<td>52 ± 7</td>
<td>49 ± 7</td>
</tr>
<tr>
<td>Waist circumference(cm)</td>
<td>142 ± 15</td>
<td>142 ± 20</td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>138 ± 21</td>
<td>141 ± 21</td>
</tr>
<tr>
<td>Diastolic BP (mm Hg)</td>
<td>76 ± 13</td>
<td>75 ± 10</td>
</tr>
<tr>
<td>Equivalent rosvastatin doses (mg)</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 5.1. Clinical characteristics of obese OSA and obese no OSA groups. Values are mean ± SD as all data was parametric. Statistical analyses – T test. There was no statistically significant difference between the two groups.

There was no significant difference in the number of patients on insulin or insulin sensitizers like metformin and glitazones. The median AHI was significantly higher in the OSA group compared to the no OSA group {13.5 (9.8 – 27.0)/hour vs 3.8 (2.2 – 4.2)/hour; p<0.0001}. There was no significant difference between the two groups in TC (5.0 vs 5.4 mmol/l), TG (1.6 vs 1.6 mmol/l), HDL-C (1.3 vs 1.3 mmol/l), LDL-C (3.1 vs 3.3 mmol/l), apoB (94 vs 107 mg/dl), apo1 (123 vs 128 mg/dl), HbA1c (6.1% vs 6%), HOMA β (203.9 vs 142.7) and HOMA-IR (6.2 vs 7.2) (Table 5.2). SdLDL apoB was significantly higher in OSA group compared to no OSA group (18.3 vs 11.7 mg/dl), p<0.05}.
HDL functionality and LDL quality: the influence of obesity, obstructive sleep apnoea and pharmacological intervention

<table>
<thead>
<tr>
<th>Biochemical characteristics</th>
<th>Obese OSA group (n=29)</th>
<th>Obese no OSA group (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apnoea hypo-apnoea index (episodes/hr)</td>
<td>13.5 (9.8 – 27.0)</td>
<td>3.8 (2.2 – 4.2)****</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.0 (4.5 – 5.6)</td>
<td>5.4 (4.9 – 6.1)</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.6 (1.2 – 1.9)</td>
<td>1.6 (1.2 – 2.2)</td>
</tr>
<tr>
<td>HDL-C (mmol/l)</td>
<td>1.3 (1.1 – 1.5)</td>
<td>1.3 (1.2 – 1.4)</td>
</tr>
<tr>
<td>LDL-C (mmol/l)</td>
<td>3.1 ± 1.2</td>
<td>3.3 ± 0.8</td>
</tr>
<tr>
<td>Apolipoprotein B100 (mg/dl)</td>
<td>94 (83 – 113)</td>
<td>107 (93 – 121)</td>
</tr>
<tr>
<td>Apolipoprotein A1 (mg/dl)</td>
<td>123 (113 – 145)</td>
<td>128 (120 – 135)</td>
</tr>
<tr>
<td>Sd LDL apoB (mg/dl)</td>
<td>18.3 (13.0 – 27.1)</td>
<td>11.7 (9.1 – 19.0) *</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>6.1 (5.7 – 6.6)</td>
<td>6.0 (5.6 – 7.3)</td>
</tr>
<tr>
<td>HOMA – IR</td>
<td>6.2 (4.3 – 10.5)</td>
<td>7.2 (3.8 – 11.2)</td>
</tr>
<tr>
<td>HOMA β</td>
<td>203.9 (139.3 – 384.2)</td>
<td>142.7 (87.6 – 215.4)</td>
</tr>
</tbody>
</table>

Table 5.2. Biochemical characteristics of obese OSA and obese no OSA groups. Values are mean ± SD or median (interquartile range). *p<0.05, *** p<0.0001. Statistical analyses – T test for parametric and Mann Whitney for non-parametric data. HDL-C – high density lipoprotein cholesterol, LDL-C – low density lipoprotein cholesterol, sdLDL apoB – small dense LDL apolipoprotein B100, HbA1c – glycated hemoglobin, HOMA-IR – homeostatic model of assessment – insulin resistance, HOMAβ – homeostatic model of assessment – insulin sensitivity.

The in vitro LPO levels at 3 hours on incubating HDL with copper in OSA group were significantly higher compared with the “no OSA” group {31 (15 – 38) vs 9 (2 – 19) nmol/ml; p = 0.005}(Figure 5.1).
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Figure 5.1. This figure demonstrates LPO generated at 3 hours on incubating HDL with copper. LPO levels in HDL from obese OSA group were significantly higher as compared to LPO generated in HDL from obese no OSA group at the end of 3 hours (p = 0.005). Statistical analyses – Mann Whitney test used as data was non-parametric. LPO – lipid peroxides

PON1 activity and apoM were significantly lower in the OSA group compared to the “no OSA” group {127 ± 73 vs 187 ± 76 nmol/ml/min; p<0.05 and 31 ± 11 vs 49 ± 23 mg/l, p<0.05 respectively} (Table 5.3). ApoA1 did not differ significantly between the OSA and “no OSA” group {123 (113 – 145) vs 128 (120 – 135) mg/dl}. PON1 activity was inversely correlated to AHI in the OSA group only (Spearman’s correlation coefficient = -0.41, p = 0.03).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Obese OSA group (n=29)</th>
<th>Obese no OSA group (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apolipoprotein A1 (mg/dl)</td>
<td>123 (113 – 145)</td>
<td>128 (120 – 135)</td>
</tr>
<tr>
<td>Paraoxonase1 activity (nmol/ml/min)</td>
<td>127 ± 73</td>
<td>187 ± 76*</td>
</tr>
<tr>
<td>Apolipoprotein M (mg/l)</td>
<td>31 ± 11</td>
<td>49 ± 23*</td>
</tr>
</tbody>
</table>

Table 5.3. Apolipoproteins and enzymes associated with HDL that promote HDL’s anti-oxidant capacity (in both groups). Values are mean ± SD or median (interquartile range). * p<0.05. Statistical analyses – T test for parametric and Mann Whitney for non-parametric data.
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TNFα and ICAM1 were significantly higher in the OSA group than in the “no OSA” group {66 (10 – 121) vs 15 (3 – 34) pg/ml; p<0.05 and 255 (234 – 271) vs 163 (151 – 185) ng/ml, p<0.0001 respectively} (Table 5.4). There was no significant difference in the levels of adiponectin between the two groups (OSA vs no OSA; 2.0 ± 0.8 vs 2.1 ± 0.7 mg/l).

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Obese OSA group (n=29)</th>
<th>Obese no OSA group (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα (pg/ml)</td>
<td>66 (10 – 121)</td>
<td>15 (3 – 34)*</td>
</tr>
<tr>
<td>ICAM-1 (ng/ml)</td>
<td>255 (234 – 271)</td>
<td>163 (151 – 185)**</td>
</tr>
<tr>
<td>Adiponectin (mg/l)</td>
<td>2.0 ± 0.8</td>
<td>2.1 ± 0.7</td>
</tr>
</tbody>
</table>

Table 5.4. Cytokines and adhesion molecules in both groups. Values are mean ± SD or median (interquartile range). * p<0.05, ***P<0.0001. Statistical analyses – T test for parametric and Mann Whitney for non-parametric data. TNFα – tumor necrosis factor alpha, ICAM-1 – intercellular adhesion molecule 1.

5.3.2. Low and high AHI groups:

The two groups were well matched for age, gender, smoking history, body mass index (BMI), waist circumference, blood pressure, equivalent rosuvastatin dose and prevalence of diabetes, hypertension and cardiovascular disease (Table 5.5).
HDL functionality and LDL quality: the influence of obesity, obstructive sleep apnoea and pharmacological intervention

<table>
<thead>
<tr>
<th>Clinical characteristic</th>
<th>High AHI group (n=20)</th>
<th>Low AHI group (n=21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>49 ± 10</td>
<td>45 ± 9</td>
</tr>
<tr>
<td>Gender Male/Female (%)</td>
<td>15/85</td>
<td>20/80</td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>30</td>
<td>20</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>65</td>
<td>50</td>
</tr>
<tr>
<td>Cardiovascular disease (%)</td>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td>Type 2 diabetes (%)</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Body Mass Index (kg/m²)</td>
<td>52 ± 6</td>
<td>50 ± 8</td>
</tr>
<tr>
<td>Waist circumference(cm)</td>
<td>143 ± 14</td>
<td>140 ± 18</td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>139 ± 23</td>
<td>139 ± 19</td>
</tr>
<tr>
<td>Diastolic BP (mm Hg)</td>
<td>76 ± 13</td>
<td>75 ± 10</td>
</tr>
<tr>
<td>Statin use (%)</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Equivalent rosuvastatin doses (mg)</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 5.5. Clinical characteristics of low AHI and high AHI groups. Values are mean ± SD as all data was parametric. Statistical analyses – T test. There was no statistically significant difference between the two groups.

There was no significant difference in the number of patients on insulin or insulin sensitizers like metformin and glitazones. There was no significant difference between the high and low AHI groups in total cholesterol (TC) (4.8 vs 5.3 mmol/l), TG (1.7 vs 1.6 mmol/l), HDL-C (1.3 vs 1.3 mmol/l), LDL-C (3.2 vs 3.2 mmol/l), apoB (90 vs 102 mg/dl), apo1 (121 vs 129 mg/dl), sdLDL apoB (17.3 vs 16.5 mg/dl), HbA1c (6.2% vs 6%), HOMA β (198.6 vs 176.6) and HOMA-IR (6.6 vs 6.9) respectively (Table 5.6).
HDL functionality and LDL quality: the influence of obesity, obstructive sleep apnoea and pharmacological intervention

<table>
<thead>
<tr>
<th>Biochemical characteristics</th>
<th>High AHI group (n=20)</th>
<th>Low AHI group (n=21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apnoea hypo-apnoea index (episodes/hr)</td>
<td>21.3 (13.5 – 45.7)***</td>
<td>4.3 (3.7 – 6.8)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>4.8 (4.5 – 5.5)</td>
<td>5.3 (4.7 – 5.7)</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.7 (1.2 – 2.0)</td>
<td>1.6 (1.1 – 2.1)</td>
</tr>
<tr>
<td>HDL-C (mmol/l)</td>
<td>1.3 (1.1 – 1.5)</td>
<td>1.3 (1.2 – 1.5)</td>
</tr>
<tr>
<td>LDL-C (mmol/l)</td>
<td>3.2 ± 1.3</td>
<td>3.2 ± 0.8</td>
</tr>
<tr>
<td>Apolipoprotein B100 (mg/dl)</td>
<td>90 (81 – 106)</td>
<td>102 (89 – 123)</td>
</tr>
<tr>
<td>Apolipoprotein A1 (mg/dl)</td>
<td>121 (113 – 136)</td>
<td>129 (122 – 138)</td>
</tr>
<tr>
<td>Sd LDL apoB (mg/dl)</td>
<td>17.3 (10.9 – 31.4)</td>
<td>16.5 (11.2 – 22.6)</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>6.2 (5.7 – 6.7)</td>
<td>6.0 (5.6 – 6.9)</td>
</tr>
<tr>
<td>HOMA – IR</td>
<td>6.6 (4.3 – 10.7)</td>
<td>6.9 (4.0 – 10.2)</td>
</tr>
<tr>
<td>HOMA – B</td>
<td>198.6 (95.5 – 390.4)</td>
<td>176.6 (104.7 – 299.9)</td>
</tr>
</tbody>
</table>

Table 5.6. Biochemical characteristics of low and high AHI groups. Values are mean ± SD or median (interquartile range). *** p<0.0001. Statistical analyses – T test for parametric and Mann Whitney for non-parametric data. HDL-C – high density lipoprotein cholesterol, LDL-C – low density lipoprotein cholesterol, sdLDL apoB – small dense LDL apolipoprotein B100, HbA1c – glycated hemoglobin, HOMA-IR – homeostatic model of assessment – insulin resistance, HOMAβ – homeostatic model of assessment – insulin sensitivity.

LPO at 3 hours on incubating HDL with copper was higher in the high AHI group than in the low AHI group {30 (14 – 43) vs 17 (6 – 31) nmol/ml; p < 0.05} (Figure 5.2).
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Figure 5.2. This figure demonstrates LPO generated at 3 hours on incubating HDL with copper. LPO levels in HDL from high AHI group were significantly higher as compared to LPO generated in HDL from low AHI group at the end of 3 hours (p < 0.05). Statistical analyses – Mann Whitney test used as data was non-parametric. LPO – lipid peroxides.

PON1 activity was significantly lower in the high AHI group compared with the low AHI group (101 ± 64 vs 186 ± 68 nmol/ml/min; p<0.0001). ApoA1 and apoM were lower in the high AHI group compared with the low AHI group but the difference was not statistically significant {121 (113 – 136) vs 129 (122 – 138) mg/dl; p = 0.3 and 31 (25 – 34) vs 37 (26 – 50) mg/l; p = 0.2 respectively} (Table 5.7).

<table>
<thead>
<tr>
<th>Protein</th>
<th>High AHI group (n=20)</th>
<th>Low AHI group (n=21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apolipoprotein A1 (mg/dl)</td>
<td>121 (113 – 136)</td>
<td>129 (122 – 138)</td>
</tr>
<tr>
<td>Paraoxanase1 activity (nmol/ml/min)</td>
<td>101 ± 64***</td>
<td>186 ± 68</td>
</tr>
<tr>
<td>Apolipoprotein M (mg/l)</td>
<td>31 (25 – 35)</td>
<td>37 (26 – 50)</td>
</tr>
</tbody>
</table>

Table 5.7. Apolipoproteins and enzymes associated with HDL that promote HDL’s anti-oxidant capacity (in both groups). Values are mean ± SD or median (interquartile range). *** p<0.0001. Statistical analyses – T test for parametric and Mann Whitney for non-parametric data.
TNFα and ICAM1 were significantly higher in the high AHI group compared with the low AHI group (87 (12.4 – 133.8) vs 15.5 (7.2 – 38.2) pg/ml; p<0.05 and 267 (237 – 280) vs 218 (157 – 245) ng/ml, p<0.0001 respectively) (Table 5.8). There was no significant difference in the levels of adiponectin between the two groups (high AHI vs low AHI; 2.1 ± 0.8 vs 2.0 ± 0.7 mg/l; p =0.5).

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>High AHI group (n=20)</th>
<th>Low AHI group (n=21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα (pg/ml)</td>
<td>87.2 (12.4 – 133.8)*</td>
<td>15.5 (7.2 – 38.2)</td>
</tr>
<tr>
<td>ICAM-1 (ng/ml)</td>
<td>267 (237 – 280)**</td>
<td>218 (157 – 245)</td>
</tr>
<tr>
<td>Adiponectin (mg/l)</td>
<td>2.1 ± 0.8</td>
<td>2.0 ± 0.7</td>
</tr>
</tbody>
</table>

Table 5.8. Cytokines and adhesion molecules in both groups. Values are mean ± SD or median (interquartile range). * p<0.05, **P=0.01. Statistical analyses – T test for parametric and Mann Whitney for non-parametric data. TNFα – tumor necrosis factor alpha, IL6 – interleukin 6, ICAM-1 – intercellular adhesion molecule 1.

5.4 Conclusion:
The capacity of HDL to protect itself from in vitro oxidation is reduced in morbidly obese patients with sleep apnoea compared to matched patients without sleep apnoea. This function of HDL diminishes with increasing severity of sleep apnoea. The significantly lower PON1 activity and apoM in the patients with increasing severity of sleep apnoea may result in reduced in vitro anti-oxidant function of HDL. PON 1 activity is negatively related to AHI only in the obese OSA group. Significantly higher TNFα and ICAM1 in the obese OSA group suggest endothelial dysfunction. It is possible that oxidative damage to PON1 in OSA may play a part in both HDL and endothelial dysfunction.
CHAPTER SIX: Extended release niacin lowers mediators of vascular inflammation but does not improve \textit{in vitro} HDL antioxidant function in statin treated dyslipidemic patients

6.1 Introduction

There is increasing evidence that apoB measurements may be of value in clinical care \cite{6, 7}. Also it has been established that oxLDL plays a key role in promoting atherosclerosis \cite{21, 22}. Lp-PLA2 circulates primarily in association with LDL \cite{366} and it has been shown that Lp-PLA2 activity and mass show continuous associations with risk of CHD, similar in magnitude to that of non-HDL cholesterol or systolic blood pressure \cite{62}. To analyse the role of apoB in the formation of the Lp-PLA2-LDL complex, Stafforini \textit{et al} tested the ability of Lp-PLA2 to bind to lipoproteins containing truncated forms of apoB. This study indicated that the carboxyl terminus of apoB played a key role in the association of Lp-PLA2 acetylhydrolase with LDL \cite{367}. LDL oxidation activates Lp-PLA2, which catalyses the hydrolysis of oxidized PC to lyso-PC and oxNEFA on the surface of oxLDL \cite{64, 65}. Lyso-PC has been shown to mediate the pro-inflammatory and pro-atherogenic effects of activated Lp-PLA2 \cite{66, 368}. The associations among Lp-PLA2, lyso-PC and proinflammatory cytokines like MCP1 in human plaques suggest that lyso-PC plays a key role in plaque inflammation and vulnerability \cite{369}. An \textit{in vitro} study demonstrated that lyso-PC could induce an increase in MCP-1 mRNA concentrations and stimulate the release of MCP-1 protein from HUVEC \cite{370}. In another \textit{in vitro} study lyso-PC stimulated MCP-1 expression at the transcriptional level in rat vascular smooth muscle cells, suggesting a molecular mechanism by which lyso-PC contributed to the atherogenicity of oxLDL \cite{371}.

Niacin has been shown to significantly reduce apoB in statin treated patients compared to placebo \cite{294}. In animal studies, it has been suggested that treatment with niacin may protect endothelial function by lowering oxLDL and down regulating the oxLDL/LOX-1 pathway \cite{372}. A placebo controlled study outcome suggested that in humans’ treatment with niacin led to significantly greater reduction in Lp-PLA2 \cite{250}. In another study it was shown that niacin was able to markedly decrease MCP1 levels in New Zealand white rabbits that had a periarterial carotid collar implanted to promote vascular
inflammation and endothelial dysfunction [373]. The addition of niacin to TNFα treated 3T3-L1 adipocytes attenuated expression of MCP1 in an in vitro study [248]. Similar results were also seen in another in vitro study on cultured human aortic endothelial cells [245, 247]. In summary LDL apoB, oxLDL, Lp-PLA2, lyso-PC and MCP1 are important constituents of a pro-atherogenic pathway and niacin has been shown to lower these parameters in different studies separately. I examined the effect of ERN/LRP on apoB, oxLDL, Lp-PLA2, lyso-PC and MCP1 as part of one single study as this has not been done previously in either animals or humans. Also past studies have shown reduction in CRP levels with niacin treatment [250, 374] but none have looked at the effect of ERN/LRP on SAA which is another systemic inflammatory marker that has been linked to cardiovascular disease (CVD) [168, 169]. Therefore I looked at the effect of ERN/LRP on SAA.

In 1975, Miller and Miller [102] reported an inverse association between plasma HDL-C concentration and CHD. It has since been postulated that HDL-C may be an independent predictor of cardiovascular events in statin-treated patients at all levels of LDL-C [104]. This is likely to be related to the diverse properties of HDL including reverse cholesterol transport, anti-inflammatory, antioxidant, antiglycation, antithrombotic, nitric oxide – inducing and antimicrobial activities [4, 105-109]. However, raising HDL-C levels has not always led to reduced cardio-vascular risk (CVR) [294, 375]. Improving HDL’s functions like its anti-oxidant capacity by therapeutic means may be more important than its cholesterol cargo. Therefore I have assessed the effect of ERN/LRP on LDL oxidizability and HDL’s in vitro anti-oxidant function in humans for the first time. I have also tried to establish the effect of ERN/LRP on various enzymes and apolipoproteins associated with HDL that play a role in HDL’s anti-oxidant function like apoA1, PON1 and apoM. Moreover I have evaluated the effect of ERN/LRP on another pro-oxidant MPO.

CETP mediates the transfer of CE from HDL and LDL to TG rich VLDL in exchange for TGs. This lipid exchange promotes the generation of smaller particles with higher density which can be pro-atherogenic [4]. Interest in the inhibition of CETP was stimulated by a 1985 publication describing a Japanese population in which a low-activity genetic variant of CETP was associated with elevated levels of HDL-C and
relatively low rates of cardiovascular disease [376]. HDL particles deliver their cholesterol load to the liver directly, via scavenger receptor B-I (SR-BI) or indirectly, via transfer to VLDL or LDL particles by the action of CETP [377]. These apoB-containing lipoproteins are taken up by the liver via the LDL-receptor [378]. It has been suggested that the transfer of cholesterol to VLDL and LDL by CETP may, in fact, be pro-atherogenic [4, 379]. On the other hand, a genetic deficiency of CETP would be expected to impair this cholesterol transport but increase HDL-C levels [376]. Previous studies have demonstrated that niacin reduces CETP activity [233-235] but for the first time I have studied the effect of ERN/LRP on CETP. In previous small studies it was suggested that treatment of dyslipidaemia with niacin might reduce cardiovascular risk [228, 229] but this was not seen in a more recent larger study [294] despite significant elevation of HDL-C achieved by ERN. Flushing has been a major cause of lack of compliance with immediate release niacin and ERN [254, 380]. Laropiprant (LRP) when combined with ERN effectively blocks flushing and improves compliance [260, 261]. Several randomised controlled trials have previously examined the effects of ERN/LRP combined with low dose statins in dyslipidaemic patients. I investigated the influence of ERN/LRP versus placebo in patients who had persistent dyslipidemia despite receiving high doses of potent statins as this more accurately reflected actual clinical practice. I have looked at the influence of ERN/LRP on various pro and anti-atherogenic properties of lipoproteins.

**Hypothesis**

Treatment with ERN/LRP will lead to a significant improvement in HDL-C and reduction in pro-atherogenic lipoproteins/apolipoproteins in patients who have persistent dyslipidaemia despite high doses of potent statins. Treatment with ERN/LRP will reduce mediators of vascular inflammation and improve HDL’s *in vitro* anti-oxidant function in these patients.

**6.2 Patients and Methods**

**6.2.1 Patients**

Patients were recruited from Central Manchester University Hospitals NHS Foundation Trust and University Hospital of South Manchester. Recruitment started in October
2010 and ended in May 2011. The study was completed in February 2012. Informed consent was obtained from all patients. The study was approved by local ethics committee. All the study patients were seen in the Cardiovascular Trials Unit at Manchester Royal Infirmary. 36 patients were recruited and 27 patients completed the study. 9 patients were lost to follow up.

6.2.2 Inclusion & exclusion criteria
Men and women aged 20-75 years with hyperlipidaemia on lipid lowering treatment (maximum tolerated statins and/or ezetimibe) but not achieving NCEP ATP III target of low density lipoprotein cholesterol (LDL-C) < 1.8 mmol/l (70 mg/l) were recruited. Patients were excluded if they were pregnant and/or breast-feeding, if they had significant renal impairment (chronic kidney disease stage 3 or more advanced: eGFR ≤ 59), alanine aminotransferase > 1.5 ULN, if they were receiving fibrates and/or Omacor, if they were allergic to niacin and if they had active peptic ulcer disease.

6.2.3 Method
The trial medication Tredaptive® (ERN/LRP) and image-matched placebo were supplied by Merck, Sharp & Dohme Ltd. LRP is an agent that reduces the flushing side effect of ERN.

Study patients were asked to attend for 5 visits (figure 6.1). Patients who fulfilled inclusion and exclusion criteria were invited for screening visit when informed consent was obtained. At the first visit, patients taking both statin and ezetimibe were asked to stop ezetimibe. Ezetimibe was specifically stopped in order to evaluate the effect of adding ERN/LRP to statin treated patients with persistent dyslipidemia and also to avoid any potential bias arising from the effect of multiple drugs.

Equivalent rosuvastatin doses were calculated by equating 40 mg simvastatin and 10 mg atorvastatin to 5 mg rosuvastatin [322, 323]. All patients had 4 weeks of receiving placebo (placebo run-in period). Patients attended for the next study visit at the end of the placebo period and were randomised to either placebo or ERN/LRP for 12 weeks (treatment period). During the treatment period, patients randomised to study medication received ERN/LRP 1g/20mg for 4 weeks followed by increased dose of ERN/LRP 2g/40mg for 8 weeks. After the first treatment period all patients repeated the placebo period (4 weeks) before the second treatment period (12 weeks). As before, they
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attended for visits at the end of the placebo period, and at the end of the treatment period. Patients who were randomised to placebo in the first treatment period received ERN/LRP in the second treatment period and vice versa. Fasting blood samples were taken at all the study visits except the screening visit. Concordance with medication was assessed by pill count at each visit.

![Study overview](image)

Figure 6.1 Study overview. Patients randomised to placebo during the first period received ERN/LRP in the second period and vice versa. In each period the ERN/LRP dose was increased from 1g/20mg to 2g/40mg after 4 weeks.

The Clinical Trials Pharmacist was responsible for the randomisation procedure. Block randomisation was carried out with equal numbers of participants per group using a computer generated list for this cross over trial. Patients and investigators were blinded to individual assignments and to the randomisation procedure.
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6.2.4 Endpoints

**Primary outcome:** To determine whether treatment with ERN/LRP leads to an increase in HDL-C compared with placebo in statin treated dyslipidaemic patients. A difference of 15% between the groups was defined as clinically significant.

**Secondary endpoints:** To find out whether treatment with ERN/LRP leads to a statistically significant decrease in LDL-C or TC or TG or non HDL cholesterol (non-HDL-C) compared to placebo in these patients.

**Descriptive analysis:** I also studied the effect of ERN/LRP on: non-HDL cholesterol, VLDL-C, VLDL-TG, apoAI, PON1, apoM, apoB, VLDL apoB, LDL apoB, buoyant LDL apoB, sdLDL apoB, total glycated apoB (gly apoB), oxLDL, Lp(a), Lp-PLA2, lyso-PC, fasting blood glucose (FBG), HbA1c, insulin, HOMA – IR, HOMA β, MCP1, MPO, SAA, HDL in vitro anti-oxidant function

6.2.5 Sample size & Power calculation

The sample size calculation was based on the primary outcome (change in HDL-C). In clinical trials niacin therapy has been associated with over 20% increase in HDL-C [4] therefore I would expect the majority (more than 90%) of patients to achieve 15% increase in HDL-C. I used these estimates to calculate sample size in Stata 11 statistical software (College Station, TX). Assuming that the mean HDL-C in patients we were planning to study was 1.2±0.2mmol/l (mean ± SD), I expected that with treatment this mean value would be increased by 15% (to 1.38mmol/l) with treatment. To achieve this difference with 90% statistical power at p<0.02, and for a paired test with subjects acting as their own controls (cross-over design); I concluded I will require 26 participants in each group. I planned to recruit 38 patients to allow for drop-outs.

6.2.6 Statistical analyses

The distribution or normality of data was determined using Kolmogorov-Smirnov test, D’Agastino and Pearson omnibus normality test and Shapiro-Wilk normality test on GraphPad Prism (GraphPad Software Inc., California, USA). Results are expressed as mean ± standard deviation (SD) for parametric data and median (interquartile range) for non-parametric data. Statistical significance of differences between groups was determined using the Student’s t-test (for parametric data) or Mann Whitney test (for non parametric data), taking p< 0.05 as statistically significant. Statistical significance
of correlations was determined using Spearman’s test, taking \( p < 0.05 \) as statistically significant. Stats direct (Stats direct, Altrincham, Cheshire, UK) was used to calculate any significant differences in period effect or treatment-period interaction. Level of significance in the results has been described as treatment effect if there was a significant difference in either period effect or treatment-period interaction. If there was no significant difference in either period effect or treatment-period interaction, then the level of significance in the results has been described as relative effectiveness of drug/placebo.

6.2.7 Isolation of lipoproteins

Blood samples were collected between 9 and 11 a.m. after participants had fasted from 10 p.m. the previous day. Serum and EDTA-plasma were isolated by centrifugation at 2000 × \( g \) for 15 min at 4°C within 2 h of collection and were maintained at that temperature until further use.

Lipoprotein fractions were prepared by sequential density gradient ultracentrifugation without the addition of EDTA in a Beckman preparative M8-55M ultracentrifuge with a 50.4Ti fixed angle rotor using 13 × 64 mm polyallomer tubes at speeds of 34,000 rpm (144,361 × \( g \)) for 22 h [333, 359]. Supernatant LDL, density range 1.019-1.063g/ml and supernatant HDL density range 1.063-1.21g/ml were isolated and the fractions dialyzed against TBS overnight at 4°C. LDL and HDL protein concentration were determined using Bicinchoninic acid [338].

6.2.8 In vitro studies

Susceptibility of lipoprotein fractions to oxidation by copper in vitro was assessed immediately after isolation by incubating 0.25 mg protein/ml of lipoprotein fractions with 5 µl copper sulphate (concentration) for 3 hours at 37°C in a Gallenkamp Economy Size 1 Incubator (Gallenkamp, Leicester, UK). Lipid peroxides (LPO) production was measured by spectrophotometer at 365 nmol at baseline and 3 hours [23]. LDL and HDL were incubated separately and together in this system.

6.2.9 Other laboratory analyses

Cholesterol and TG were determined using CHOD-PAP and GPO-PAP methods respectively, with reagents from ABX Horiba-UK, Northampton, UK. HDL-C was measured by a direct second generation homogeneous method (Roche Diagnostics,
Burgess Hill, UK). LDL-C was estimated using Friedewald Formula. ApoB and apo A1 were assayed immunoturbidimetrically. A Cobas Mira auto-analyzer (ABX Horiba-UK, Northampton, UK) was employed for all these assays. Serum paraoxonase 1 activity (PON1) was determined by a semi-automated micro-titre plate method using paraoxon (\(O,O\)-Diethyl \(O\)-(4-nitrophenyl) phosphate) as a substrate and read by spectrophotometer at 405 nm [324].

Sd LDL apoB concentration (\(d = 1.044 – 1.063\) g/ml) was determined as previously described. HbA1c and fasting blood glucose were measured using the standard laboratory methods of the Department of Clinical Biochemistry, Central Manchester University Hospitals NHS Foundation Trust. Ox-LDL was measured in plasma by a direct ELISA sandwich technique with a kit from Merckodia (Upsala, Sweden). Total glycerol apoB was assayed in plasma using Glyacor ELISA kits from bioGnosis Ltd, Hailsham, UK. Insulin was determined in plasma using Mercodia ELISA kits from Diagenics Ltd. Lp-PLA2 mass was determined in plasma using USCN Lifescience ELISA kits from Hölzel Diagnostika Handels GmbH. Lyso-PC was measured in serum using Azwell lyso-PC enzymatic assay kits from Cosmo Bio, Japan. MCP-1 was measured in serum using DuoSet® ELISA development kits from R&D Systems (Abingdon, UK). MPO was determined in plasma by ELISA using kits from R&D Systems Ltd. SAA was assayed by ELISA using kits from Invitrogen Ltd, Paisley, UK. HOMA \(\beta\) was calculated using the formula \(\text{HOMA } \beta = (20 \times \text{insulin mU/l})/\text{(glucose mmol/l–3.5)}\). HOMA-IR was calculated using the formula \(\text{HOMA IR} = (\text{insulin mU/l X glucose mmol/l})/22.5\). Apo M was assayed in serum using Bluegene E01A0522 kits from Hölzel Diagnostika Handels GmbH, Köln, Germany. (Lp(a) was determined using ELISA kits from Diagenics Ltd. CETP activity was measured using a fluorometric method using assay kits (ab65383) from Abcam, UK.

### 6.3 Results

In this study patients acted as their own controls and twenty seven patients completed all five visits. Two thirds of these patients were males. 10% were current smokers and 10% had diabetes. Nearly half of the patients had hypertension, history of ischaemic heart disease and heterozygous familial hypercholesterolemia. The average BMI was 31kg/m\(^2\).
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and the average of equivalent rosuvastatin doses was 35mg (simvastatin 40mg = atorvastatin 10mg = rosuvastatin 5mg) [322, 323] (table 6.1).

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>57 ± 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (M, F)</td>
<td>18, 9</td>
</tr>
<tr>
<td>Smoker n (%)</td>
<td>3 (10)</td>
</tr>
<tr>
<td>Type 2 diabetes n (%)</td>
<td>3 (10)</td>
</tr>
<tr>
<td>Hypertension n (%)</td>
<td>13 (48)</td>
</tr>
<tr>
<td>Known IHD n (%)</td>
<td>11 (40)</td>
</tr>
<tr>
<td>HeFH n (%)</td>
<td>11 (40)</td>
</tr>
<tr>
<td>BMI (kg/m²) mean</td>
<td>31 ± 4</td>
</tr>
<tr>
<td>Systolic BP mean (mmHg)</td>
<td>135 ± 14</td>
</tr>
<tr>
<td>Diastolic BP mean (mmHg)</td>
<td>76 ± 10</td>
</tr>
<tr>
<td>Statin type (R, A, S)</td>
<td>15, 11, 1</td>
</tr>
<tr>
<td>Equivalent rosuvastatin doses (mg)</td>
<td>35 ± 12</td>
</tr>
<tr>
<td>Hormone replacement therapy in post menopausal females n (%)</td>
<td>1/7 (15)</td>
</tr>
</tbody>
</table>


6.3.1 Endpoints

6.3.1.1 In vivo: Treatment with ERN/LRP was associated with a 15% improvement in HDL-C levels compared to placebo (1.5 vs 1.3 mmol/l, p<0.0001). ERN/LRP therapy compared to placebo resulted in significant reductions in TC (5.1 vs 5.7 mmol/l, p = 0.005), TG (1.0 vs 1.5 mmol/l, p = 0.01), LDL-C (2.6 vs 3.3 mmol/l, p = 0.01), non-HDL-C (3.4 vs 4.2 mmol/l, p = 0.0002), VLDL-TG (0.6 vs 0.7 mmol/l, 0.007), total apoB (99 vs 128 mg/dl, p < 0.0001), apoB/apoAI ratio (0.8 vs 1.0, p = 0.001) and Lp(a) (199 vs 286 U/l, p < 0.0001) (table 6.2 and 6.3). The reduction in total apoB was mainly...
due to a significant reduction in buoyant LDL apoB (67.8 vs 89.1 mg/dl, p = 0.0006). There was a small reduction in sdLDL apoB with ERN/LRP treatment (15.8 vs 17.9 mg/dl, p = 0.2) but this did not reach significance (table 6.3). CETP activity was also significantly reduced after ERN/LRP treatment (19.8 vs 26.1 nmol/ml/hr, p=0.02) (table 6.2).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Post placebo (n=27)</th>
<th>Post ERN/LRP (n=27)</th>
<th>p value for treatment effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDL-C (mmol/l)</td>
<td>1.3 (1.1 – 1.6)</td>
<td>1.5 (1.2 – 1.7)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Total Cholesterol (mmol/l)</td>
<td>5.7 (4.7 – 6.6)</td>
<td>5.1 (4.1 – 6.6)</td>
<td>0.005</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.5 (1.1 – 2.2)</td>
<td>1.0 (0.8 – 1.7)</td>
<td>0.01</td>
</tr>
<tr>
<td>LDL-C (mmol/l)</td>
<td>3.3 (2.6 – 4.4)</td>
<td>2.6 (2.1 – 4.2)</td>
<td>0.01</td>
</tr>
<tr>
<td>Non-HDL cholesterol (mmol/l)</td>
<td>4.2 (3.3 – 5.2)</td>
<td>3.4 (2.7 – 4.6)</td>
<td>0.0002</td>
</tr>
<tr>
<td>VLDL-TC (mmol/l)</td>
<td>0.5 (0.3 – 0.7)</td>
<td>0.3 (0.2 – 0.7)</td>
<td>0.08</td>
</tr>
<tr>
<td>VLDL-TG (mmol/l)</td>
<td>0.7 (0.6 – 1.5)</td>
<td>0.6 (0.4 – 1.1)</td>
<td>0.007</td>
</tr>
<tr>
<td>VLDL-TG/TC ratio</td>
<td>1.8 (1.2 – 2.5)</td>
<td>1.9 (1.4 – 2.4)</td>
<td>0.9</td>
</tr>
<tr>
<td>ApoB/ApoAI ratio</td>
<td>1.0 (0.7 – 1.1)</td>
<td>0.8 (0.6 – 0.9)</td>
<td>0.001</td>
</tr>
<tr>
<td>CETP activity (nmol/ml/hr)</td>
<td>26.1 (19.6 – 32.3)</td>
<td>19.8 (16.4 – 25.7)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Table 6.2 Basic lipoprotein results on treatment with ERN/LRP and placebo. Values are in median (interquartile range). Mann Whitney test used as all data non-parametric. HDL-C – high density cholesterol, LDL-C – low density lipoprotein cholesterol, VLDL-TC – very low density lipoprotein total cholesterol, VLDL-TG – very low density lipoprotein triglycerides, ERN – extended release niacin, LRP – laropiprant, CETP – cholesteryl ester transport protein.
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<table>
<thead>
<tr>
<th>Variable</th>
<th>Post placebo (n=27)</th>
<th>Post ERN/LRP (n=27)</th>
<th>p value for treatment effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total ApoB (mg/dl)</td>
<td>128 (99 – 158)</td>
<td>99 (83 – 121)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>VLDL ApoB (mg/dl)</td>
<td>10.7 (7.3 – 16.9)</td>
<td>8.6 (6.0 – 11.8)</td>
<td>0.08</td>
</tr>
<tr>
<td>LDL ApoB (mg/dl)</td>
<td>110.5 (90.2 – 140.4)</td>
<td>96.0 (75.2 – 113.3)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Buoyant LDL ApoB (mg/dl)</td>
<td>89.1 (70.8 – 118.8)</td>
<td>67.8 (61.3 – 98.0)</td>
<td>0.0006</td>
</tr>
<tr>
<td>sdLDL ApoB (mg/dl)</td>
<td>17.9 (10.4 – 22.5)</td>
<td>15.8 (9.7 – 25.7)</td>
<td>0.2</td>
</tr>
<tr>
<td>Total gly apoB (mg/dl)</td>
<td>1.8 (1.3 – 3.1)</td>
<td>1.5 (1.1 – 2.7)</td>
<td>0.4</td>
</tr>
<tr>
<td>oxLDL (mg/dl)</td>
<td>1850 (1330 – 2515)</td>
<td>1540 (1035 – 2295)</td>
<td>0.01</td>
</tr>
<tr>
<td>gly apoB/ LDL ApoB (%)</td>
<td>1.6%</td>
<td>1.5%</td>
<td></td>
</tr>
<tr>
<td>oxLDL/LDL ApoB (ratio)</td>
<td>16.7</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Lp(a) (U/l)</td>
<td>286 (123 – 928)</td>
<td>199 (66 – 726)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Lp-PLA2 (ng/ml)</td>
<td>730 (589 – 813)</td>
<td>509 (398 – 657)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Lyso-PC (µmol/l)</td>
<td>285.5 (262.5 – 354.0)</td>
<td>257.2 (230.7 – 335.6)</td>
<td>0.007</td>
</tr>
</tbody>
</table>

Table 6.3 Pro-atherogenic lipoproteins, apo-lipoproteins and enzymes on treatment with ERN/LRP and placebo. Values are in median (interquartile range). Mann Whitney test used as all data non-parametric. ApoB – apolipoprotein B, VLDL – very low density lipoprotein, LDL – low density lipoprotein, sdLDL – small dense LDL, oxLDL – oxidized LDL, gly – glycated, Lp(a) – lipoprotein(a), Lp-PLA2 – lipoprotein associated phospholipase A2

There was a significant reduction of ox LDL (1540 vs 1850 mg/dl, p = 0.01), Lp-PLA2 (509 vs 730 ng/ml, p<0.0001), lyso-PC (257.2 vs 285.5 µmol/l, p = 0.007), MCP1 (188.4 vs 205.6 pg/ml, p = 0.01) and SAA (16.2 vs 17.6 mg/l, p = 0.03) with ERN/LRP treatment compared to placebo (table 6.3 and 6.4).
HDL functionality and LDL quality: the influence of obesity, obstructive sleep apnoea and pharmacological intervention

<table>
<thead>
<tr>
<th>Variable</th>
<th>Post placebo (n=27)</th>
<th>Post ERN/LRP (n=27)</th>
<th>p value for treatment effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAA (mg/l)</td>
<td>17.6 (11.1 – 36.4)</td>
<td>16.2 (9.9 – 56.6)</td>
<td>0.03</td>
</tr>
<tr>
<td>MCP1 (pg/ml)</td>
<td>205.6 (155.8 – 268.3)</td>
<td>188.4 (136.5 – 246.5)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Table 6.4 Markers of systemic inflammation and vascular inflammation on treatment with ERN/LRP and placebo. Values are in median (interquartile range). Mann Whitney test used as all data non-parametric. SAA – serum amyloid A, MCP1 – monocyte chemotactic protein 1

Treatment with ERN/LRP did not affect apoA1, PON1 activity, apoM levels and MPO (table 6.5).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Post placebo (n=27)</th>
<th>Post ERN/LRP (n=27)</th>
<th>p value for treatment effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoAI (mg/dl)</td>
<td>130 (117 – 152)</td>
<td>132 (119 – 147)</td>
<td>0.75</td>
</tr>
<tr>
<td>PON1 activity (nmol/ml/min)</td>
<td>144 (86 – 179)</td>
<td>147 (56 – 187)</td>
<td>0.4</td>
</tr>
<tr>
<td>ApoM (mg/l)</td>
<td>30 (27 – 36)</td>
<td>31 (23 – 38)</td>
<td>0.8</td>
</tr>
<tr>
<td>MPO (ng/ml)</td>
<td>92 (72 – 119)</td>
<td>90 (74 – 103)</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Table 6.5 Anti-oxidant proteins associated with HDL on treatment with ERN/LRP and placebo. Values are in median (interquartile range). Mann Whitney test used as all data non-parametric. ApoAI – apolipoprotein AI, PON1 – paraoxonase 1, ApoM – apolipoprotein M, MPO – myeloperoxidase.

ERN/LRP compared to placebo did not have any effect on HbA1c, HOMA β, HOMA-IR but did cause a significant increase in fasting blood glucose levels (5.4 vs 5.1 mmol/l, p = 0.01) (table 6.6). There was a significant reduction in mean platelet count with ERN/LRP compared to placebo (211 vs 237*10⁹/l, p<0.001). Treatment with ERN/LRP did not have any significant effect on median ALT or mean serum bilirubin or mean creatinine compared to placebo.
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<table>
<thead>
<tr>
<th>Variable</th>
<th>Post placebo (n=27)</th>
<th>Post ERN/LRP (n=27)</th>
<th>p value for treatment effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBG (mmol/l)</td>
<td>5.1 (4.8 – 5.7)</td>
<td>5.4 (4.9 – 6.0)</td>
<td>0.01</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.8 (5.5 – 6.0)</td>
<td>5.8 (5.6 – 6.2)</td>
<td>0.2</td>
</tr>
<tr>
<td>Insulin (mU/l)</td>
<td>10.2 (6.8 – 20.5)</td>
<td>12.7 (8.6 – 18.3)</td>
<td>0.5</td>
</tr>
<tr>
<td>HOMA𝛽</td>
<td>117.3 (89.7 – 208.0)</td>
<td>140.0 (98.0 – 195.0)</td>
<td>0.6</td>
</tr>
<tr>
<td>HOMA - IR</td>
<td>2.8 (1.5 – 5.1)</td>
<td>2.9 (2.2 – 4.7)</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Table 6.6 Glycaemic control and β cell function related parameters on treatment with ERN/LRP and placebo. Values are in median (interquartile range). Mann Whitney test used as all data non-parametric. HbA1c – glycated haemoglobin, FBG – fasting blood glucose, HOMAβ – homeostatic model assessment of β cell function, HOMA IR - homeostatic model assessment of insulin resistance.

6.3.1.2 In vitro: There was no significant difference in LPO generated at 3 hours on incubating LDL or LDL+HDL with copper on treatment with ERN/LRP and placebo (144 vs 125 nmol/ml, p = 0.2 and 44 vs 41 nmol/ml, p = 0.4 respectively) (table 6.7).

<table>
<thead>
<tr>
<th>Mixture</th>
<th>Post placebo (n=27)</th>
<th>Post ERN/LRP (n=27)</th>
<th>p value for treatment effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL LPO (3-0hr) (nmol/ml)</td>
<td>125 (77 – 159)</td>
<td>144 (106 – 165)</td>
<td>0.2</td>
</tr>
<tr>
<td>LDL+HDL LPO (3-0hr) (nmol/ml)</td>
<td>41 (15 – 70)</td>
<td>44 (25 – 54)</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Table 6.7 Lipid peroxides (LPO) generated at 0-3 hours on incubating LDL or LDL+HDL with copper on treatment with ERN/LRP and placebo (in vitro). Values are in median (interquartile range). Mann Whitney test used as all data non-parametric. LDL – low density lipoprotein, HDL – high density lipoprotein.

6.3.2 Adverse events

23 adverse events were reported whilst patients were on ERN/LRP (table 6.8):
These included 12 flushing, 1 diarrhoea, 2 skin rash, 1 hypotension, 1 blurred vision, 2 joint pain and 1 paraesthesiae, 1 ALT > 4*ULN, 1 impaired fasting glycaemia, 1 case of increased risk of type 2 diabetes based on change in % HbA1c. The patient with raised
ALT was asymptomatic and had a normal abdominal ultrasound. The high ALT resolved after stopping the trial drug. 23 adverse events were reported whilst patients were on placebo (table 6.8): These included 2 flushing, 2 diarrhoea, 3 chest tightness/pain, 2 headache, 1 skin rash, 3 joint pain, 1 sinusitis, 1 fatigue, 1 lower limb oedema, 1 thrombophlebitis, 2 oesophageal acid reflux, 1 dry cough, 1 pain at previous shingles site, 1 exacerbation of asthma and 1 elevated ALP which resolved spontaneously.

**Serious adverse events (SAE):** There were 2 SAE during the ERN/LRP phase needing hospitalization. Neither had any long-term or permanent consequences (1 angina and 1 abdominal pain). There were 2 SAE during the placebo phase needing hospitalization. Neither had any long-term or permanent consequences (1 allergic reaction to another drug and 1 chest pain). There was 1 SAE during the washout phase (angina).

**Withdrawal from study drug:** 5 patients withdrew from the study while they were receiving ERN/LRP (2 flushing, 1 SAE of abdominal pain and 2 patients were untraceable). Therefore the withdrawal rate due to flushing was 5%. 4 patients withdrew from the study when they were receiving placebo (1 diarrhoea, 1 SAE of chest pain and 2 unknown reasons). Two patients withdrew from the study during the initial 4 week washout phase (reason unknown).
HDL functionality and LDL quality: the influence of obesity, obstructive sleep apnoea and pharmacological intervention

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ERN/LRP</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment-related clinical AE</td>
<td>22</td>
<td>20</td>
</tr>
<tr>
<td>Treatment-related serious clinical AE</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Treatment-related laboratory AE</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Discontinued because of treatment-related AE</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Discontinued because of serious treatment-related AE</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>Specific AEs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flushing</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Skin rash</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Hypotension</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Blurred vision</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Chest pain</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Headache</td>
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</tr>
<tr>
<td>Joint pain</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Paraesthesiae</td>
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<td>0</td>
</tr>
<tr>
<td>Fatigue</td>
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</tr>
<tr>
<td>Sinusitis</td>
<td>0</td>
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</tr>
<tr>
<td>Lower limb oedema</td>
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</tr>
<tr>
<td>Thrombophlebitis</td>
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<tr>
<td>Oesophageal acid reflux</td>
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<td>2</td>
</tr>
<tr>
<td>Dry cough</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Exacerbation of asthma</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Pain at previous shingles site</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><strong>Laboratory based AEs</strong></td>
<td></td>
<td></td>
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<tr>
<td>Elevated ALP</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>ALT &gt; 4*ULN</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Impaired fasting glycemia</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Increased risk of diabetes based on HbA1c</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 6.8 Summary of adverse events, serious adverse events and discontinuations
6.4 Conclusion

In this study treatment with ERN/LRP resulted in a significant improvement in HDL-C and reduction in pro-atherogenic lipoproteins/apolipoproteins in patients who had persistent dyslipidaemia despite high doses of potent statins. For the first time I have shown that ERN/LRP reduces mediators of vascular inflammation but does not influence HDL’s capacity to impede LDL oxidation \textit{in vitro} or any other markers of HDL functionality like PON1 activity, apoAI, MPO or apoM. This could mean that improving HDL functionality is more relevant than increasing its cholesterol cargo. Withdrawal rates due to flushing were 5\% as a result of combining laropiprant with niacin.
CHAPTER SEVEN: DISCUSSION AND CONCLUSION

7.1. Discussion

7.1.1 HDL and paraoxonase 1

I had aimed to evaluate the protection offered by HDL to LDL against in vitro oxidation in dyslipidemic patients treated with statins. My study shows that, HDL appears to protect LDL from in vitro oxidation in these patients. None of the previous studies that assessed this effect included HDL in the in vitro studies. HDL-C is associated with lower levels of conjugated dienes in plasma from healthy people and those with CHD. Human HDL infused into hypercholesterolaemic rabbits also exhibits the capacity to lower the plasma levels of conjugated dienes [143, 144]. This effect, which persisted for several hours, was not due to chain-breaking antioxidants or transition metal chelation, but to enzymatic activity present on HDL [139]. These observations have since been repeated by others in a variety of experimental systems and have proved relatively uncontroversial [145, 381].

The results from my study also suggest that in these patients the capacity of HDL to protect itself and LDL from oxidation in vitro is significantly better in individuals with higher serum PON1 activity. For the first time I have demonstrated that serum PON1 activity correlates with HDL’s antioxidant capacity in vitro and that high PON1 HDL poses better antioxidant capacity. These results provide evidence that PON1 associated with HDL plays an important role in promoting HDL’s in vitro anti-oxidant function.

A recent meta-analysis of the relationship between PON1 activity and CHD susceptibility which studied 9853 cases and 11408 controls showed that on pooled analysis CHD patients had 19% lower PON1 activity than did controls (p<10^{-5}) [382]. In studies of macrophages in cell culture PON1 exhibits a variety of potentially atheroprotective properties such as reducing macrophage oxidative stress and the ability of macrophages to oxidize LDL, inhibit cholesterol synthesis and promote cholesterol efflux [383]. Overall, the body of evidence to support PON1’s role in HDL’s antioxidant function is strong, in particular that from transgenic and PON1 knockout mice. PON1 transgenic mice demonstrated an improved protection of LDL against oxidation [384], but avian HDL which lacks PON1 protein failed to protect LDL against Cu^{2+} induced...
in-vitro oxidation [385], and HDL from PON1 knockout mice lost its capacity to protect against LDL oxidation induced by a co-culture model of artery wall cells [120]. In clinical studies the serum concentrations of phospholipid peroxidation derivatives have been shown to correlate inversely with PON1 activity and low activity of PON1 has also been associated with accelerated atherosclerosis [109, 144]. The mechanism by which PON1 retards LDL oxidation is unproven but appears to involve the hydrolysis of the truncated oxidised fatty acids from phospholipid, cholesteryl ester and triglyceride hydroperoxides resulting in the production of lysolipids, cholesterol, diglycerides and oxidised fatty acids. These lysolipids and oxidised fatty acids produced by PON1 are themselves potentially proatherogenic but do not appear to be so when produced on HDL [386]. Un-metabolised lipid hydroperoxides are highly inflammatory, inducing the production of MCP1 by arterial cells which attracts monocytes into the arterial intima at the very start of atherosclerotic process [21]. In the presence of PON1, lipid hydroperoxide concentrations are reduced, MCP1 production inhibited and the atherosclerotic process attenuated [212, 387].

The results from my study demonstrate that PON1 activity may also protect ApoB from glycation in vivo. Oxidation is known to accompany glycation and it has been argued that glycation should more properly be described as glycoxidation [75]. In keeping with this, it has previously been reported that when LDL is glycated in vitro, small quantities of LPO are also generated even under the conditions imposed where there is no external source of oxygen free radicals [161]. It has recently been suggested that HDL is able to impede in vitro LDL apoB glycation and PON1 may be important for this function of HDL [76]. My result is consistent with these in vitro experiments from our group.

In my study inspite of there being a significant difference in glycated apoB (in vivo) between the two groups, there was no difference in oxLDL (in vivo) as glycation has a minor contribution to oxidation. Similarly there are significant differences in LPO formation (in vitro) but not in vivo oxLDL between the two groups, as during the in vitro studies the lipoproteins were exposed to much more potent oxidizing conditions as compared to in vivo conditions. Therefore PON1 may be equally important in prevention of glycation and oxidation.
HDL functionality and LDL quality: the influence of obesity, obstructive sleep apnoea and pharmacological intervention

I also looked at many factors that affect PON1 activity but was unable to determine the exact causes that may have contributed to the difference in PON1 activity between the two groups. There was no difference in statins used in the low and high PON1 groups. There was no significant difference in other factors including age, gender, BMI, alcohol consumption, hormone replacement therapy in post-menopausal women, smoking, diabetes, menopause, thyroid disease, chronic renal or liver failure, apoA1, oxLDL, Lp-PLA2, use of aspirin or omega 3 fatty acids. Genetic analysis may throw some more light on this as PON1 activity depends on PON1 polymorphism as well. PON1 activity is the highest in individuals with RR192 genotype than in those with QQ192 [213]. Other factors that may affect PON1 activity are dietary contents and exercise. These factors were not assessed in this study. Therefore PON1 activity represents a potential therapeutic target for future in high risk patients.

Under physiological conditions, paraoxonase1 degrades metabolites of polyunsaturated fatty acids and homocysteine (Hcy) thiolactone, among other compounds. By detoxifying both oxidized low-density lipoprotein and Hcy thiolactone, PON1 protect against atherosclerosis and coronary artery diseases, as has been illustrated by many types of in vitro and in vivo experimental evidence. The abundance and/or activity of PON1 can be regulated by lipoproteins and their metabolites, biological macromolecules, pharmacological treatments, dietary factors, and lifestyle [388]. Therefore it would be plausible to measure PON1 activity to evaluate individual patient’s CVR and effect of interventions. Potential problems here will include:

1. Lack of uniformity around which PON1 activity is most clinically relevant i.e. arylesterase or lactonase or paraoxonase. Measuring any of these routinely may not be practical.
2. Interventions or therapies that have been shown to increase PON1 activity are already in extensive use and have other parameters as their primary targets.
3. Targeting non-HDL-C, apoB, apoB/apoA1 ratio, Lp-PLA2, homocysteine and hsCRP in order to reduce CVR has a more favorable evidence base as compared to PON1 elevation.

7.1.2 Obstructive sleep apnoea and lipoproteins
HDL functionality and LDL quality: the influence of obesity, obstructive sleep apnoea and pharmacological intervention

### 7.1.2.1 Anti-oxidant function of HDL:

The groups were well matched with low probability of confounding due to age, gender and BMI. In keeping with previous studies I was able to demonstrate that the presence and increasing severity of OSA leads to reduction in PON1 activity [196, 202]. Another study had demonstrated HDL dysfunctionality in OSA patients by its inability to prevent *in vitro* LDL oxidation and a lower PON1 activity in the OSA group. The degree of dysfunctionality of HDL and levels of oxidised LDL were directly related to AHI, which reflects the severity of OSA [193]. I have shown that the capacity of HDL to protect itself from *in vitro* oxidation is reduced in morbidly obese patients with sleep apnoea compared to matched patients without sleep apnoea. This function of HDL diminishes with increasing severity of sleep apnoea. The previous studies were not matched for basic characteristics like BMI, gender and age. I recruited age, gender and BMI matched patients in order to avoid bias. I have partially elucidated the causes of HDL dysfunctionality in these patients. The significantly lower PON1 activity may contribute to HDL dysfunction. PON1 was lower in the OSA and the high AHI groups compared to the ‘obese no OSA’ and low AHI groups respectively. PON1 showed an inverse relation to AHI. These results suggest that PON1 activity is increasingly affected by the oxidative stress created by the presence and increasing severity of OSA. As PON1 is known to play an important role in HDL’s antioxidant function, it is likely that the diminished PON1 activity due to OSA contributed to HDL’s reduced antioxidant function.

ApoM and apoA1 also play a role in HDL’s anti-oxidant function through distinct pathways. I have shown that apoM concentrations were lower in the OSA and high AHI groups. This may also be contributing to the diminished anti-oxidant function of HDL. There was no significant difference in ApoA1 between the two groups. Similar result was obtained in a previous study [193], suggesting that ApoA1 concentration may not be affected by the excessive oxidative stress in sleep apnoea.

The current study showed that OSA does not effect the levels of adiponectin. This result is similar to another recent study which looked at the influence of obesity and OSA on adiponectin. In this study adiponectin level was associated with obesity but not OSA [389].
7.1.2 Mechanisms of PON1 and TNFα interaction from literature: I have for the first time reported a possible relation between PON1 activity and TNFα in OSA patients. Whether the relationship between PON1, TNFα and OSA is causative, remains to be elucidated.

There is evidence from literature that PON1 activity and TNFα may be related. It has been shown that adenovirus based over-expression of human PON1 in apolipoprotein E knock-out mice may be associated with reduced TNFα levels [390]. A study on atherosclerosis in rheumatoid arthritis found a correlation between PON1 activity and serum TNFα levels [391]. In another study in patients with rheumatoid arthritis, TNFα antagonist therapy led to enhanced PON1 levels, heightened anti-oxidant capacity of HDL and a reduced inflammatory status [392]. But there could be other pathways as well based on previous work done in this field. It could be that reduced PON1 activity leads to increased action of TNFα as demonstrated in a study where HDL obtained from coronary disease patients had low PON1 activity. This led to activation of endothelial protein kinase C beta 2 (PKC β2) pathways and loss of endothelium’s ability to resist to TNFα mediated expression of adhesion molecules [393].

There is also a possibility that TNFα regulates PON1 expression as shown in studies based on murine and human hepatoma cell lines. Similar results were obtained in animals studies *in vivo*. It was suggested that this effect was possibly via nuclear factor-kappaB (NF-kB) and nuclear receptor peroxisome proliferator- activated receptor-alpha (PPAR-alpha) dependent mechanisms. The reduced PON1 activity led to diminished antioxidant capacity [394, 395].

7.1.3 Niacin

7.1.3.1 Effect on HDL-C and anti-oxidant function of HDL

In this cohort HDL-C at baseline was 1.3 mmol/l. and treatment with ERN/LRP led to a significant increase in HDL-C compared to placebo. There was no difference in ApoA1 levels after either ERN/LRP or placebo. These findings may suggest that treatment with ERN/LRP leads to an increase in HDL cholesterol content rather than number of particles. There is evidence from previous studies that treatment with niacin promotes HDL mediated reverse cholesterol transport [238, 239] and lowers CETP activity [233,
Both these actions of niacin will result in an increase in cholesterol content of HDL rather than the number of particles. My results also show that ERN/LRP significantly reduces CETP activity explaining the increase in HDL-C. ApoA1, PON1 and apoM play a role in HDL’s anti-oxidant function [396]. In this study there was no difference with treatment in apoA1, apoM levels or PON1 activity. This explains the lack of difference in HDL’s ability to protect LDL from in vitro oxidation on treatment with ERN/LRP or placebo. Therefore improving HDL functionality may be more important that its cholesterol content.

In a previous study treatment with niacin increased HDL-C and ApoA1 levels and reduced PON1 activity and LPO [397] but the baseline HDL-C in that study was lower at 0.96 mmol/l compared to my study’s baseline HDL-C level.

7.1.3.2 Effect on VLDL

There is evidence from previous work that niacin decreases VLDL output from the liver by reducing delivery NEFA to the liver [230] or by inhibiting DGAT2 [231]. DGAT2 activity and NEFA are important for TG synthesis and VLDL lipidation [398]. My results showed that treatment with ERN/LRP was associated with reduction in VLDL-TG. There was also a trend towards reduction in VLDL-C and VLDL apoB on treatment with ERN/LRP although these did not reach significance. This might indicate that there was a greater reduction in VLDL particle size compared to particle number with ERN/LRP. Similar results were obtained in a previous study where cholesterol associated with lipoprotein subclasses was quantified by vertical auto profile II (VAP II) [399].

7.1.3.3 Effect on LDL

My results show that ERN/LRP significantly lowered LDL apoB and buoyant LDL apoB. There was reduction in sdLDL apoB but it was not statistically significant inspite of the reduction in CETP. Previous studies have shown significant reduction in sdLDL apoB with niacin treatment [277, 278] and this study may not have adequate power to evaluate this. Also results similar to my study were obtained in a previous study where cholesterol associated with lipoprotein subclasses was quantified by VAP II [399]. SdLDL is the most important determinant of glyLDL. Lack of significant reduction in sdLDL could explain the same for glyLDL in my study.
VLDL is a precursor of LDL; the reduction in VLDL apoB on treatment with ERN/LRP explains the reduction in LDL apoB. Also ERN may increase LDL uptake by the liver through an as yet unidentified mechanism. Therefore there may be four possible mechanisms for greater reduction in buoyant LDL apoB as opposed to sdLDL apoB: 1) The study may not be powered to detect effect of ERN/LRP on sdLDL apoB which forms a minority part of total LDL apoB (as compared with buoyant LDL apoB); 2) ERN/LRP through an unknown mechanism may be increasing expression of hepatic LDL receptors which are much more efficient in accepting buoyant LDL as compared to sdLDL; 3) ERN/LRP reduces hepatic VLDL output and VLDL size. Buoyant LDL is the predominant successor of VLDL; 4) 40% of the subjects had HeFH, which is characterised by lesser preponderance for buoyant LDL to convert to sdLDL as a result of lower VLDL TG content and lower CETP activity (as compared to patients with diabetes dyslipidemia or mixed dyslipidemia). SdLDL is more vulnerable to atherogenic modification as compared to buoyant LDL, but it is important to note that buoyant LDL also contributes to atherogenesis as seen in HeFH patients with premature CAD.

A recent study has provided functional evidence that genetically increased hepatic sortilin expression both reduces hepatic apoB secretion and increases LDL catabolism, providing dual mechanisms for the association between increased hepatic sortilin expression and reduced plasma LDL-C levels in humans [400]. It is possible that niacin increases hepatic sortilin expression. Further research is needed to explore this.

In previous studies anti-inflammatory effects of niacin were thought to be independent of its lipid lowering functions [249, 401]. In my study niacin reduced all constituents of a distinct pro-atherogenic mechanism involving oxLDL, Lp-PLA2, lyso-PC and MCP1. The significant reduction in oxLDL, Lp-PLA2, lyso-PC and MCP1 with ERN/LRP treatment could be explained by its ability to reduce apoB levels or number of LDL particles significantly. Also I have shown that ERN/LRP treatment leads to reduction in SAA. Hence niacin may be able to produce anti-inflammatory effects through multiple pathways.

7.1.3.4 Safety: More patients on ERN/LRP experienced flushing compared to placebo but this did not lead to a higher overall discontinuation rate in this study. This could be
attributed to the action of laropiprant. The overall safety of ERN/LRP was comparable to placebo.

7.1.3.5 Role of niacin in the future

LDL-C lowering with statins remains first-line treatment in CVD but there is mounting evidence that the residual CV risk of 60-70% is also modifiable. This risk may be reduced by optimizing treatment targets based on apo-B measurement [402], but HDL-C also represent an attractive therapeutic target. Statins have a limited effect on HDL-C, whereas niacin reduces triglycerides and all atherogenic apo-B containing particles as well as increasing HDL-C. Therefore, as over the past decade focus has shifted to HDL-C as an additional treatment target, there has been a steady increase in research elucidating protective effects of HDL-C and identifying drugs that are able to improve the anti-atherogenic properties of HDL.

Niacin has been shown to prevent CV events and reduce mortality in initial small scale studies, but the results of two large scale trials were disappointing. The future role of niacin will be defined by these two studies AIM HIGH and HPS2-THRIVE. The results from the AIM HIGH study were contrary to previous evidence and patients on the treatment arm had more ischaemic strokes compared with placebo although this did not reach significance. The addition of ERN to patients with an average LDL-C of 1.8mmol/l was found to have no additional positive effect on cardiovascular outcomes. I speculate that the failure of combining ERN with statin in reducing CVD endpoints compared with statin monotherapy may be attributable to trial design, which led to smaller than anticipated differences in HDL-C between the placebo and treatment groups and the low pre-randomisation mean LDL-C of participants, which limits the potential for additional benefit. However, the possibility remains that there is no additional benefit to be gained. Although the results from HPS2-THRIVE have not been published as yet the trial design and intervention in this study was similar to AIM-HIGH. Again LDL-C was lowered to <2 mmol/l with simvastatin ± ezetimibe in order to then assess the impact of elevating HDL-C on CV mortality and morbidity by introducing ERN/LRP. HPS2-THRIVE also did not show any benefit of elevating HDL-C by ERN/LRP in patients with an LDL-C ≤ 2 mmol/l (major vascular events 13.3% vs 13.9%, P=0.29). The main distinction between the two studies was that in AIM-HIGH
patients with HDL-C below 1.03 mmol/l in men and 1.29 mmol/l in women were selected whereas there were no such restrictions in the HPS2-THRIVE study. At this stage it is difficult to separate the role played by ERN and LRP in the final results of HPS2-THRIVE. But also, on the contrary there may be some merit in stating that elevating HDL-C with niacin in patients with LDL-C at target does not reduce cardiovascular events.

In contrast to these large scale studies, I recruited patients who had not reached the NCEP-ATP III targets for LDL-C with current statin therapy. I have subsequently shown that in spite of elevating HDL-C, ERN/LRP did not improve HDL’s anti-oxidant function in vitro nor potential markers of its functionality like PON1 activity, MPO, apoM and apoAI in these patients. Whereas it makes a significant impact on the pro-atherogenic lipoproteins and mediators of inflammation. This could mean that niacin may be initiated in patients with high LDL-C despite the use of potent statins. It is worth mentioning here that the only niacin study that recruited patients with high LDL-C and looked at CV events was HATS [37] which did show significant benefit by adding niacin to simvastatin compared to statin alone (24 % first CV events in placebo but 3% in Niacin + simvastatin group (p=0.03)). There was significant reduction in LDL-C in this study which could have contributed to the positive outcome. A recent systematic review included results from AIM HIGH as well as all previous randomized control trials that had assessed the clinical efficacy of niacin. This review and meta-analysis demonstrated that niacin reduces CVD events and that this may occur through a mechanism not reflected by changes in HDL-C [403].

The mode of action of niacin is particularly useful in combined dyslipidaemia in metabolic syndrome and diabetes. The impact on glycaemic control of niacin seems to be attenuated by use of ERN but additional modification of glycaemic therapy may be necessary and should be anticipated. The effect of fibrates on TG when combined with statins is similar to the combination of niacin and statins. But recent studies on fibrates combined with statins with the hope of additional benefit from triglyceride lowering have been disappointing [404, 405]. Also it has been reported that fibrates have a paradoxical HDL lowering action in some circumstances [406].

7.1.3.6 CETP inhibitors: CETP inhibitors anacetrapib, dalcetrapib and evacetrapib
HDL functionality and LDL quality: the influence of obesity, obstructive sleep apnoea and pharmacological intervention

have been shown to increase HDL-C by up to 138.8%, 30.9% and 128.8% [285, 407, 408]. The first oral CETP inhibitor to be assessed in major clinical trials was torcetrapib. Despite promising results in phase I and II studies, increases in HDL-C of 72% and decreases in LDL-C of 25% and triglycerides of 9%, the large-scale phase III study Investigation of Lipid Level Management to Understand its Impact in Atherosclerosis Events (ILLUMINATE) trial was terminated early because the torcetrapib and atorvastatin group experienced increased all-cause mortality and cardiovascular events compared with recipients of atorvastatin alone [409]. The negative outcome for torcetrapib has been attributed to CETP-independent angiotensin-aldosterone activation and adverse effects on blood pressure. The possibility that CETP inhibition may be pro-atherogenic was not supported by post-hoc analysis. CVD rates were lower in the subgroup of patients treated with torcetrapib who showed an increase in HDL-C above the median compared with those whose HDL-C increased under the median [410]. In another post-hoc analysis the authors reported a significant improvement in mean plasma glucose and glycated haemoglobin (HbA1c) levels, with a significant decrease in serum insulin levels and HOMA-IR suggesting enhanced insulin sensitivity [411]. In another study, HDL functionality was assessed in vitro, with HDL isolated from torcetrapib-treated patients showing a normal or enhanced promotion of cholesterol efflux from macrophages [412].

The second CETP inhibitor to progress to phase III studies was dalcetrapib. Dalcetrapib associates differently with CETP and has no angiotensin-aldosterone-stimulating or hypertensive effects. Disappointingly, Dalcetrapib was recently discontinued from clinical development following an interim analysis of the dal-OUTCOMES trial (efficacy and safety of dalcetrapib in patients with recent acute coronary syndrome) showing no effect on cardiovascular events [413]. Anacetrapib has been shown to have robust effects on HDL-C and LDL-C in the phase III study DEFINE (Determining the Efficacy and tolerability of CETP Inhibition with AnacEtrapib) [285]. Anacetrapib remains in clinical development. The issue of whether CETP inhibition reduces the risk of cardiovascular disease may well be settled by these results [414, 415]. At present there is no evidence to suggest any harm from complete CETP inhibition with anacetrapib or its modulation with dalcetrapib [407, 415]. Results from ongoing trials
assessing the impact of CETP inhibitors on CV morbidity or mortality are awaited. Thus the search for optimal therapy to raise HDL-C and lower CV outcomes as a supplementary treatment to statins goes on.

Most current guidelines agree that low HDL-C is associated with high CV risk and therefore HDL-C should be included in CV risk assessment. Definitive HDL-C targets and therapeutic means to reach these targets are as yet not included in most of these guidelines. Even though, the evidence for the favorable effects of niacin therapy on atherogenic apoB containing particles is evident, to date there is no evidence to confirm cardiovascular benefits from HDL-C increase associated with niacin therapy. This is likely because HDL’s function does not improve and the increase of HDL-C is mediated through inhibition of CETP activity. It is noteworthy that partial inhibition of CETP activity by Dalcetrapib failed to reduce cardiovascular events in a large trial.

7.2 Conclusion:

7.2.1 In statin treated dyslipidemic patients, HDL protects LDL against oxidation in vitro. In these patients the capacity of HDL to protect itself and LDL from oxidation in vitro is significantly better in individuals with higher serum PON1 activity. These results provide evidence that PON1 associated with HDL plays an important role in HDL’s in vitro anti-oxidant function. In the same cohort PON1 activity may also protect ApoB from glycation in vivo. The reasons for variation in PON1 activity amongst this group of well matched statin treated dyslipidemic patients is not evident and needs further research and exploration. PON1 activity may play a role in modifying residual risk in high risk populations through protecting LDL from glycation and oxidation. This needs further investigations.

7.2.2 The capacity of HDL to protect itself from in vitro oxidation is reduced in morbidly obese patients with sleep apnoea compared to matched patients without sleep apnoea. This function of HDL diminishes with increasing severity of sleep apnoea. The significantly lower PON1 activity and apoM in the patients with increasing severity of sleep apnoea may result in reduced in vitro anti-oxidant function of HDL. PON 1 activity is negatively related to AHI only in the obese OSA group. Significantly higher TNFα and ICAM1 in the obese OSA group suggest endothelial dysfunction. It is
possible that oxidative damage to PON1 in OSA may play a part in both HDL and endothelial dysfunction.

7.2.3 Treatment with ERN/LRP resulted in a significant improvement in HDL-C and reduction in pro-atherogenic lipoproteins/apolipoproteins in patients who had persistent dyslipidaemia despite high doses of potent statins. For the first time I have shown that ERN/LRP reduces mediators of vascular inflammation but does not influence HDL’s capacity to impede LDL oxidation in vitro or any other markers of HDL functionality like PON1 activity, apoAI, MPO or apoM. This could mean that improving HDL functionality is more relevant than increasing its cholesterol cargo. Withdrawal rates due to flushing were 5% as a result of combining laropiprant with niacin.

7.3 Limitations and Future direction:

7.3.1 Establishing the role of gene polymorphisms on PON1 activity in statin treated dyslipidemic patients as the largest effect on PON1 activity is through genetic polymorphisms [416].

To study the influence of PON1 on the in vitro oxidation of LDL and HDL’s capacity to impede this in healthy non-statin treated volunteers.

Organizing a randomized control trial comparing statin treated to statin naïve dyslipidemic patients where LDL with and without HDL is incubated during the in vitro studies.

Developing pharmacological therapies that target PON1 activity.

7.3.2 I selected patients with similar age, gender and BMI to reduce confounding. There may be a case to recruit another group matched for age and gender but with normal BMI (18.5 – 24.9 kg/m²). This may assist in dissecting out the effect of obesity without OSA when compared to healthy individuals on all the parameters considered in this study. It is worth noting that previous studies have shown that PON1 activity is reduced and TNFα levels are elevated due to obesity [417-419].

In both groups 40% of patients were treated with statins. The study had insufficient power to investigate any modifying effect of statins. There were visible differences in percentage of smokers and prevalence of CVD between “OSA” and “no OSA” groups but these were not statistically significant. Further recruitment with a view
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to iron out these minimal differences is a possibility especially with the knowledge that matched cohorts with high incidence of CVD have lower PON1 activity.

It would have been prudent to measure levels of LPO in serum but our group’s experience from previous work is that this measurement is unreliable (unpublished work).

Future work would also include studying the effect of interventions like CPAP or weight loss after bariatric surgery on these parameters.

7.3.3 Studying the effect of ERN on other HDL functions like cholesterol efflux is desirable and also on LDL’s atherogenicity by assessing its ability to form foam cells when incubated with macrophages.

Conducting a large scale randomised control trial to evaluate the effect of niacin vs placebo on patients with high LDL-C despite statin treatment.

Evaluating the effect of ERN on HDL proteomics
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