HDL- STRUCTURE AND FUNCTION

IN RELATION TO CARDIOVASCULAR DISEASE

By

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Abstract

Coronary heart disease (CHD) is the most common cause of death in Western societies. This disease affects both men and women and accounts for approximately 500,000 deaths annually in the U.S.A. alone.

For a number of years, plasma concentration of HDL cholesterol (HDL-C) was found to correlate inversely with the incidence of coronary heart disease and atherosclerosis. In addition, other studies in humans showed that higher plasma HDL-C levels correlates with slower progression of atherosclerotic lesions and possible stabilization of unstable atherosclerotic plaque. These findings have led to the suggestion that raising HDL-C will protect from the disease. One of the first trials demonstrating the potential benefit of raising HDL-C levels was the Helsinki Heart Study. In this randomized trial, 4081 men with dyslipidemia received gemfibrozil or placebo and five years later it was seen that the drug reduced CHD risk. Gemfibrozil treatment increased HDL-C levels by 11% but also reduced total plasma cholesterol levels by 10%, LDL-C cholesterol levels by 11% and triglyceride levels by 35%. Although all fibrates have been revealed to raise HDL-C significantly, their overall effect on all-cause mortality and cardiac mortality remains debatable.

More recent studies have suggested that the relationship between HDL and cardiovascular risk is more complex than first thought and extends beyond consideration of levels of total HDL-C in plasma. In particular, Mendelian randomization studies challenged the existing view on HDL-C and cardiovascular risk and prompted a discussion as to whether low HDL-C is a causal risk factor for the development of heart disease.

In parallel, research interest has intensified in studies aimed at better understanding the many biological functions of HDL and the partner proteins and receptors with which it interacts. There have been a number of studies over recent years indicating that HDL can fail to function effectively in subjects at risk for coronary diseases. It is important therefore to attempt to understand if abnormalities in HDL function are associated with variation in CVD risk. This is

especially true in light of the failure of recent trials that raise total HDL to reduce risk of myocardial infarction and CVD.

A feature that appears to be related to the atheroprotective functions of HDL is the relative level and distribution of HDL subpopulations in different individuals. Although HDL is unusually regarded as a single entity in clinical settings, studies using non- denaturing two-dimensional electrophoresis have revealed a number of HDL particles with distinct shape, size and composition. Along the same lines, administration of statins - a medication proven to reduce CHD risk - to patients increases specific subpopulation of HDL suggesting that some of the atheroprotective properties of statins may be mediated by increasing selected HDL subpopulations.

The overall objective of the present work was to examine in details the relationship of HDL oxidation potential, the ability of HDL to protect LDL from oxidation, and the abundance of the major antioxidant enzyme, PON1, to atherosclerosis in a cross section of subjects recruited from across the social economic spectrum in the West of Scotland (the pSoBid study). PSoBid is valuable as a means of testing these properties of HDL because of its mix of males and females, wide age range, and the fact that it focused in recruiting people at extremes of social deprivation with widely varying lifestyles.

The population has been well characterized in terms of classical risk factors and this thesis takes the investigation to a new level of detail with respect to HDL.

The major questions addressed were:

1. Is HDL oxidation (measured by three factors; time at half maximum (T1/2max), maximum velocity of oxidation (Vmax), or maximum amount of oxidized HDL measured by optical density) related to a commonly used index of atherosclerosis?
2. Is HDL antioxidant potency to protect LDL from oxidation related to its protective effect in atherosclerosis risk?
3. Is HDL PON1 activity related to atherosclerotic marker?
4. If some HDL subclasses, rather than HDL-C, are particularly related to atherosclerosis?

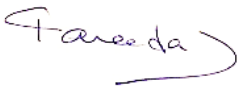
Our major findings revealed that:

1. HDL is more readily oxidized in those subgroups associated with less atherosclerosis.
2. The susceptibility of HDL to oxidation was also related to lifestyle factors associated with less atherosclerotic disease such as moderate alcohol intake, not smoking, active exercise habit and high intake of fruits and vegetables.
3. For HDL structure, %HDL2b was inversely associated with atherosclerosis while %HDL3b was directly associated with atherosclerosis.
4. Comparing the two factors which were inversely associated with atherosclerosis, HDL oxidation and the distribution of HDL subpopulations, our results revealed that HDL oxidation potential was more important than the percentage HDL subfraction distribution in relation to atherosclerosis.
5. HDL mediated inhibition of LDL oxidation was not found to be associated with carotid atherosclerosis nor did it appear to be related to major risk factors.

Our interpretation for atherosclerosis is that HDL particles, which are very abundant in the circulation, might play a sacrificial role in that they are oxidized first and therefore have the capacity to prevent LDL oxidation in vivo.

Author's Declaration

Unless otherwise stated by acknowledgement or reference to published literature, the presented work in this thesis is the author's own, as approved by the thesis committee and the Graduate office and has not been submitted for a degree at another institution.

Faridah Alkandari  Date: 12-06-2017

The findings of some of the studies have been published as follows:

Abstract:

Abstract. Preβ1-HDL as Part of the Lipoprotein Spectrum and Vascular Disease. Atherosclerosis, 2014; 236 (2): E307 (**Appendix 5**).

Conferences communication(s)

Poster- Preβ1-HDL and pSoBid study. Heart UK 28th Annual Conference 2014 (**Appendix 6**).

The main finding of this study are being prepared for a paper to be submitted to an international journal.

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"O my Lord! advance me in knowledge"

Holy Quran (20:114)

''When a person dies, his deeds come to an end except for three things: ceaseless charity; a knowledge which is beneficial, or a virtuous descendent who prays for him''

Prophet Mohammed (PBUH)

To my Parents Amnah & Mohammad

With utmost love, respect and appreciation

To my husband Adnan who supported me during my educational journeys all the way from Bachelor to PhD

For my Kids

And to all my brothers and sisters

for their constant encouragement and moral support

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List of Abbreviations

2D	2-Dimentional
AAPH	2,2'-Azobis (2-methylpropionamidine) dihydrochloride
ABCA1	ATP-binding cassette protein A1
ABCG1	ATP-binding cassette protein G1
Acyl-CoA	Acyl-coenzyme A
AF	Arterial fibrillation
Apo	Apolipoprotein
APOA-I	Apolipoprotein A-I
Apo-B	Apolipoprotein-B
ATP	Adenosine triphosphate
BCG	Bromocresol green
BIP	Bezafibrate Infraction Prevention
BMI	Body mass index
BPI	Bactericidal permeability- increasing protein
CV	Coefficients of variance
CAD	Coronary artery disease
CE	Cholesterol ester
CETP	Cholesterol ester transfer protein
CHD	Coronary heart disease
CI	Confidence interval
cIMT	Carotid intima-media thickness
CL	Control limit
CM	Chylomicron
CMRs	Chylomicron remnants
CRP	C-reactive protein
CVD	Cardiovascular disease
EDTA	Ethylenediaminetetraacetic Acid
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
FC	Free cholesterol
FED	Fish Eye Disease
F.F.As	Free fatty acids
FER-HDL	Fractional rate of cholesterol esterification
FLD	Familial LCAT deficiency
GC	Gas Chromatography
GC-MS	Gas chromatography- mass spectrophotometry
GGE	Gradient gel electrophoresis
GSPx	Glutathione selenoperoxidase
HDL	High density lipoproteins
HDL-C	High-density lipoprotein cholesterol

HDLR	HDL receptor
HHS	Helsinki Heart Study
HL	Hepatic lipase
HMG-CoA	3-hydroxymethyl-3-methylglutaryl coenzyme A
HMW	High molecular weight
HPLC	High-performance liquid chromatography
HSCIC	Health and Social Care Information Centre
IDL	Intermediate density lipoproteins
LBP	Lipopolysaccharide (LPS)-binding protein
LCAT	Lecithin: cholesterol acyltransferase
LCL	Lower control limit
L.D	Least deprived
LDL	Low density lipoproteins
LDL-C	LDL-cholesterol
LDLR	LDL receptor
Li-Hep	Lithium Heparin
LPL	Lipoprotein lipase
LpPLA2	Lipoprotein-associated phospholipase A2
LPS	Lipopolysaccharide
LRP	LDL receptor-related protein
LXR	Liver X receptor
LXRE	LXR response element
M.D	Most deprived
MI	Myocardial infraction
Min	Minutes
MTP	Microsomal triglyceride transfer proteins
NMR	Nuclear magnetic resonance
oxLDL	Oxidized low-density lipoprotein
PA	Phosphatidic acid
PAD	Peripheral arterial disease
PAF-AH	Platelet-activating factor acetylhydrolase
PBS	Phosphate buffer saline
PC	Phosphotidylcholine
PE	Phosphotidylethanolamine
PEG	Polyethylene glycol
PG	Phosphotidylglycerol
PI	Phosphotidylinositol
PL	Phospholipid
PLA2	Phospholipase A2
PLTP	Phospholipid transfer protein
PON	Paraxonase enzyme
PON1	Paraxonase-1 enzyme
PPAR-α	Peroxisome proliferator-activated receptor alpha

PS	Phosphotidylserine
PUFA	Polyunsaturated fatty acids
RCT	Reverse cholesterol transport
rHDL	Reconstituted High density lipoprotein
RR	Relative risk
SAA	Serum amyloid A
SBP	Systolic blood pressure
sdLDL	Small dense LDL
Sf	Svedberg floatation
SIP	Shingosine-1-phosphate
SIMD	Scottish Index of Multiple Deprivation
SR-B1	scavenger receptor B1
T1/2max	Time at half maximum
T2DM	Type 2 diabetes mellitus
TICE	Trans-intestinal excretion of plasma-derived cholesterol
TBAR	Thiobarbituric acid- reactive substances
TBBL	Thiobutil butyrolactone
TC	Total cholesterol
TD	Tangier Disease
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TLC	Thin layer chromatography
tPA	Tissue plasminogen activator
TG	Triglyceride
UCL	Upper control limit
UKPDS	UK prospective diabetes study
VA-HIT	Veterans Affairs High-density lipoprotein Intervention Trial
VLDL	Very low density lipoproteins
Vmax	Maximum velocity

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1 Introduction and Literature Review

1.1 General Lipoprotein Physiology

1.1.1 Lipoprotein Composition and Classes

Lipids are an important component of living cells. Lipids are defined chemically as organic substances that are insoluble in water and soluble in alcohol, ether, and chloroform (Akoh & Min, 2008). They include fat, waxes, sterols, fat-soluble vitamins (such as vitamins A, D, E, and K), monoglycerides, diglycerides, triglycerides (TGs), phospholipids (PLs), and others (Ekuni, Battino, Tomofuji & Putnins, 2014). Lipids are considered as one of the macronutrients alongside carbohydrate and protein (Akoh & Min, 2008). The vast majority of dietary lipids consist of triglycerides (> 95%) (Alters & Schiff, 2013), and the remaining are PLs, free fatty acids (F.F.As) cholesterol and fat-soluble vitamins. Biologically and clinically, the most important plasma lipids are cholesterol and triglycerides. Cholesterol and TG are almost insoluble in the aqueous bloodstream; therefore they are transported via their attachment to circulating lipoproteins to enable the organism to transport them to the peripheral tissues for storage or metabolism (Gurr, Harwood & Frayn, 2002 ; Hegele, 2009).

Lipoproteins are spherical particles (**Figure1.1**) consisting of a core of hydrophobic lipids, which are mainly cholesterol ester and triglycerides, surrounded by an amphipathic shell of free cholesterol, PLs and one or more specific proteins called apolipoproteins which function both to stabilize the lipoprotein and to promote its further metabolism (Ginsberg H. N, Zhang Y. L & A., 2005).

Plasma lipoproteins are classified into four major classes based on their hydrated density or Svedberg floatation rate (Sf); chylomicrons (CM), very low-density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL) (Gurr, Harwood & Frayn, 2002). **Table 1.1** summarizes the major lipoproteins along with their features. Chylomicron remnants (CMRs) and intermediate density lipoproteins (IDL) are also included in this classification. They are formed by the degradation of CM and VLDL particles respectively. Each lipoprotein class comprises a family of particles that vary slightly in density, size, migration, during electrophoresis, and protein composition. The density of a lipoprotein is determined by the relative concentrations of lipids and proteins and by the diameters of the broadly spherical particles. Although preparative/ analytical ultracentrifugation remains a

frequent and valuable technique for isolating all serum lipoproteins (Brousseau T, Clavey V, Brad & Fruchart, 1993; Ginsberg H. N, Zhang Y. L & A., 2005), lipoproteins can also be separated according to their behavior on column chromatography, high-performance liquid chromatography (HPLC) and electrophoresis. Apolipoproteins (apo) or apoproteins are important regulators of lipoprotein metabolism through their influence on the transport and redistribution of lipid among various cells and tissues (**Table 1.2**). This is achieved either by their role as a cofactor for enzymes of lipid metabolism or through their role in lipoprotein particle assembly (Mahley, Innerarity, Rall & Weisgraber, 1984).

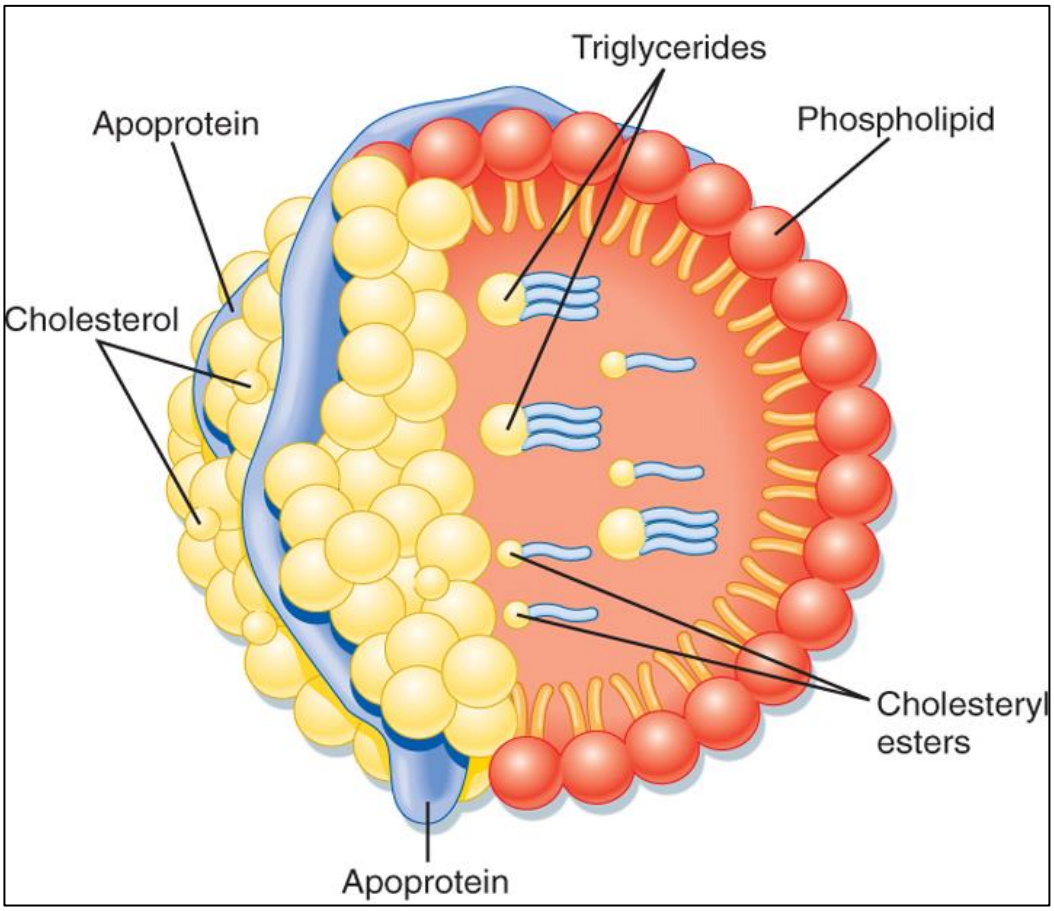


Figure 1.1 The lipoprotein particle. The external monolayer of the particle contains free cholesterol, phospholipids, and apolipoproteins. The very hydrophobic cholesterol esters and Triglycerides concentrate within the particle core. Lipoproteins also carry fat-soluble vitamins. Apoprotein, apolipoprotein. [From: (Baynes, 2005)].

Table 1.1 Characteristics and percentage content of the various lipoprotein particles relative to total weight. [Source: (Ginsberg H. N, Zhang Y. L & A., 2005; Gurr, Harwood & Frayn, 2002 ; Packard & Shepherd, 1997; Wills 2014) .

							Composition (%)				
Lipoprotein particle	**Diameter (Angstroms)**	**Density g.mL^{-1}**	**Flotation rate (Sf)**	**Site of Synthesis**	***Major apolipo-proteins**	**Mobility by electrophoresis**	**Protein**	**Cholesterol**	**TG**	**PL**	**Function**
CM	80–1000	<0.95	>400	Gut	A-I, A-II, A-IV, B-48, C-I, C-II, C-III, E	Remains at origin	2	2-7	80-95	3-9	Transport dietary fat
VLDL	30-80	0.95–1.006	20-400	Liver	B-100, C-I, C-II, C-III, E	Pre-beta	8	5-15	55-80	10-20	Transport endogenous fat
LDL	18-25	1.019–1.063	0-12	Peripheral tissue capillaries	B-100	Beta	22	40-50	5-15	20-25	Transport cholesterol to periphery
HDL	5-12	1.063–1.21	-	Gut/ Liver	*A-I, A-ll, A-IV, C-I, C-II, C-III, D, E	Alpha & Pre-beta (for some nascent HDLs)	40	15-25	5-10	20-30	Reverse transport of cholesterol

* Only major apolipoproteins are mentioned. TG, Triglyceride; PL, phospholipids

Table 1.2 Characteristics of major apolipoproteins [Source: (Ramasamy, 2014)].

Apo	M.Wt	Lipoproteins	Metabolic functions	Synthesis
ApoA-I	28,016	HDL, CM	Structural component of HDL, LCAT activator	Liver, intestine
ApoA-II	17,414	HDL, CM		liver
ApoA-IV	46,465	HDL, CM	Involved in chylomicron assembly and secretion	Intestine in humans
ApoA-V		HDL, CM, VLDL	Effects on plasma TG concentrations are complex and variable. Activator of intravascular hydrolysis by LPL. Modulates hepatic TG metabolism	Predominantly in the liver
ApoB-48	264,000	CM	Necessary for assembly and secretion of chylomicrons from the small intestine	intestine
ApoB-100	540,000	VLDL, IDL, LDL	Necessary for assembly and secretion of VLDL from liver. Structural protein of VLDL, IDL and LDL. Ligand for LDL receptor	Liver
ApoC-I	6630	CM, VLDL, IDL, HDL	ApoC-I inhibits lipoprotein binding to its receptors. Potent inhibitor of cholesteryl ester transfer protein.	liver
ApoC-II	8900	CM, VLDL, IDL, HDL	Activator of lipoprotein lipase	
ApoC-III	8800	CM, VLDL, IDL, HDL	Inhibits lipoprotein lipase; increase VLDL secretion. ApoC-III can also stimulate several processes involved in atherogenesis and vascular inflammation. Interferes with remnant lipoprotein clearance.	Synthesized in the Liver and to a lesser extent in the intestine
ApoE	34,145	CM, VLDL, IDL, HDL	LDL receptor ligand for LDL and chylomicron remnants. Ligand for LRP. Role in reverse cholesterol transport	Predominantly in the liver

TG, triglyceride; Apo, Apolipoprotein; M.Wt, Molecular weight in Daltons.

1.2 Lipoprotein Metabolism

1.2.1 Exogenous Pathway: Transport of Dietary Lipids

In the small intestine, dietary TGs are hydrolyzed by the action of pancreatic lipases to form monoacylglycerols and fatty acids. These are then emulsified by bile acids along with dietary cholesterol to form micelles which are absorbed by the enterocytes of the intestine. Dietary lipids once absorbed are largely re-esterified. Dietary cholesterol is esterified in the enterocyte to form cholesterol esters (Rader & Hobbs, 2012). After absorption, fatty acids are converted to their acyl-coenzyme A (acyl-CoA) derivatives. Acyl-CoA then reacts with 2-monoacylglycerol to form TGs (Puri D, 2011). In the endoplasmic reticulum, the new TGs are incorporated with apolipoprotein B (ApoB), isoform B-48, cholesteryl esters (CEs), PLs, and cholesterol to form chylomicrons (CMs). The newly synthesized chylomicrons consist of a core of CE and TGs, with a surface of unesterified cholesterol and PL, and apolipoproteins B-48 (Rader & Hobbs, 2012). Nascent CMs (**Figure 1.2**) are then transferred to the left brachiocephalic vein of blood circulation via the intestinal lymphatic system. In the blood circulation, chylomicrons acquire apoC-II, apoC-III and apoE apolipoproteins (Ginsberg H. N, Zhang Y. L & A., 2005) and before they reach the liver, they are lipolysed by lipoprotein lipase (LPL) which is attached to heparan sulfate proteoglycans on the endothelial capillaries surrounding the adipose tissues, heart, and skeletal muscles. LPL hydrolyses TGs of the chylomicrons, in a process where ApoC-II acts as a cofactor, releasing FFAs and 2-monoacylglycerol. FFAs are then taken up by adjacent myocytes or adipocytes and either oxidized for energy usage or re-esterified and stored as TGs. In addition, some TGs are transferred to HDL and LDL in exchange for cholesterol ester. This exchange is catalyzed by CE transfer protein enzyme (CETP). On the other hand, cholesterol, PL and ApoC-II of the outer surface of the chylomicron particles are also dissociated and transferred to other particles such as HDL (Rader & Hobbs, 2012).

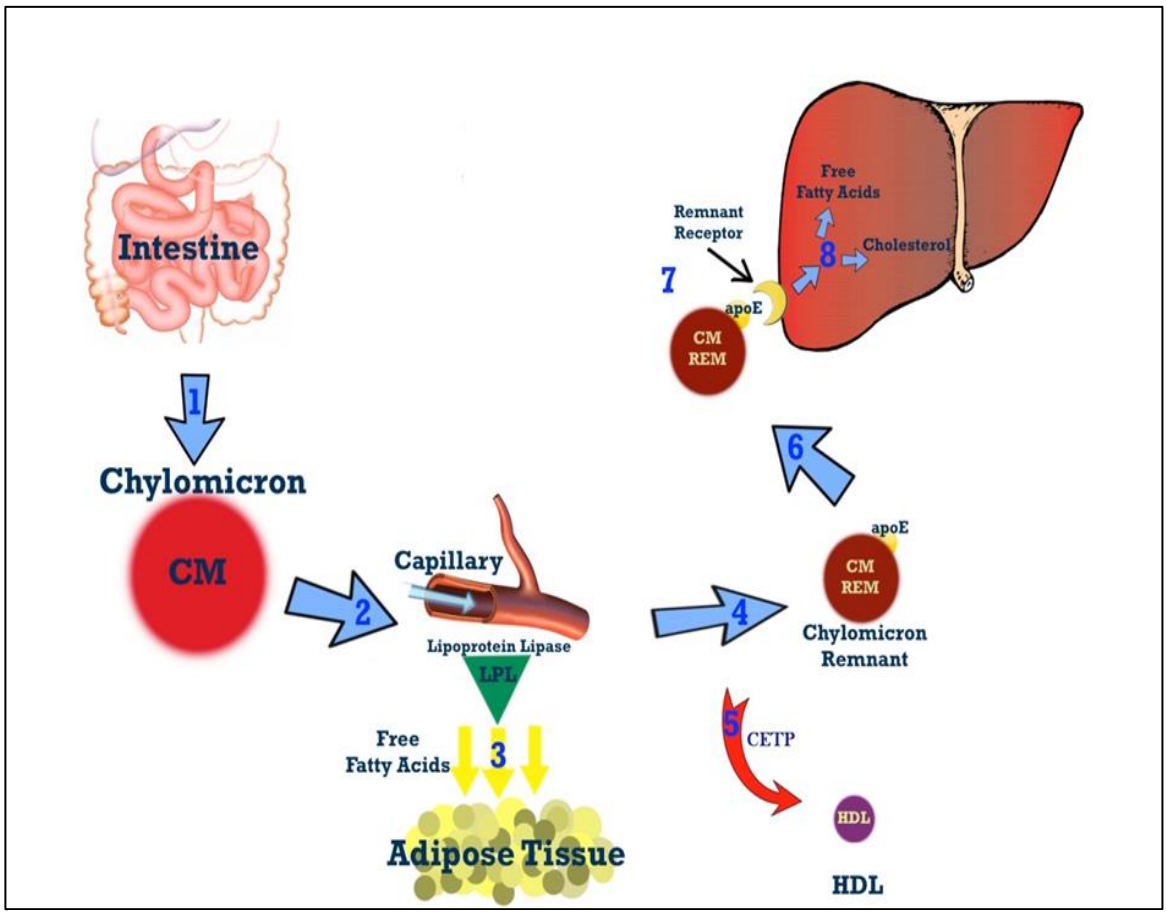


Figure 1.2 Exogenous pathway of lipoprotein metabolism. Hydrolysed TGs and cholesterol enter the enterocytes (intestinal cells) where cholesterol is esterified and new TGs are formed. CM formed from the incorporation of TGs, cholesterol ester, apoB-48, PL and cholesterol. Nascent CM enters the blood stream via the lymphatic system. CMs then acquire apoC-II, apoC-III and apoE then processed by LPL attached to the endothelial capillaries. LPL hydrolyses TG to form FFAs and 2-monoacylglycerol. FFAs are taken up by adipocytes or myocytes for energy expenditure or stored as TGs. Some TGs is also exchanged with cholesterol esters with other lipoproteins, like HDL, with the aid of CETP. PLs, cholesterol and apoC-II also detached for CM and transferred to other particles such as HDL. CM remnant (CMr) is taken up by the hepatic receptor LDLR. TG, triglyceride; CM , chylomicron ; CM REM, chylomicron remnant ; LPL, Lipoprotein lipase ;FFAs, free fatty acids ; CETP, cholesteryl ester transfer protein enzyme; PL, phospholipids[Source:(Aswar, http://www.slideshare.net/silky1/lipid-metabolism-and-hypolipedemic-drugs)].

These two processes produce smaller chylomicron remnants (CMRs) which retain both apoB-48 and apo-E from the chylomicron. CMRs are rapidly removed from the circulation and taken up by hepatic LDL receptor (LDLR) and LDL receptor-related protein (LRP) in a process that requires Apo-E (Redgrave, 2004). TGs from the CMRs are stored in the liver or repackaged with cholesterol and the Apo-B, isoform B-100, into VLDL. In normal individuals, postprandial TGs levels return to baseline within 8-10 hours after an intake of dietary fat (Meyer et al., 2008).

1.2.2 Endogenous Pathway: Transport of Hepatic Lipids

In the endogenous pathway of lipoprotein metabolism, TGs are distributed from the liver to other tissues. The liver assembles and secretes TG-rich VLDL (**Figure 1.3**) particles which transport TGs from the liver to peripheral tissues. VLDL is synthesized and secreted from the liver in a process that is apoB-100 dependent. VLDL particles are very similar to chylomicrons in protein composition with apoB-100, isoform of apoB, rather than apoB-48. In normal individuals, TG appear to be nearly the sole lipid in the core of nascent VLDL (there is ~1 mg of cholesterol for every 5 mg of TG) (Rader & Hobbs, 2012; Vega & Grundy, 2012). The packaging of hepatic TGs with apoB-100, cholesterol esters, PLs, and vitamin E to form nascent VLDL requires the action of the enzyme microsomal triglyceride transfer protein (MTP) (Sundaram & Yao, 2010). In the plasma, VLDL acquires multiple copies of apoE and apolipoproteins of the C series. TGs of VLDL are like CMs lipolysed into F.F.As and glycerol by LPL and its cofactor apoC-II. As VLDL shrink in size they are ultimately converted into VLDL remnants or intermediate density lipoproteins (IDL) which contains similar amounts of cholesterol ester and a smaller amount of TGs (Vega & Grundy, 2012). IDL TGs are in turn hydrolyzed by hepatic lipase (HL) to produce low density lipoprotein (LDL) or they are removed by the interaction of apolipoprotein E with the LDLR on the surface of the liver.

LDL transports cholesterol primarily to hepatocytes but also to peripheral tissues. ApoB-100 is responsible for the recognition and uptake of LDL by the LDL receptor, which clears approximately 60-80% of LDL in normal individuals. However, if LDL is oxidized, it can enter the macrophage through the scavenger receptors, CD36 and SR-A, on the surface of the macrophage or be taken up by vascular smooth muscle cells. When these macrophages become burdened with CEs, they transform into foam cells, which is a major step in the development of atherosclerosis. As LDL becomes lipid depleted, small dense LDL (sdLDL) is

produced. sdLDL has lower affinity for LDLR but is highly susceptible to oxidative modification. Thus sdLDL are believed to be more atherogenic than larger LDL particles (Soares & Costa, 2009).

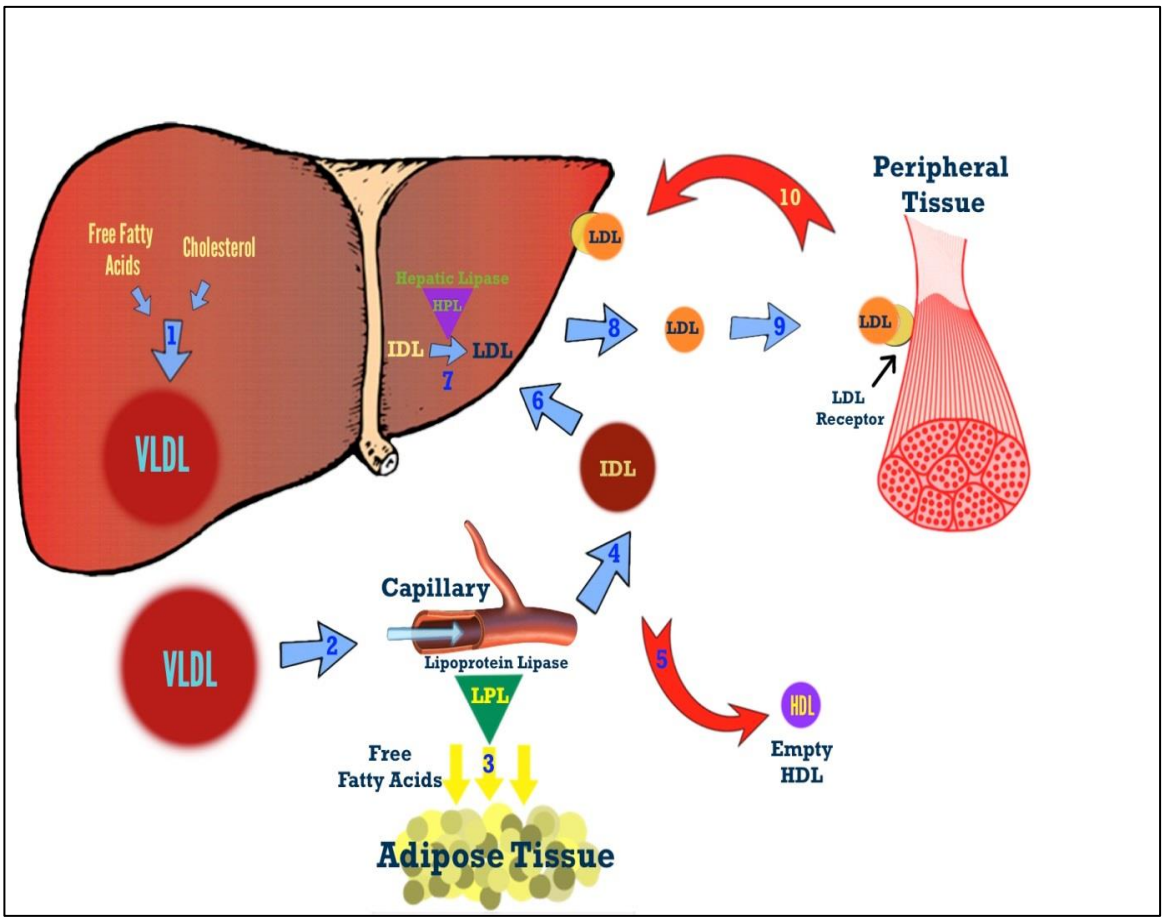


Figure 1.3 Endogenous pathway of lipoprotein metabolism. VLDL is secreted from the liver**.** In the plasma, VLDL acquires multiple copies of apoE and apolipoproteins of the C series. TGs of VLDL are lipolysed into free fatty acids and glycerol by LPL and its cofactor apoC-II. VLDL shrink in size converted into VLDL remnant or IDL . IDL TGs are hydrolysed by HL to produce LDL or they are removed by the interaction of apolipoprotein E with the LDL receptor on the liver surface. ApoB-100 is responsible for the recognition and uptake of LDL by the LDL receptor, which clears approximately 60-80% of LDL in normal individuals. TGs, triglycerides; LPL, lipoprotein lipase; HL, hepatic lipase. [Source: (Aswar, http://www.slideshare.net/silky1/lipid-metabolism-and-hypolipedemic-drugs)].

1.2.3 HDL Metabolism

HDLs are the smallest and most dense of the lipoproteins and the metabolism of these particles is somewhat more complex than that of the other major lipoprotein classes. HDL is thought to play a key role in reverse cholesterol transport (RCT), an important step that relieves peripheral cells from a cholesterol load.

HDL Production

Although most of the HDLs arise as discoidal particles, the majority of the HDL particles circulating in human plasma are spherical particles that contain a core of neutral lipids (CEs and a small amount of TG) surrounded by a surface monolayer that consist of PLs, apolipoproteins, and limited amount of unesterified cholesterol (Rye & Barter, 2014).

The discoidal particles are either generated in the liver before secretion into the extracellular space (Hamilton, Williams, Fielding & Havel, 1976) or assembled in the circulation from individual lipid and apolipoproteins constituents (Hara & Yokoyama, 1991). Discoidal HDLs consist of a PL bilayer surrounded by ≥2 apolipoprotein A-I molecules. These particles obtain unesterified cholesterol from cell membranes and other plasma lipoproteins. The unesterified cholesterol in discoidal HDL particles partitions between the PL acyl chains (Lund-Katz & Phillips, 1984).

Biogenesis of discoidal HDL

There are 4 main apolipoproteins in human HDLs, in order of decreasing abundance, are apolipoproteins A-I (apoA-I), apolipoproteins A-II (apoA-II), apolipoprotein A-IV (apoA-IV), and apolipoprotein E (apoE) (Rye & Barter, 2014). ApoA-I is produced in the liver and intestine. Hepatic apoA-I is first synthesized as a preprotein that is cleaved intercellularly by a single peptidase (Rye & Barter, 2014) **(Figure 1.4A)**. The resulting propeptide is secreted before cleavage by bone morphogenic protein-1 in a process that is facilitated by procollagen C-proteinase enhancer-2 (Chau, Fielding & Fielding, 2007) (Zhu, Gardner, Pullinger, Kane, Thompson & Francone, 2009). It has been established, by in vitro studies

that ≤45% of apoA-I is lipidated before it is secreted from hepatocytes (Ji, Wroblewski, Cai, de Beer, Webb & van der Westhuyzen, 2012). The initial lipidation of apoA-I occur in the endoplasmic reticulum and is independent of the ATP-binding cassette transporter AI (ABCA1). Further lipidation of apoA-1 takes place in the Golgi and at the plasma membrane in processes that are dependent on a dimeric form of ABCA1 (Maric, Kiss, Franklin & Marcel, 2005).

ApoA-I is also secreted from the liver into the extracellular space in a lipid-free or lipid-poor form. After secretion from the liver, the C-terminal domain of lipid–free apoA-I combine with an extracellular loop of ABCA1 in a process that commence the biogenesis of discoidal HDLs (Vedhachalam et al., 2004). It has been determined by recent studies that the ABCA1-dependent export of lipids from adipocytes and the intestine to apoA-I also makes a significant contribution to HDL biogenesis (Chung, Sawyer, Gebre, Maeda & Parks, 2011).

ABCA1 exports PLs from cell membranes to lipid-free/lipid-poor apoA-1 in the extracellular space, forming a discoidal PL/apoA-I complex that further accept cholesterol from cell membranes in a process that is dependent on ABCA1 as well (**Figure 1.4B**) (Wang, Silver, Thiele & Tall, 2001). Lipid-free/lipid-poor apoA-I can also form discoidal complexes with the PLs and cholesterol that dissociate from the surface of triglyceride-rich lipoproteins that are undergoing lipoplysis by LPL (Patsch, Gotto, Olivercrona & Eisenberg, 1978).

ApoA-II, the second most abundant HDL apolipoproteins, is also synthesized in the liver, where it attains PLs and cholesterol to form discoidal HDLs (Forte, Bielicki, Goth-Goldstein, Selmek & McCall, 1995). As apoA-II has high affinity for lipid and does not circulate in a lipid-free form, discoidal (A-II) HDLs are mostly assembled in the liver (Gillard, Lin, Massey & Pownall, 2009). It has been shown , from in vitro studies, that apoA-II accept the cholesterol and PLs that are released from cell membranes through ABCA1 as effectively as apoA-I (Remaley et al., 2001).

ApoA-IV is the third most abundant HDL apolipoproteins and has a much lower affinity for lipid than either apoA-I or apoA-II (Rye & Barter, 2014). ApoA-IV is synthesized mainly in the intestine and enters the circulation as a component of chylomicrons (Karathanasis, Yunis & Zannis, 1986) .Hydrolysis of chylomicron triglycerides by LPL depletes the particles of the core lipids which will generate redundant surface constituents, including apoA-IV, that

dissociates from chylomicrons and are subsequently incorporated into the HDL fraction (Rye & Barter, 2014).

Like apoA-I and apoA-II, apoE is synthesized predominantly in the liver, where it associates with VLDLs before it is secreted to the extracellular space (Gillard, Lin, Massey & Pownall, 2009). Once in the circulation, apoE is incorporated into the HDL fraction subsequent to the hydrolysis of VLDL triglycerides by LPL. Several reports indicated that the lipidation of apoE is also dependent on ABCA1, with in vivo and in vitro studies indicating that the N-terminal domain of the apolipoproteins is important for this interaction (Petropoulou, Gantz, Wang, Rensen & Kypreos, 2011).

It is important to mention that loss-of-function mutations in the ABCA1 gene, which reduce the export of cellular cholesterol and PLs to apolipoproteins, inhibit HDL biogenesis. This is exemplified by the low levels or complete absence of HDLs in people with Tangier disease (TD) (Rye & Barter, 2014). ABCA1 gene transcription and cholesterol efflux to apoA-I are both markedly enhanced when the nuclear liver X receptor (LXR) complex is activated by oxysterols and peroxisome proliferator- activated receptor α/ɣ ligands (Chawla et al., 2001). More recently, activation of the VLDL receptor and the apoE receptor 2 have also been shown to increase ABCA1 mRNA levels and protein expression, as well as cholesterol efflux to apoA-I (Chen et al., 2012). On the other hand, macrophage ABCA1 gene expression and the efflux of cholesterol to apoA-I are suppressed by members of the oxysterol-binding protein-related protein family (Yan et al., 2008).

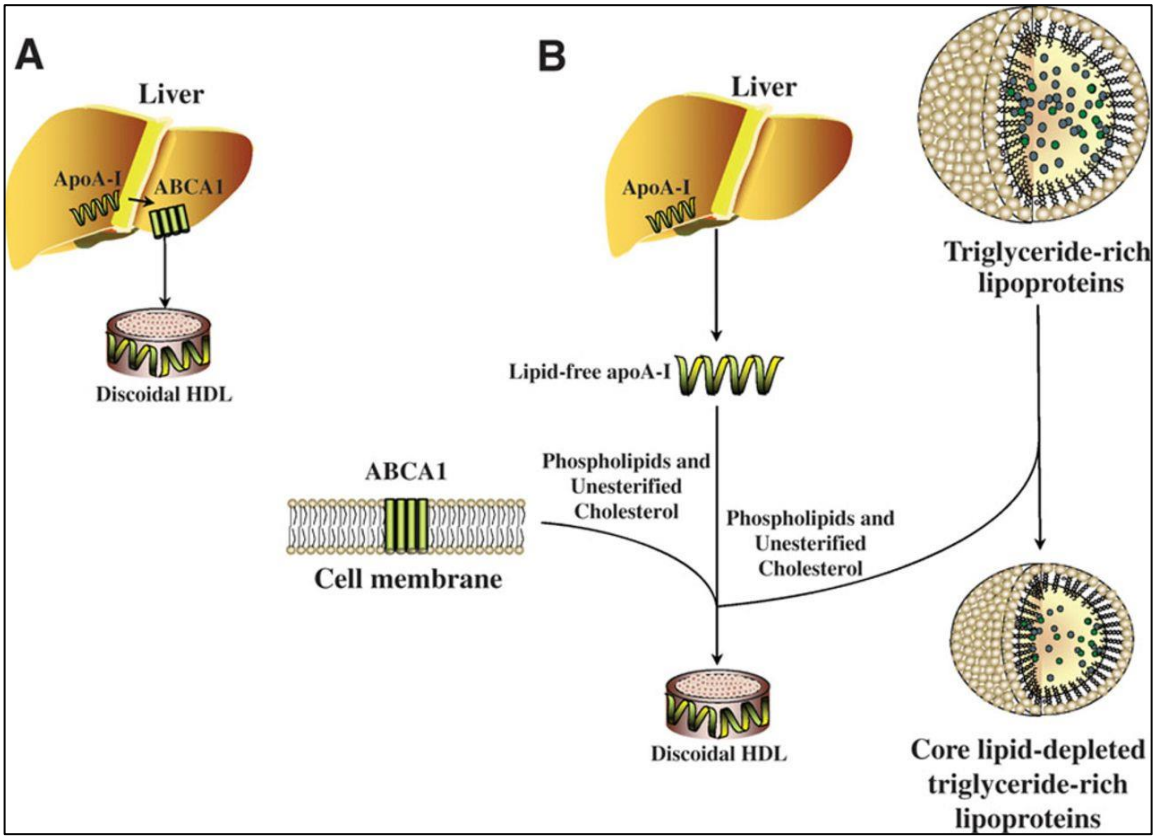


Figure 1.4 Biogenesis of apolipoprotein A-I (apoA-I)–containing discoidal high-density lipoproteins (HDLs). **A**, ApoA-I is synthesized mainly in the liver. A proportion of the apoA-I in hepatocytes interacts with ABCA1 to acquire PLs and unesterified cholesterol, generating discoidal HDLs that are secreted into the extracellular space. **B**, ApoA-I is secreted from the liver into the extracellular space in a lipid-free or lipid-poor form. This apoA-I accepts PLs and unesterified cholesterol from cell membranes that express ABCA1, forming discoidal HDLs. Discoidal HDLs are also generated when lipid-free apoA-I in the plasma acquires PLs and unesterified cholesterol that are shed from the surface of triglyceride-rich lipoproteins that are being hydrolyzed by lipoprotein lipase. ABCA1, ATP-binding cassette transporter A1; apoA-I, apolipoproteinA-I; PL, phospholipid [Source: (Rye & Barter, 2014)]

Biogenesis of Spherical HDLs

As mentioned, ABCA1 plays a critical role in the biogenesis of discoidal HDLs. Nonetheless, this represents only the first step in the formation of mature, spherical HDLs (Rye & Barter, 2014).

The discoidal HDL particles that are either secreted from the liver or assembled in the plasma are rapidly converted into mature spherical HDLs by Lecithin: cholesterol acyltransferase (LCAT). LCAT is an HDL associated enzyme which generates almost all of the CEs in plasma. Evidence of the importance of LCAT in the formation of mature, spherical HDLs comes from studies of people with LCAT deficiency, in which HDLs are predominantly discoidal (Asztalos et al., 2007). Results from in vitro studies have indicated that lipid-free (or lipid-poor) apoA-I can also be incorporated directly into preexisting spherical HDLs that are increasing in size as a consequence of their interaction with LCAT (Liang, Rye & Barter, 1996).

LCAT enzyme mediates the formation of cholesterol esters in human plasma by the transfer of the *sn*-2 acyl chain of the phosphotidylcholine in discoidal HDLs (**Figure 1.5**). This produces fatty acid groups that are transferred by LCAT to the 3-hydroxyl group of cholesterol in a reaction that generates CEs and lysophosphotidylcholine. Since CEs are extremely hydrophobic, they partition into the inner core of the discoidal HDLs, which are thereby converted into spherical particles. The lysophosphotidylcholine that is generated by the LCAT reaction associates with albumin. The LCAT reaction depletes discoidal HDLs of unesterified cholesterol. This creates a concentration gradient whereby additional cholesterol is transferred from other lipoproteins and cell membranes to the HDL surface, resulting in persisting cholesterol esterification and the continuing generation of HDL CEs (Rye & Barter, 2014).

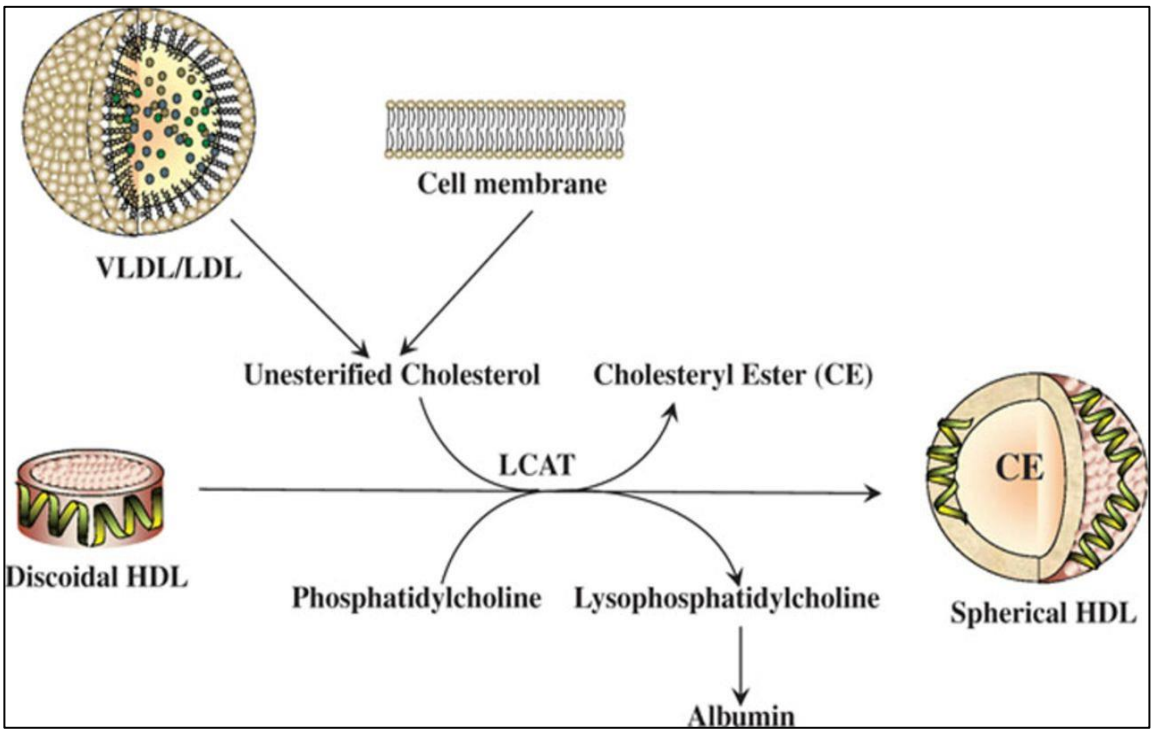


Figure 1.5 Biogenesis of spherical high-density lipoproteins (HDLs). Lecithin: cholesterol acyltransferase (LCAT) hydrolyses PLs in discoidal HDLs, generating fatty acyl groups and lysophosphatidylcholine. The fatty acyl groups are transferred to unesterified cholesterol, generating cholesteryl esters that partition into the particle core. The lysophosphatidylcholine associates with albumin. The LCAT reaction depletes discoidal HDLs of unesterified cholesterol, establishing a concentration gradient whereby additional cholesterol is transferred from very-low-density lipoprotein (VLDLs), low-density lipoprotein (LDLs), and cell membranes to the HDL surface for subsequent esterification by LCAT. LCAT, lecithin: cholesterol acyltransferase; PLs, phospholipids. [Source: (Rye & Barter, 2014)]

Studies of human plasma have confirmed that the acyl ester hydrolase and cholesterol transesterification activities of LCAT are controlled by apolipoprotein cofactors. ApoA-1 is the main activator of LCAT (Fielding, Shore & Fielding, 1972), with the N-terminal domain of the apolipoprotein being responsible for this action in vitro and in vivo (Scott et al., 2001). In vitro studies of discoidal reconstituted HDLs (rHDLs) consisting of apoA-IV and apoE complexed with phosphatidylcholine have proven that these apolipoproteins also stimulate LCAT, but with much lower catalytic efficiencies than apoA-I (Rye, Bright, Psaltis & Barter, 2006). The indication regarding whether LCAT generates spherical apoE-containing HDLs that also contain apoA-I is paradoxical (Rye & Barter, 2014). A study showing that HDLs that contain apo-IV are also deficient in apoA-I is consistent with LCAT interacting specifically with apoA-IV- comprising discoidal HDLs (Ehnholm et al., 1998).

The HDLs in normal plasma have been classified on the basis of their apolipoprotein content into those that contain apoA-I, but not apoA-II, and those that contain apoA-I as well as apoA-II (Cheung & Albers, 1984). In vitro studies of reconstituted HDL (rHDL) have proven that apoA-II is not a cofactor for LCAT (Durbin & Jonas, 1999).

The PL composition of discoidal HDLs regulated their ability to act as substrates for the LCAT reaction. For example, the presence of sphingomyelin in discoidal HDLs prevents cholesterol esterification by LCAT (Subbaiah, Horvath & Achar, 2006). This is because of a strong interaction between unesterified cholesterol and sphingomyelin in the discoidal HDLs, which decreases the amount of cholesterol available for esterification by LCAT (Rye & Barter, 2014). The kinetics of LCAT reaction is also controlled by the length and unsaturation of the PL acyl chains in discoidal HDLs (Jonas, Zorich, Kezdy & Trick, 1987).

Regulation of HDL Subpopulation Distribution

The HDL fraction in human plasma consists of various subpopulations of particles that are continually being remodeled and interconverted by plasma factors.

HDLs circulating in human plasma have been classified on the basis of hydrated density into 2 main subfractions: HDL2 and HDL3. HDL that contain apoA-I, but not apoA-II, are mainly found in the HDL2 fraction, whereas HDLs that contain apoA-I as well as apoA-II are generally associated with smaller and denser particles in the HDL3 subfraction. An association of HDL2 and HDL3 levels with a decreased risk of myocardial infraction has been reported in the

Physicians' Study (Stampfer, Sacks, Salvini, Willett & Hennekens, 1991). This association was different in other studies which has revealed that the inverse relationship between HDL2 subfraction and the progress of ischemic heart disease in participants in the Quebec Cardiovascular Study is greater than that of HDL3 (Lamarche, Moorjani, Cantin, Dagenais, Lupien & Despres, 1997).

Non-denaturing gradient gel electrophoresis is frequently used to separate HDLs on the basis of size into 5 different subpopulations of particles. In order of decreasing size, these are HDL2b, HDL2a, HDL3a, HDL3b, and HDL3c (Blanche, Gong, Forte & Nichols, 1981). HDL can also be resolved on the basis of surface charge into particles that migrate to a pre-β-position or α-position through agarose gel electrophoresis. Lipid-free apoA-I, lipid-poor apoA-I, and most discoidal HDLs migrate to a pre-β-position, whereas spherical HDLs exhibit α-migration (Castro & Fielding, 1988).

The subpopulation distribution of HDLs is extensively regulated by multiple plasma factors, including 2 members of the bactericidal permeability-increasing protein and lipopolysaccharide-binding protein family: CETP and phospholipid transfer protein (PLTP), 2 members of the TG lipase gene family, HL and endothelial lipase (EL), and the group IIA secretory phospholipase A_2 ($sPLA_2$) (Rye & Barter, 2014).

CETP promotes the transfers of CEs out of HDLs and the transfer of TG out from VLDLs which generate HDLs that are enriched in TG. Because a TG molecule is significantly larger than a CE molecule, the substitution of CEs with TG increases HDL size (Rye, Hime & Barter, 1995).

Activity of CETP decreases plasma HDL cholesterol levels and reduces HDL particle size (Rye & Barter, 2014). The rate of CETP-mediated transfers of core lipids between HDLs and TG-rich lipoproteins appears to be independent of HDL apolipoprotein composition (Rye & Barter, 1994).

CETP-mediated remodeling of HDLs into large and small particles is also altered by PL composition. HDL particles that contain PLs with long, polyunsaturated sn-2 phospholipid acyl chains are remodeled more comprehensively by CETP than those with shorter, more saturated sn-2 acyl chains (Rye et al., 2002). This could be because of the long,

polyunsaturated phospholipid acyl chains destabilizing the particle structure and excluding apoA-I from the surface (Rye et al., 2002).

In vitro studies have established that **PLTP** transfers PLs between HDLs and VLDLs, as well as between particles within the HDL fraction. PLTP also remodels HDLs into large and small particles by processes that are accompanied by the dissociation of lipid-free or lipid-poor apoA-I (Tu, Nishida & Nishida, 1993). The PLTP-mediated dissociation of apoA-I from HDLs has been reported to stimulate HDL biogenesis by enhancing cholesterol and PL efflux from cells that express ABCA1. It is also confirmed that PLTP is incapable of remodeling HDLs when its PL transfer activity is repressed, indicating that there is a great interdependence between the lipid transfer and HDL remodeling function of PLTP (Huuskonen, Olkkonen, Ehnholm, Metso, Julkunen & Jauhiainen, 2000).

Studies with spherical apoA-I-containing rHDLs have clarified the mechanism by which PLTP remodels HDLs into large and small particles. The process involves an initial particle fusion, with following rearrangement of the unstable fusion product via 2 distinct pathways **(Figure 1.6).** The first pathway (pathway 1) produces small HDL particles without dissociation of lipid-free or lipid-poor apoA-I (Settasatian et al., 2001). The second pathway (pathway 2) involves the dissociation of lipid-free/lipid-poor apoA-I from the fusion product and the formation of large spherical HDLs (Settasatian et al., 2001).

PLTP also remodels spherical apoE-containing rHDLs into large and small particles by a mechanism that involves sequential particle fusions and rearrangements of the fusion products in processes that do not involve the dissociation of apoE **(Figure 1.7)** (Settasatian et al., 2001).

The remodeling of HDLs by PLTP is regulated by both the apolipoprotein and the core lipid composition of the particles (Rye & Barter, 2014). The incorporation of apoA-II into apoA-I-containing HDL particles isolated from human plasma prevents both the PLTP-mediated remodeling of HDLs into large and small particles and the detachment of apoA-I (Pussinen, Jauhiainen & Ehnholm, 1997).

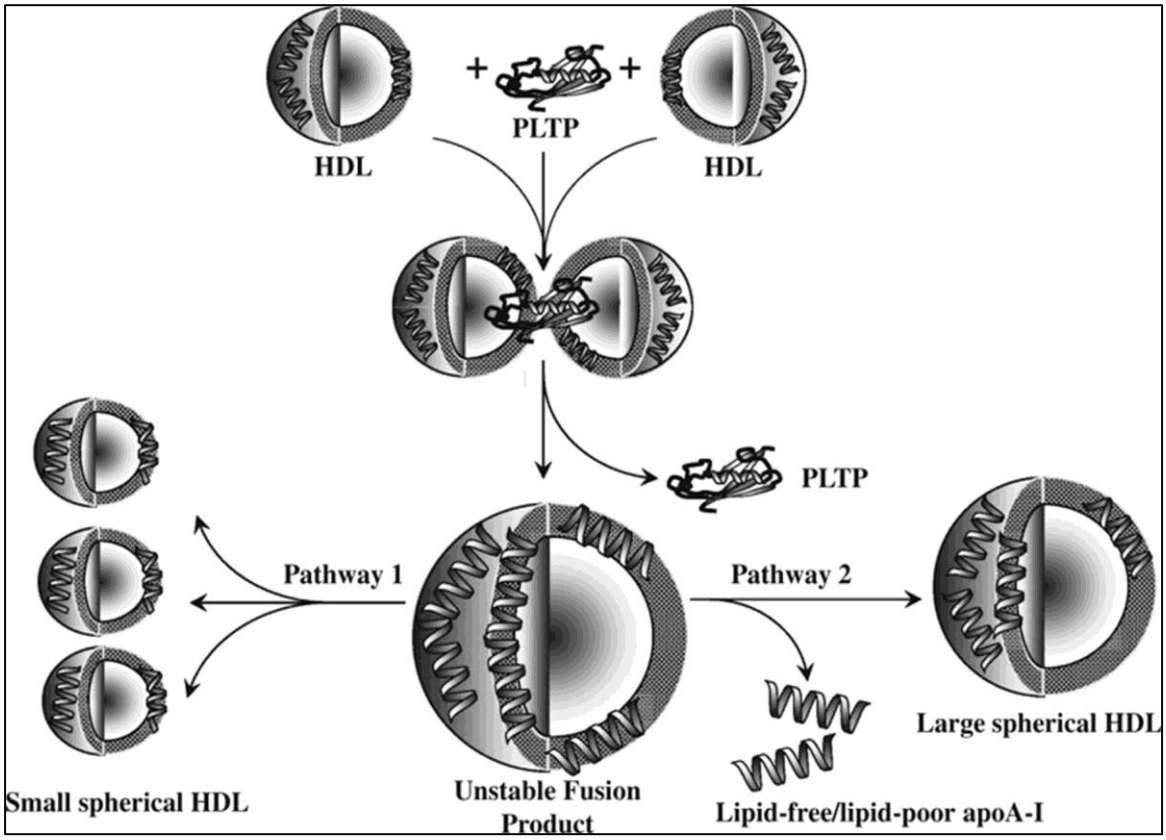


Figure 1.6 Remodeling of apolipoprotein A-I (apoA-I)–containing high-density lipoproteins (HDLs) by PLTP. PLTP interacts with apoA-I–containing HDLs to generate a large, unstable fusion product. A proportion of the fusion products is remodeled into small HDL particles in a process that does not cause the dissociation of apoA-I (pathway 1). The fusion product is also remodeled into a more stable large particle in a process that involves the dissociation of lipid-free/lipid-poor apoA-I (pathway 2).PLTP, phospholipid transfer protein. [Source: (Rye & Barter, 2014)]

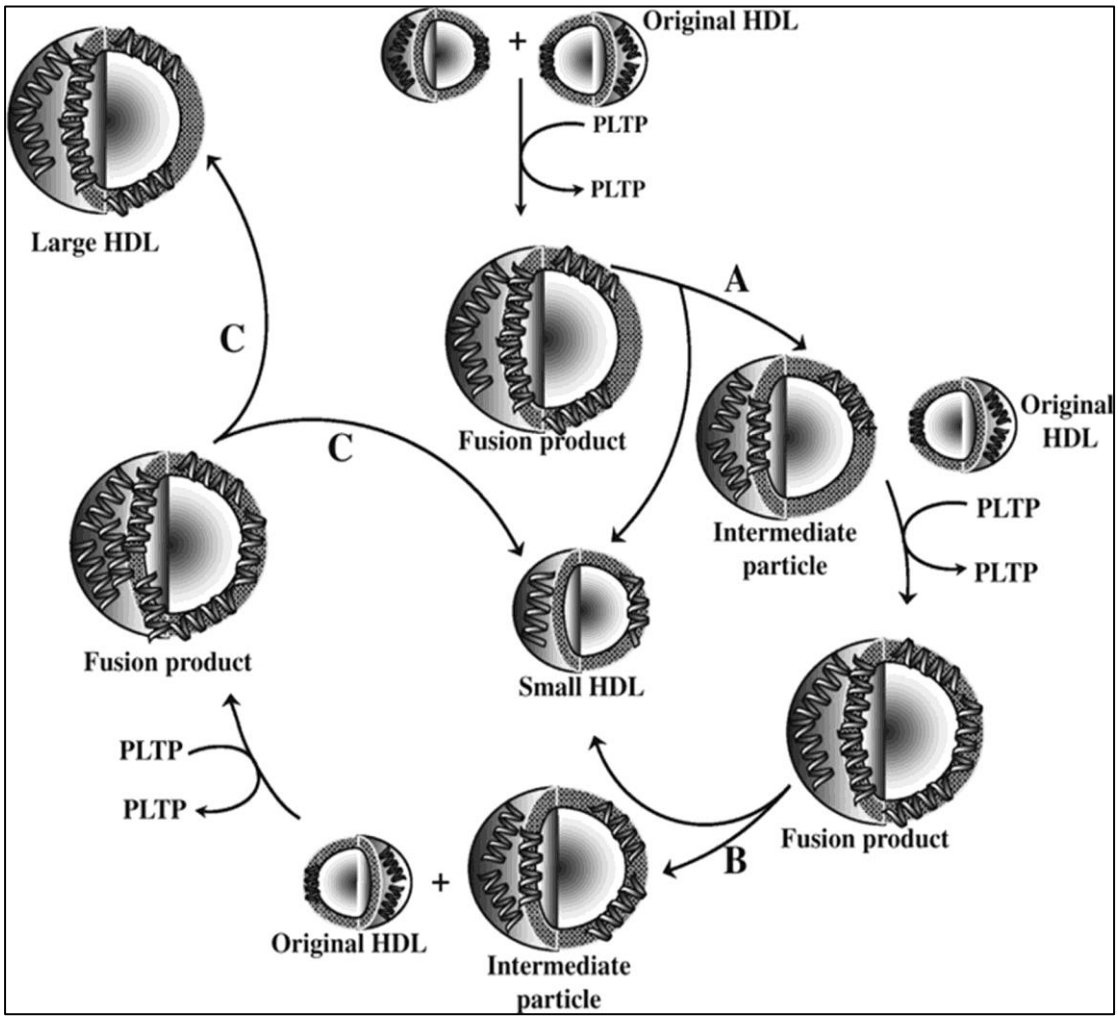


Figure 1.7 Remodeling of apolipoprotein E (apoE)–containing high-density lipoproteins (HDLs) by PLTP. PLTP remodels spherical apoE-containing HDLs into large and small particles. A, The remodeling is initiated by particle fusion, followed by rearrangement of the fusion product into a small HDL particle and an unstable intermediate particle. B, PLTP promotes the fusion of the intermediate particle with an original HDL particle to generate another fusion product that rearranges into a small HDL particle and a second intermediate particle. C, In the final step, PLTP promotes the fusion of the second intermediate particle with an original HDL particle to generate another fusion product that rearranges into large and small HDL particles. PLTP, phospholipid transfer protein. [Source: (Rye & Barter, 2014)]

Although **HL** has a significant phospholipase activity, it preferentially hydrolyses HDL triglycerides. HDL particles that become TG-enriched after interaction with CETP and VLDLs are excellent substrates of HL (Rye & Barter, 2014). The hydrolysis of HDL TGs by HL produces small core lipid-depleted particles and promotes the dissociation of lipid-free/lipid-poor apoA-I **(Figure 1.8)** (Barrans et al., 1994). This is also aligned with the results of human genetic studies showing that polymorphisms in the HL gene that decrease its activity are associated with increased HDL2 levels (Carr, Ayyobi, Murdoch, Deeb & Brunzell, 2002), and results of animal studies in which transgenic overexpression of human HL in rabbits and mice decreases HDL levels (Fan et al., 1994).

The apolipoprotein and PL composition of HDLs both influence on the reactivity with HL. For example, the presence of apoA-II in HDLs inhibits the HL-mediated hydrolysis of both PLs and TG in vitro (Hime, Barter & Rye, 1998) and in vivo (Zhong, Goldberg, Bruce, Rubin, Breslow & Tall, 1994). On the other hand, apoA-I, irrespective of whether it is present in HDLs that also contain apoA-II, enhances the HL-mediated hydrolysis of HDL PLs and TGs (Hime, Barter & Rye, 2001). Moreover, the rate at which HL hydrolyses PLs and TGs in rHDLs is considerably greater in particles that contain apoE compared with particles that contain apoA-I (Hime, Drew, Hahn, Barter & Rye, 2004).

Although **endothelial lipase** (EL) and HL belong to the same gene family, EL has a very different substrate specificity from that of HL. HL favorably hydrolyses HDL TGs, whereas EL has a preference for HDL phospholipids and low TG lipase activity (Jaye et al., 1999). EL also differs from HL in its ability to remodel HDLs. The phospholipase activity of EL modestly decreases rHDL size in vitro in a process that is not accompanied by the dissociation of lipid-free or lipid-poor apoA-I (Jahangiri, Rader, Marchadier, Curtiss, Bonnet & Rye, 2005). This reflects the inability of EL to hydrolyze TG and reduce the core lipid content of the particles (Rye & Barter, 2014).

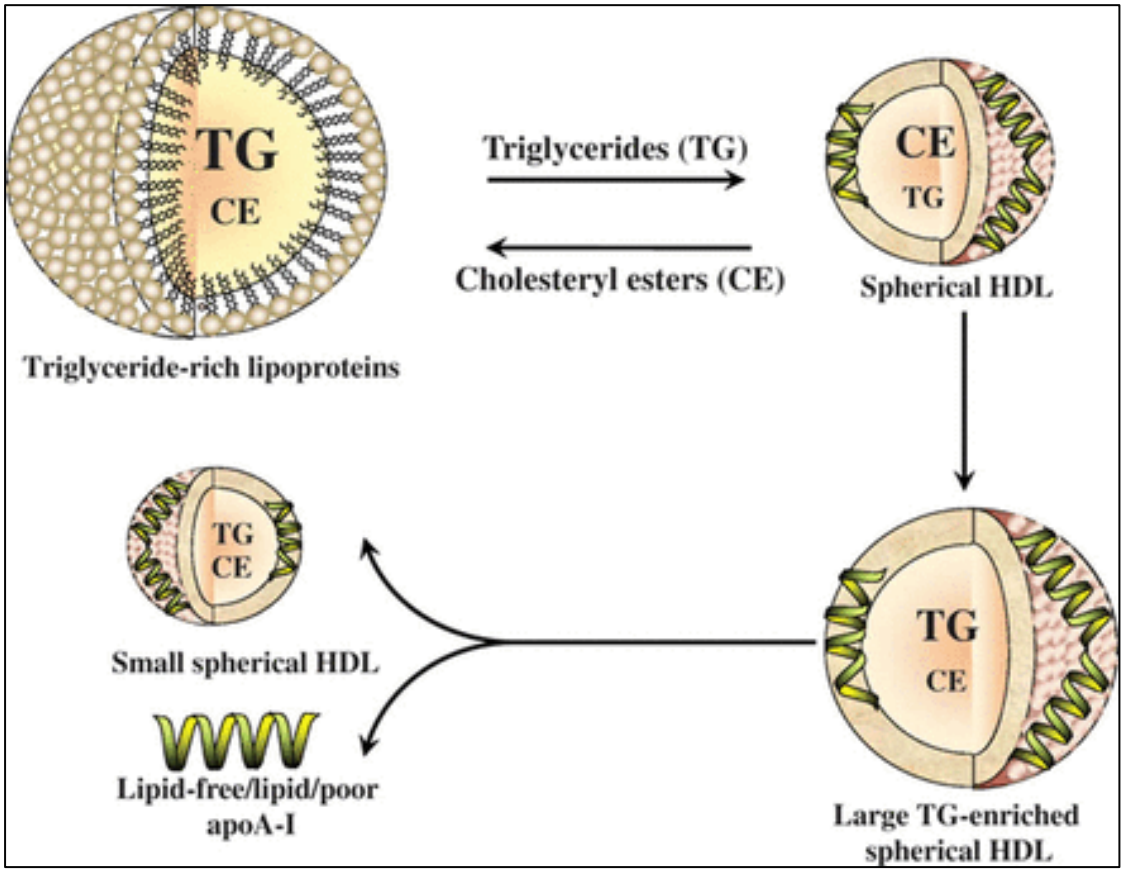


Figure 1.8 Remodeling of high-density lipoproteins (HDLs) by cholesteryl ester transfer protein (CETP) and hepatic lipase. HDL particles that have become TG-enriched by interacting with CETP and very-low-density lipoproteins are substrates for hepatic lipase. Hepatic lipase hydrolyses the TGs in TG-enriched HDLs, generating small core lipid–depleted HDL particles from which lipid-free/lipid-poor apolipoprotein A-I dissociates. TG, Trigyceride; CE, cholesterol ester. [Source: (Rye & Barter, 2014)].

In vitro studies have established that the phospholipase activity of EL is regulated by both the apolipoprotein and PL composition of HDLs, with spherical apoA-II-containing rHDLs being weak substrates for EL in vitro (Caiazza, Jahangiri, Rader, Marchadier & Rye, 2004). This was also confirmed in mice transgenic for human apoA-I and human A-II (Broedl, Jin, Fuki, Millar & Rader, 2006).

The specificity of EL for HDL phospholipids is also distinct from that of HL, with EL preferentially hydrolyzing PLs with long polyunsaturated sn-2 acyl chains as opposed to HL, which has a preference for PLs with short saturated acyl chains (Duong, Psaltis, Rader, Marchadier, Barter & Rye, 2003).

The group IIA **$sPLA_2$** is an acute phase protein that associates with HDLs under inflammatory conditions and hydrolyses HDL phospholipid sn-2 acyl ester bonds. Transgenic overexpression of $sPLA_2$ in mice increases the fractional catabolic rate of HDLs (Tietge et al., 2000). It also reduces HDL cholesterol levels (Tietge et al., 2000) and particle size. $sPLA_2$ deficiency in mice, by contrast, does not affect plasma HDL levels (Burton et al., 2002).

HDL Catabolism

There is compelling evidence to suggest that the clearance of intact HDL particles from the circulation is minimal and that most HDL components are catabolized individually (Rye & Barter, 2014). The major sites of catabolism of the protein components of HDL particles are the liver and kidney (Sriram et al., 2011).

CEs are selectively removed from HDLs when they bind to hepatic scavenger receptor B1 (SR-B1). In addition, CEs are also selectively removed from HDLs by CETP, which transfers them to other lipoprotein particles. On the other hand, the lipid-free and lipid-poor apoA-I that dissociates from HDL particles as a consequence of CETP-mediated core lipid transfers to other lipoproteins may be cleared from the circulation by the endocytic receptors, megalin and cubulin, which are expressed in the kidney (Rye & Barter, 2014).

SR-B1 promotes the selective uptake of CEs from HDLs into the liver and steriodegenic tissues (Brundert et al., 2005). In vitro studies have indicated that HL facilitates this process by a process that involves the binding of HDLs to the cell surface (Lambert, Chase, Dugi,

Bensadoun, Brewer & Santamarina-Fojo, 1999). The significance of SR-B1 in regulating plasma HDL cholesterol levels is confirmed from reports of mice in which hepatic overexpression of SR-B1 is associated with decreased levels of HDL cholesterol (Kozarsky, Donahee, Rigotti, Iqbal, Edelman & Krieger, 1997) and of SR-B1-null mice that have increased HDL cholesterol levels (Rigotti, Trigatti, Penman, Rayburn, Herz & Krieger, 1997). In vitro studies have indicated that the apolipoprotein composition of HDLs regulate the selective uptake of CEs by SR-B1, although the results are conflicting (Rye & Barter, 2014).

Moreover, HDL particles can be removed from the circulation by holoparticle HDL receptor, HDLR, Indeed, hepatic cells rapidly take up holo-HDL particles which are accumulated in endosomal components and can be subsequently transferred to mutivesicular bodies and, to a minor degree, to alysosomes for degradation (Rohrl et al., 2010).

The overall HDL metabolism is shown in **Figure 1.9**

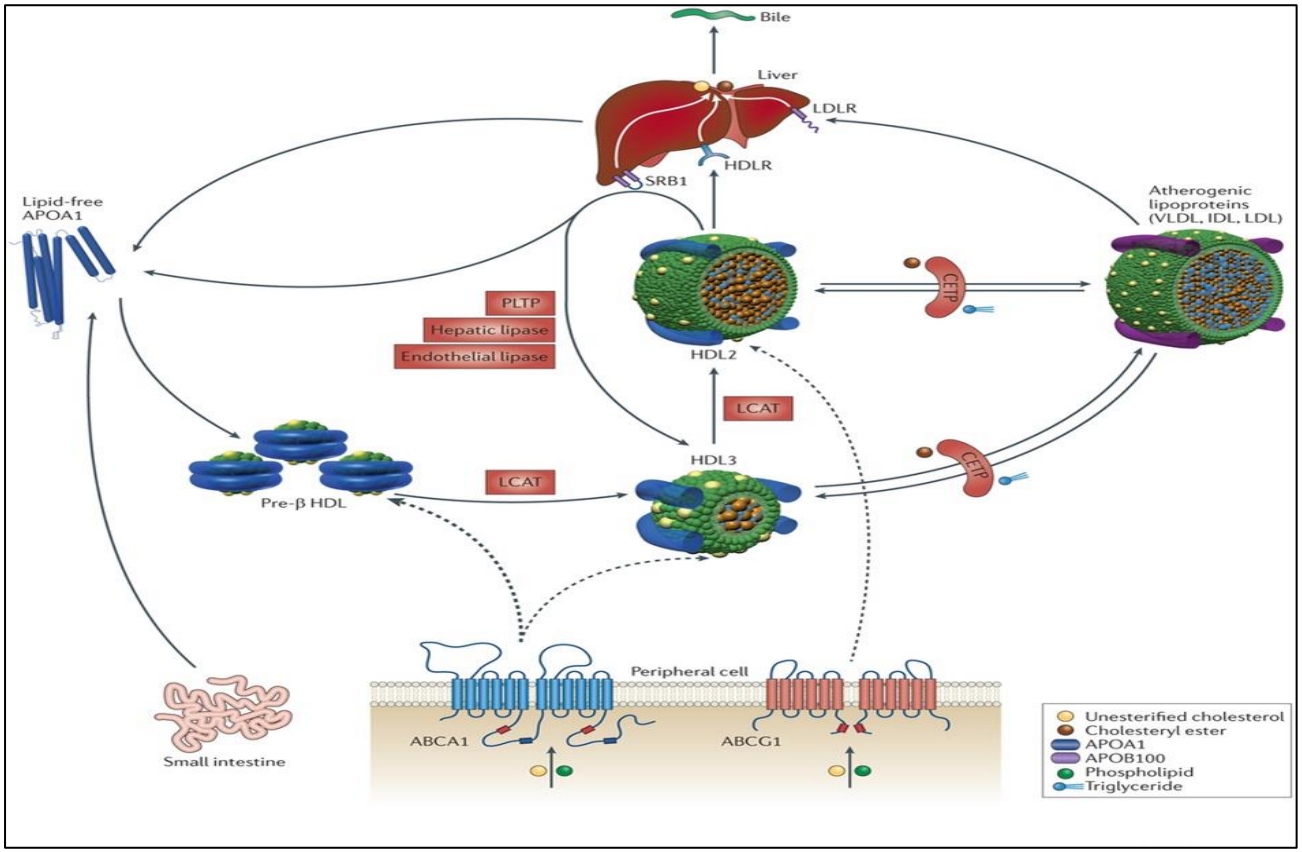


Figure 1.9 HDL metabolism. Lipid free apoA-I are synthesized by the intestine and hepatocytes. Once in the circulation, this lipid poor HDL triggers free cholesterol efflux and PL release from cellular membranes via membrane protein (ABCA1). Lipid poor, discoid HDLs undergo remodeling under the action of LCAT which mediates esterification of unesterified cholesterol and produce large spherical HDL particles with a neutral lipid core of cholesteryl ester and TG. HDL particles gain access surface material including unesterified cholesterol released during the lipolysis of the TG-rich lipoproteins via PLTP resulting in HDL2. CETP facilitates the exchange of cholesteryl esters from HDL2 for TGs in apoB containing lipoproteins results cholesterol ester-depleted and TG-enriched HDL particles. These TG-enriched particles are subject to hydrolysis of their TG and PL content by the action of HL. Those two actions on HDL2, from CETP and from HL, result in the formation of small, dense HDL3 particles and lipid-free or lipid-poor apoA-1 which can interact in further lipidation cycles with ABCA1. In addition to SRB1- mediated uptake of cholesterol and cholesterol esters from HDL by hepatocytes, HDL can be catabolized in the liver through its uptake as holoparticles by an HDL receptor (HDLR) which is still unidentified. APOA1, apolipoprotein A-I; PL, phospholipid; LCAT, lecithin: cholesterol acyltransferase; PLTP, phospholipid transfer protein, CETP, cholesterol ester transfer protein; [From: Kingwell et al 2014 (Kingwell, Chapman, Kontush & Miller, 2014)].

1.3 High Density Lipoprotein Composition

HDLs are the smallest and densest of the plasma lipoproteins (Barter & Rye, 1996). They comprise a heterogeneous group of lipoprotein particles with density ranging from 1.063 to 1.21 $g.mL^{-1}$ and small size, 8-10 nm. Like other plasma lipoproteins, they are composed of a core of CE and triglycerides surrounded by an amphipathic layer of free cholesterol, PLs and proteins (Barter & Rye, 1996).

1.3.1 Proteomes

As HDL is highly rich in proteins compared to other lipoprotein species, HDL apoproteins are considered the major building blocks of the HDL particle. The many proteins found on HDL have been divided into several major subgroups; apolipoproteins, enzymes, lipid transfer proteins, acute-phase response proteins, complement components, proteinase inhibitors and other protein components (**Table 1.3** and **Table 1.4**) (Kontush, Lindahl, Lhomme, Calabresi, Chapman & Davidson, 2015). The discovery of minor proteins involved in complement regulation and protection from infection and acute-phase response , like serum amyloid A (SAA) which is a major positive acute phase reactant, has abolished the traditional view that only apolipoproteins and enzymes have a biological importance in HDL (Benditt, Eriksen & Hanson, 1979). Recently, almost 200 proteins have been identified within the HDL proteome and about half of them have been independently confirmed in three separate studies (Shah, Tan, Long & Davidson, 2013).

Essentially, all HDL particles are believed to contain apoA-I (Asztalos & Schaefer, 2003a; Schaefer, Santos & Asztalos, 2010). Apolipoprotein A-I (apoA-I: M_r 28 kDa) is the major protein moiety of HDL which accounts for about 70% of total HDL protein mass with apolipoprotein A-II being the second most abundant protein, accounting for 15-20% (Kontush & Chapman, 2011). Other proteins include the enzyme LCAT (Glomset, 1968), (Calabresi & Franceschini, 2010) and CETP (Brousseau et al., 2005; Brousseau et al., 2009).

Moreover, other diverse proteins has been revealed by mass spectrometry, like proteins involved in heme metabolism, platelet regulation, vitamin binding, or immunity (Shah, Tan, Long & Davidson, 2013) which suggest that the vast range of HDL proteins collaborate to form subspecies of particles with possible range of individual functions.

Table 1.3 Major Component of HDL Proteome (part 1) [Source:(Kontush, Lindahl, Lhomme, Calabresi, Chapman & Davidson, 2015)].

	Protein	M_r, kDa	Major Function	Number of proteomic studies in which the protein was detected*
Apolipoproteins	ApoA-I	28	Major structural and functional apolipoprotein, LCAT activator	14
	ApoA-II	17	Structural and functional apolipoprotein	13
	ApoA-IV	46	Structural and functional apolipoprotein	14
	ApoC-I	6.6	Modulator of CETP activity, LCAT activator	12
	ApoC-II	8.8	Activator of LPL	12
	ApoC-III	8.8	Inhibitor of LPL	14
	ApoC-IV	11	Regulates TG metabolism	6
	ApoD	19	Binding of small hydrophobic molecules	11
	ApoE	34	Structural and functional apolipoprotein, ligand for LDLR and LRP	13
	ApoF	29	Inhibitor of CETP	8
	ApoH	38	Binding of negatively charged molecules	8
	ApoJ	70	Binding of hydrophobic molecules, interaction with cell receptors	11
	ApoL-I	44/46	Trypanolytic factor of human serum	14
	ApoM	25	Binding of small hydrophobic molecules	12
Enzymes	LCAT	63	Esterification of cholesterol to cholesterol esters	4
	PON1	43	Calcium-dependent lactonase	12
	PAF-AH (LpPLA2)	53	Hydrolysis of short-chain oxidized phospholipids	
	GSPx-3	22	Reduction of hydroperoxides by glutathione	

*Only proteins detected in more than 50 % of 14 proteomic studies (Shah, Tan, Long & Davidson, 2013) are listed together with seven others previously known to be associated with HDL (apoC-IV, apoH, LCAT, PAF-AH, GSPx-3, PLTP, CETP).

Table 1.4 Major Component of HDL Proteome (part 2). [Source:(Kontush, Lindahl, Lhomme, Calabresi, Chapman & Davidson, 2015)].

	Protein	M_r, kDa	Major Function	Number of proteomic studies in which the protein was detected*
Lipid transfer proteins	PLTP	78	Conversion of HDL into larger and smaller particles, transport of LPS	5
	CETP	74	Heteroexchange of CE and TG and homoexchange of PL between HDL and apoB-containing lipoproteins	3
Acute-phase proteins	SAA1	12	Major acute-phase reactant	10
	SAA4	15	Minor acute-phase reactant	10
	Alpha-2-HS-glycoprotein	39	Negative acute-phase reactant	9
	Fibrinogen alpha chain	95	Precursor of fibrin, cofactor in platelet aggregation	10
Complement components	C3	187	Complement activation	9
Proteinase inhibitors	Alpha-1-antitrypsin	52	Inhibitor of serine proteinases	11
	Hrp	39	Decoy substrate to prevent proteolysis	10
Other proteins	Transthyretin	55	Thyroid hormone binding and transport	12
	Serotransferrin	75	Iron binding and transport	10
	Vitamin D-binding protein	58	Vitamin D binding and transport	10
	Alpha-1B-glycoprotein	54	Unknown	9
	Hemopexin	52	Heme binding and transport	8

* Only proteins detected in more than 50 % of 14 proteomic studies (Shah, Tan, Long & Davidson, 2013) are listed together with seven others previously known to be associated with HDL (apoC-IV, apoH, LCAT, PAF-AH, GSPx-3, PLTP, CETP).

Apolipoprotiens

ApoA-I is the chief structural and functional HDL protein which accounts for approximately 70% of total HDL protein. Almost all HDL particles are believed to contain apoA-I (Kontush, Lindahl, Lhomme, Calabresi, Chapman & Davidson, 2015). Major functions of apoA-I involve interaction with cellular receptors, stimulation of LCAT and providing HDL with multiple anti-atherogenic activities. Circulating apoA-I represents a typical amphipathic protein that lacks glycosylation or disulfide linkages and contains 8 alpha-helical amphipathic domains of 22 amino acids and two repeats of 11 amino acids (**Figure 1.10**). As a consequence, apoA-I binds avidly to lipids and possesses potent detergent-like properties. ApoA-I readily moves between lipoprotein particles and is also found in chylomicrons and VLDL. As for many plasma apolipoproteins, the main sites for apoA-I synthesis and secretion are the liver and small intestine (Kontush, Lindahl, Lhomme, Calabresi, Chapman & Davidson, 2015).

ApoA-II is the second most abundant HDL apolipoprotein which represents approximately 15–20 % of total HDL protein. Approximately half of HDL particles in human serum contain apoA-II (Duriez & Fruchart, 1999). ApoA-II is more hydrophobic than apoA-I and more strongly associated with HDL (Kalopissis, Pastier & Chambaz, 2003) and circulates as a homodimer composed of two identical polypeptide chains (Puppione et al., 2009; Shimano, 2009) connected by a disulphide bridge at position 6 (Brewer, Lux, Ronan & John, 1972). ApoA-II equally forms heterodimers with other cysteine-containing apolipoproteins (Hennessy et al., 1997) and is predominantly synthesised in the liver but also in the intestine (Gordon, Budelier, Sims, Edelstein, Scanu & Strauss, 1983).

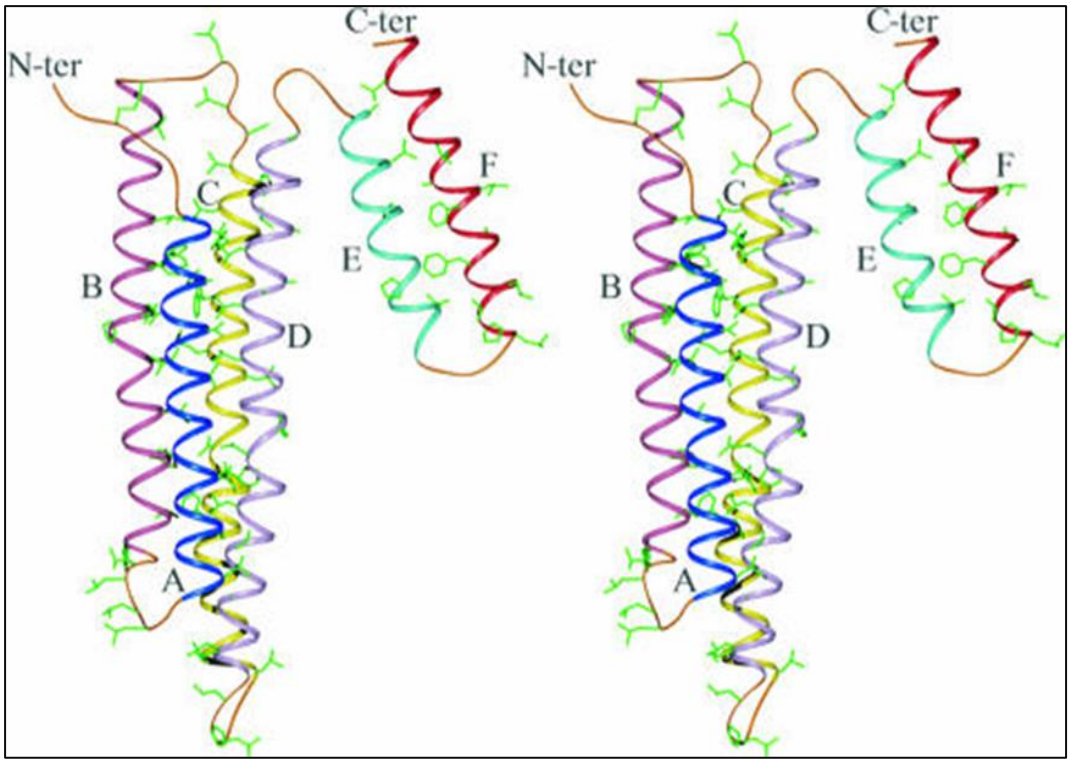


Figure 1.10 Overall stereoview of apoA-1 structure. Mature apoA-1 represent a typical amphipathic structure of 8 alpha-helical amphipathic domains of 22 amino acids and two replications of 11 amino acids which makes apoA-1 a potent detergent and form stable micellar complexes with PL, cholesterol, TGs, and cholesteryl esters. [Source: (Ajees, Anantharamaiah, Mishra, Hussain & Murthy, 2006)].

Enzymes

LCAT catalyses the esterification of cholesterol to cholesterol esters in plasma lipoprotein, primarily in HDL but also in apoB-containing particles. About 75% of plasma LCAT activity is associated with HDL. In plasma, LCAT is closely associated with apoD, which frequently co-purify (Holmquist, 2002).

Human **paraxonases** (PON) are calcium-dependent lactonases PON1, PON2 and PON3 (Goswami, Tayal, Gupta & Mallika, 2009). PON1 is a widely studied enzyme because of its protective role against poisoning by metabolites of specific organophosphate insecticides and its potential role in vascular disease (Ceron, Tecles & Tvarijonaviciute, 2014). In the circulation, PON1 is almost exclusively associated with HDL. Human PON1 is mainly synthesized in the liver and to a lesser extent in the kidney and colon (Mackness, Beltran-Debon, Aragones, Joven, Camps & Mackness, 2010). Originally, interest in this enzyme came from its function in detoxification, but more recent research has focused on other clinical aspects such as a defensive role in vascular disease as well as its use as a biomarker of disease processes such as: (a) oxidative stress, since PON1 protects against oxidation (James, 2006); (b) inflammation, being considered PON1 as a negative acute phase protein (Novak, Vavrova, Kodydkova, Hynkova, Zak & Novakova, 2010) and (c) liver diseases, because PON1 is synthesized in this organ (Mogarekar & Talekar, 2013).

PON1 is a 6-bladed β-propeller with a unique active-site lid, which is also involved in HDL biding (**Figure 1.11**). PON1's 3D- structure has been solved and thereby affords a model for HDL-binding having a catalytic calcium ion (Aharoni & Tawfik, 2004).

Platelet- activating factor acetyl hydrolase (PAF-AH) equally termed lipoprotein-associated phospholipase A2 (LpPLA2) is a calcium-independent, N-glycosylated enzyme, which degrades PAF by hydrolysing the sn-2 ester bond to yield biologically inactive lyso-PAF (Mallat, Lambeau & Tedgui, 2010). The enzyme cleaves PL substrates with a short residue at the sn-2 position and thus can hydrolyse proinflammatory oxidised short-chain PLs; however, it is inactive against long-chain non-oxidised PLs. PAF-AH is synthesized throughout the brain, white adipose tissue and placenta. Macrophages represent the central source of

the circulating enzyme (McIntyre, Prescott & Stafforini, 2009). Plasma PAF-AH circulates in association with LDL and HDL particles, with the majority of the enzyme bound to small, dense LDL and to lipoprotein (a) (Tselepis, Dentan, Karabina, Chapman & Ninio, 1995).

Plasma **glutathione selenoperoxidase 3** (GSPx-3), also called **glutathione peroxidase 3**, is distinct from two other members of the GSPx family termed GSPx-1 and GSPx-2 which represent erythrocyte and liver cytosolic enzymes. All GSPx enzymes protect biomolecules from oxidative damage by catalysing the reduction of hydrogen peroxide, lipid peroxides and organic hydroperoxide, in a reaction involving glutathione. Human GSPx-3 is a homotetrameric protein containing selenium as selenocysteine residue at position 73. Human GSPx-3 is synthesised in the liver, kidney, heart, lung, breast and placenta. In plasma, GSPX-3 is exclusively associated with HDL (Chen, Liu, Greiner & Holtzman, 2000).

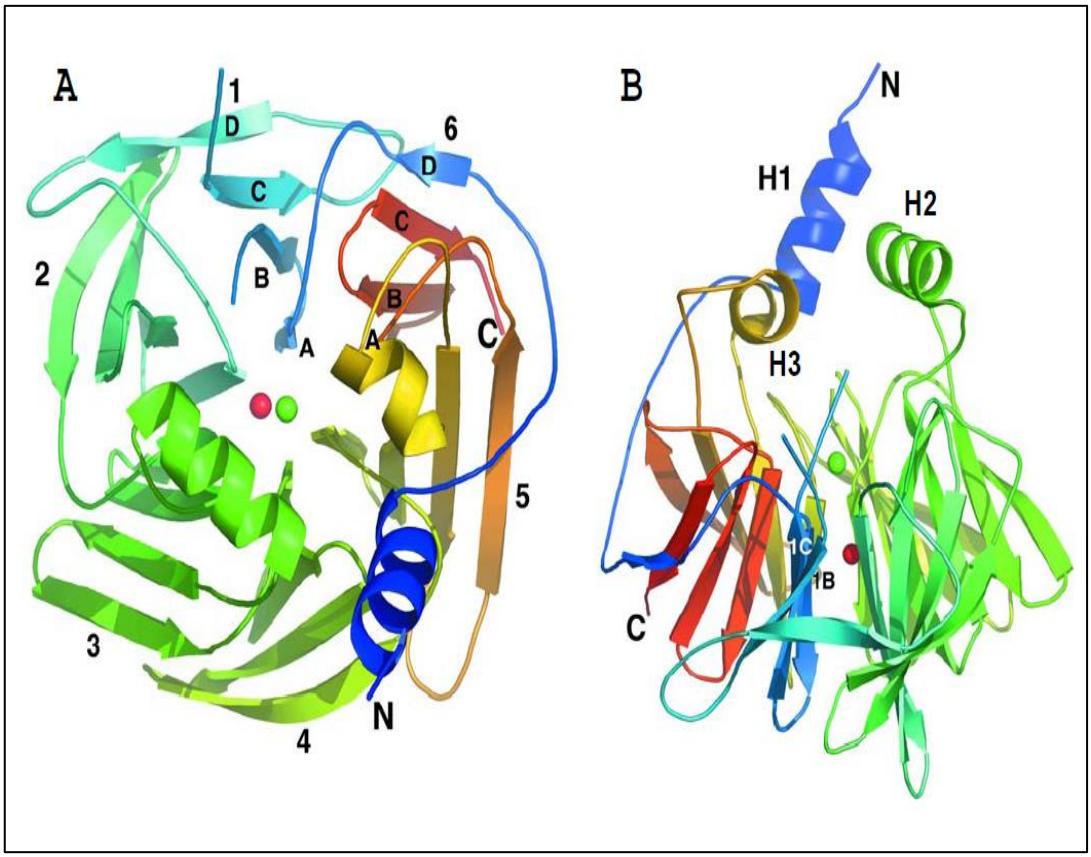


Figure 1.11 The overall structure of paraxonase 1 (PON1). (A) A view of the 6-bladed β-propeller from its top. Shown are the N-termini, the six blades (labelled 1-6) each of which is comprised of 4 β-strands (labelled A-D), and the two calcium atoms in the central tunnel of the propeller (green and red spheres). (B) A side view of the propeller, including the three helices at the top of the propeller (H1-H3). The top calcium ion (green sphere) is a key part of PON1's active site, and the three helices comprise an active-site lid. Helixes H1 (the N-termini of PON1) and H2 are also thought to participate in the binding to PON1 to HDL. [Source: (Aharoni & Tawfik, 2004)].

Lipid Transfer Proteins

PLTP belongs to the bactericidal permeability- increasing protein (BPI)/lipopolysaccharide (LPS)-binding protein (LBP)/Plunc superfamily of proteins. PLTP is produced in the placenta, pancreas, lung, kidney, heart, liver, skeletal muscle and brain. In the circulation, PLTP is primarily associated with HDL and converts it into larger and smaller particles. PLTP also plays a role in extracellular PL transport and can bind LPS. PLTP is a positive acute-phase reactant with a potential role in the innate immune system (Kontush, Lindahl, Lhomme, Calabresi, Chapman & Davidson, 2015).

CETP equally belongs to the BPI/LBP/Plunc superfamily and contains multiple N-glycosylation sites. It is primarily expressed by the liver and adipose tissue. In the circulation, CETP shuttles between HDL and apoB-containing lipoproteins and facilitates the bidirectional transfer of CEs and TG between them (Kontush, Lindahl, Lhomme, Calabresi, Chapman & Davidson, 2015).

Acute-Phase Response Proteins

Positive **acute-phase response proteins**, whose plasma concentrations are markedly elevated by acute inflammation, form a large family of HDL-associated proteins (Heinecke, 2009; Vaisar et al., 2007). Under normal conditions, the content of such proteins in HDL is however much lower as compared to apolipoproteins. On the other hand, plasma levels of several HDL apolipoproteins, such as apoA-I and apoA-IV, are reduced during the acute-phase response (Navab et al., 2004); such proteins can therefore be deliberated as negative acute-phase response proteins.

Serum amyloid A (SAA) proteins, major acute-phase reactants, are secreted during the acute phase of the inflammatory response. In humans, three SAA isoforms, SAA1, SAA2 and SAA4, are produced predominantly by the liver. SAA1, the chief member of this family, is predominantly carried by HDL in human, rabbit and murine plasma (Cabana, Lukens, Rice, Hawkins & Getz, 1996). In the circulation, SAA1 does not exist in a free form and associates with non-HDL lipoproteins in the absence of HDL (Cabana et al., 2004).

LBP is an acute-phase glycoprotein capable of binding the lipid A moiety of LPS of Gram-negative bacteria and facilitating LPS diffusion (Wurfel, Kunitake, Lichenstein, Kane & Wright, 1994). LBP/LPS complexes appear to interact with the CD14 receptor to enhance cellular responses to LPS. LBP also binds PLs, thereby acting as a lipid exchange protein (Yu, Hailman & Wright, 1997), and belongs to the same BPI/LBP/Plunc protein superfamily as PLTP and CETP (Kontush, Lindahl, Lhomme, Calabresi, Chapman & Davidson, 2015).

1.3.2 Lipidomes

Besides proteins, HDL contains multiple molecular species of lipids (**Table 1.5**) (Kontush, Lindahl, Lhomme, Calabresi, Chapman & Davidson, 2015).

Phospholipids

Phosphotidylcholine (PC) is the principal plasma PL that accounts for 32-35 mol% of total lipids in HDL (wiesner, Leidl, Boettcher, Schmitz & Liebisch, 2009). PC is a structural lipid, consistent with its even distribution across HDL subpopulations (Kontush, Lindahl, Lhomme, Calabresi, Chapman & Davidson, 2015).

LysoPC is an essential PL subclass in HDL (1.4-8.1 mol% of total lipids). It is derived from the regulated degradation of PC by phospholipases, including LCAT, consistent with the preferential association of the latter with HDL particles (Kontush et al., 2007). More specifically, LCAT was described earlier to associate mainly with small, dense HDL particles, which are also enriched in lysoPC by nearly twofold as compared to large, light HDL (Camont et al., 2013). LysoPC is also formed by the hydrolytic action of LpPLA2 on oxidized PC or by secreted PLA2 under pro-atherogenic conditions, such as oxidative stress and inflammation, and hence constitutes a potential biomarker of inflammation (Kontush, Lindahl, Lhomme, Calabresi, Chapman & Davidson, 2015).

Phosphotidylethanolamine (PE) is moderately abundant in HDL (0.7- 0.9 mol% of total lipids), and its content tends to increase with increasing HDL hydrated density (Camont et al., 2013; wiesner, Leidl, Boettcher, Schmitz & Liebisch, 2009).

Plasmalogens contain a vinyl ether-linked fatty acid essential for their specific antioxidative properties (Maeba & Ueta, 2003). PC-plasmalogens are the most abundant species in HDL (2.2-3.5 mol%) but represent less than 10% of total PC (Ståhlman et al., 2013).

Phosphotidylinositol (PI), **phosphotidylserine (PS)**, **phosphotidylglycerol (PG)**, **phosphatidic acid (PA)** and **cardiolipin** are negatively charged PLs present in HDL which may significantly control its net surface charge . The content of these lipids can therefore modulate lipoprotein interactions with lipases, membrane proteins, extracellular matrix and other protein components. Such interactions are largely charge-dependent (Rosenson et al., 2011).

PI, similarly to PE, is moderately abundant in HDL (0.5-0.8 mol %) and tends to be enriched in small, dense HDL.

PS is a minor negatively charged PL component of HDL (0.016-0.030 mol %). This PL was very recently reported to be highly enriched (34-fold) in the small, dense HDL3c subpopulation relative to large, light HDL2 (Camont et al., 2013) as well as in small discoid preβ HDL and small nascent HDL formed by ABCA1 (up to 2.5 mol % total lipids). Interestingly, small dense HDL also displayed potent biological activities which correlated positively with PS content in HDL (Camont et al., 2013). This lipid could consequently, in part, account for enhanced functionality of HDL3c (Kontush, Lindahl, Lhomme, Calabresi, Chapman & Davidson, 2015).

PA, a second messenger, is both a common metabolic precursor and an enzymatic product of PL metabolism. This negatively charged lipid is present in very low abundance in HDL (0.006-0.009 mol%) but, similarly to PS, is enriched in small, dense HDL (by more than threefold (Camont et al., 2013). This observation might reflect favored association of PA with apoL-I which is equally enriched in small, dense HDL (Kontush & Chapman, 2011).

PG is a metabolic precursor of cardiolipin present in HDL in very low amounts (0.004-0.006 mol %). PG tends to be enriched in small, dense particles (Camont et al., 2013).

Cardiolipin is a minor anionic PL present in trace amounts in HDL (0.08-0.2 mol%) This lipid with potent anticoagulant properties may contribute to the effects of lipoproteins on coagulation and platelet aggregation (Deguchi, Fernandez, Hackeng, Banka & Griffin, 2000).

Together, these data indicate that although negatively charged lipids represent minor HDL constituents (0.8 mol % of total lipids), they are highly enriched in small, dense HDL, consistent with the elevated surface electronegativity of this subpopulation (Rosenson et al., 2011).

Isoprostanes are well established as biomarkers of oxidative stress and are predominantly associated with HDL (Kontush & Chapman, 2011).

Sphingolipids

Sphingomyelin, a structural lipid which enhances surface lipid rigidity (Rye , Hime & Barter ,1996) , is the major sphingolipid in circulating HDL (5.6-6.6 mol % of total lipids) (Camont et

al., 2013; Ståhlman et al., 2013) (**Table 1.5**), which largely originates from triglyceride-rich lipoproteins and only to a minor extent from nascent HDL (Nilsson & Duan, 2006).

Among lysosphingolipids, **shingosine-1-phosphate (SIP)** is principally interesting as this bioactive lipid plays key roles in vascular biology (Lucke & Levkau, 2010). More than 90% of circulating sphingoid base phosphates are found in HDL and albumin-containing fractions (**Table 1.5**) (Kontush & Chapman, 2011). Interestingly, SIP associate preferentially with small, dense HDL particles (up to tenfold enrichment compared to large, light HDL) (Kontush et al., 2007).

Ceramide is a sphingolipid intermediate involved in cell signaling, apoptosis, inflammatory responses, mitochondrial function and insulin sensitivity (Lipina & Hundal, 2011). This lipid is poorly transported by HDL, which carries only 25 mol% of total plasma ceramide (wiesner, Leidl, Boettcher, Schmitz & Liebisch, 2009), and constituents only between 0.02 and 0.097 mol% of total HDL (Camont et al., 2013; Ståhlman et al., 2013) (**Table 1.5**). Similarly to sphingomyelin, this product of shingomyelin hydrolysis is enriched in large, light HDL.

Neutral Lipids

Unesterified (free) **sterols** are located in the surface lipid monolayer of HDL particles and control its fluidity. HDL sterols are dominated by cholesterol, reflecting the key role of lipoproteins in cholesterol transport through the body. Other sterols are present in lipoproteins at much lower levels are exemplified by minor amounts of lathosterol, ergosterol, phytospherols (β-sitosterol, campesterol), oxysterols and estrogens (largely circulating as esters) (Kontush & Chapman, 2011). Free cholesterol, whose affinity for sphingomyelin is now well established, tends to preferentially associate with large, light HDL (Kontush, Lindahl, Lhomme, Calabresi, Chapman & Davidson, 2015).

CEs are largely (up to 80%) formed in plasma HDL, through transesterification of PL and cholesterol catalyzed by LCAT. These highly hydrophobic lipids from the lipid core of HDL and contribute up to 36 mol% of total HDL lipid (Camont et al., 2013; Ståhlman et al., 2013) (**Table 1.5**). Most of HDL CE is accounted for by cholesteryl linoleate (Kontush, Lindahl, Lhomme, Calabresi, Chapman & Davidson, 2015). Studying the CE molecular species distribution through HDL subpopulations, using gas chromatography, displayed very similar profiles between HDL2 and HDL3 particles (Vieu et al., 1996).

HDL-associated **TG** are dominated by species containing oleic, palmitic and linoleic acid moieties (Kontush, Lindahl, Lhomme, Calabresi, Chapman & Davidson, 2015) and represent around 3 mol% of total HDL lipids (Camont et al., 2013; Ståhlman et al., 2013) (**Table 1.5**). Similarly to CE, TAG species profile is conserved between HDL2 and HDL3 (Vieu et al., 1996).

These data illustrate the power of lipidomics to deliver essential information on the metabolism and function of lipoproteins relevant for the development of cardiovascular disease, which can in turn provide novel biomarkers of cardiovascular risk (Kontush, Lindahl, Lhomme, Calabresi, Chapman & Davidson, 2015).

Table 1.5 Major Components of the HDL lipidome

Lipid Class	HDL content in mol % of total lipids
Phospholipids	*37.4–49.3*
Phosphatidylcholine	32–35
PC-plasmalogen	2.2–3.5
LysoPC	1.4–8.1
Phosphatidylethanolamine	0.70–0.87
PE-plasmalogen	0.54–0.87
Phosphatidylinositol	0.47–0.76
Cardiolipin	0.077–0.201
Phosphatidylserine	0.016–0.030
Phosphatidylglycerol	0.004–0.006
Phosphatidic acid	0.006–0.009
Sphingolipids	*5.7–6.9*
Sphingomyelin	5.6–6.6
Ceramide	0.022–0.097
Hexosyl Cer	0.075–0.123
Lactosyl Cer	0.037–0.060
S1P d18:1	0.015–0.046
S1P d18:0	0.007
SPC d18:1	0.001
Neutral lipids	*46.7–54.0*
Cholesteryl esters	35–37
Free cholesterol	8.7–13.5
Triacylglycerides	2.8–3.2
Diacylglycerides	0.17–0.28
Minor lipids	
Free fatty acids	16:0, 18:0, 18:1[a]
Isoprostane-containing PC	ND (IPGE2/D2-PC (36:4))[a]

Data are shown for HDL obtained from normolipidemic healthy subjects according to (Deguchi, Fernandez, Hackeng, Banka & Griffin, 2000; Kontush et al., 2007), (wiesner, Leidl, Boettcher, Schmitz & Liebisch, 2009), (Camont et al., 2013) , (Ståhlman et al., 2013), (Pruzanski, Stefanski, de Beer, de Beer, Ravandi & Kuksis, 2000), (Sattler et al., 2010), (Argraves et al., 2011). SPC sphingosylphosphorylcholine; S1P sphingosine-1-phosphate; IPGE2 isoprostaglandin E2. [a] no quantitative data available, major molecular species identified.[Source: (Kontush, Lindahl, Lhomme, Calabresi, Chapman & Davidson, 2015)]

1.4 HDL Subclasses

HDL is a complex macromolecule comprised of lipids and proteins. It is the most abundant lipoprotein per unit volume of plasma and exists at the highest (micromolar) levels compared with other lipoproteins (Jeyarajah, Cromwell & Otvos, 2006; Kontush & Chapman, 2006) . On the other hand, HDL particles are a vastly heterogeneous lipoprotein family consisting of several subclasses differing in their physiochemical properties, composition, shape, density, size, charge, intravascular metabolism and biologic actions (**Figure 1.12** and **Table 1.6**) (Kontush & Chapman, 2011). The outstanding heterogeneity of HDL particles predominantly results from the extremely dynamic structure of apoA-1, which permits different conformations as a function of the amount of bound lipid and particle dimension (Davidson & Thompson, 2007). Another key role of HDL heterogeneity is HDL capacity to bind and carry distinct proteins as some HDL proteins possess a high alpha helical content which allows it to interact with surface lipids while other HDL particles may tend to form specific protein-protein complexes (Vaisar, 2009).

The classic method for separation of lipoprotein subfractions is by density. In 1954, Gofman and colleagues has described the difference in HDL subclass using flotation rate in high salt solutions in analytical ultracentrifugation (Delalla & Gofman, 1954). Two major HDL subclasses were identified: HDL2 which is a less dense (1.063–1.125 $g.mL^{-1}$), relatively lipid rich form and HDL3 which is more dense (1.125–1.21 $g.mL^{-1}$), relatively protein-rich form. Those two classes could also be separated by rate-zonal ultracentrifugation (Franceschini, Tosi, Moreno & Sirtori, 1985), single vertical spin ultracentrifugation (Kulkarni, Marcovina, Krauss, Garber, Glasscock & Segrest, 1997) or a precipitation method (Gidez, Miller, Burstein, Slagle & Eder, 1982). Although density gradient ultracentrifugation methods are time consuming and require expensive instruments, they remain the most precise techniques and are considered as the gold standard for lipoprotein separation even after 50 years.

According to size, three distinct subclasses for HDL3 and two for HDL2 could also be revealed by non-denaturing polyacrylamide gradient gel electrophoresis (GGE): HDL3c, 7.2-7.8 nm diameter; HDL3b 7.8-8.2 nm; HDL3a, 8.2-8.8 nm; HDl2a, 8.8-9.7 nm; and HDL2b, 9.7-12.0 nm (Nichols, Krauss & Musliner, 1986). Equally, those subclasses could also be isolated by

isopycnic density gradient ultracentrifugation (Chapman, Goldstein, Lagrange & Laplaud, 1981; Kontush, Chantepie & Chapman, 2003).

Surface charge and shape is another important property that has been used to separate HDL subclasses. Agarose gel electrophoresis, which has been used for decades as a standard laboratory technique to separate lipoproteins, has allowed the analytical separation of HDL into α-migrating particles (which have the same mobility as alpha-globulin and represent the majority of circulating HDL) and preβ-migrating particles (which migrate similarly to pre-beta globulin) comprised of nascent discoidal and poorly lipidated HDL. If agarose gel is stained with Coomassie blue or with anti-apolipoprotein A-I antibodies, the relative protein content of the two HDL subclasses can be determined (Favari et al., 2004).

Maximum resolving power was achieved by a 2-dimentional (2D) electrophoretic method. This method allowed the identification of 12 distinct apoA-1 containing HDL subclasses; Preβ subclasses (preβ_1 and preβ_2), α (α_1, α_2, α_3, and α_4) and preα (preα_1, preα_2, preα_3). This method splits HDL according to charge in the first run and according to size in the second run (conducted at right angles to the first run). Gels are then stained with apolipoprotein-specific antibodies, typically with anti-apoA-I antibodies, allowing the recognition of distinct HDL subclasses (Asztalos & Schaefer, 2003a; Asztalos & Schaefer, 2003b). Electroimmunodiffusion technique in agarose gels allows the separation HDL into two major subclasses according to their major apolipoprotein composition, those containing only apoA-I (LPA-I) and those containing both apoA-I and apoA-II (LpA-I: A-II) (Fruchart & Ailhaud, 1992; Warden, Hedrick, Qiao, Castellani & Lusis, 1993).

The plasma preβ-HDL concentration can be also measured using a sandwich enzyme immunoassay (Miida et al., 2003). The assay employs a monoclonal antibody which unambiguously recognizes apoA-I bound to preβ-HDL.

Nuclear magnetic resonance (NMR) spectroscopy separates HDL into subfractions of three different sizes: small, medium, and large (Otvos, 2002). It measures the particle concentrations of these subfractions after differentiating them by their unique magnetic resonance properties. These properties are distinguished by the terminal lipid methyl group protons, which are independent of chemical compositional differences related to fatty acid, CE, or triglyceride content. Although characterization of HDL subpopulations by NMR is now common, the technique requires broader validation (Martin, Jones & Toth, 2014).

Increasing awareness of HDL heterogeneity in the last decade has led to the exploration of the relationship between HDL subclasses and cardiovascular risk, as well as acknowledgement of a need to consider the clinical effects of HDL modifying drugs. Although HDL subclasses have been identified/ isolated by different techniques, the number and nomenclature of HDL subclasses are not uniform among the different techniques. Moreover, some techniques measure HDL subclass concentrations, others define the percentage distribution of HDL subclass relative to the total or characterize the HDL distribution by average particle diameter. It has been suggested a classification of HDL by physical properties, which incorporates terminology from several methods and outlines five HDL subclasses, termed very large, large, medium, small and very small HDL (Rosenson et al., 2011). The proposed nomenclature by physical properties could help in defining the relationship between HDL subclasses and cardiovascular risk.

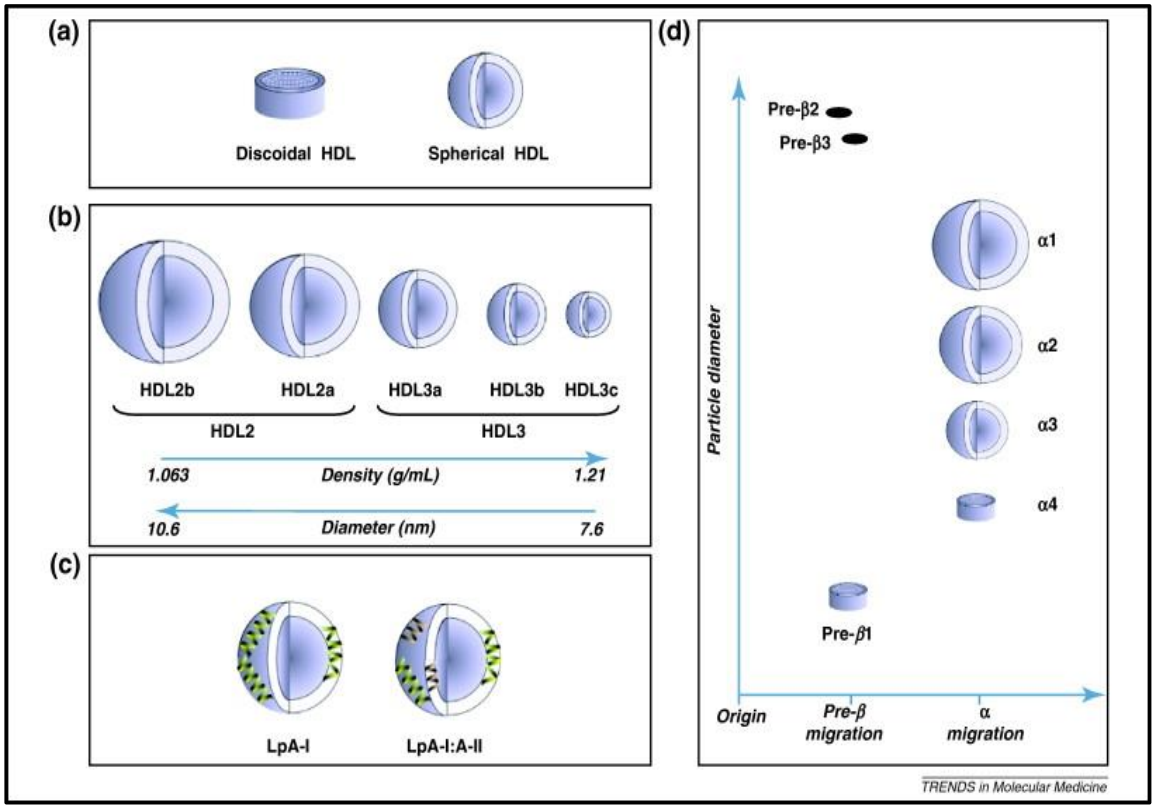


Figure 1.12 Heterogeneity of high-density lipoprotein (HDL) particles. Major subpopulations of HDL particles differing in shape (a), density and size (b), apolipoprotein composition (c) and electrophoretic mobility (d) are shown as revealed by gel electrophoresis (a, d), ultracentrifugation (b) and immunoaffinity chromatography (c). Using two-dimensional gel electrophoresis, particles are separated by size in the vertical dimension and by charge in the horizontal dimension into particles of pre-β, α and pre-α mobility. [Adapted from Rye et al. (Rye, Bursill, Lambert, Tabet & Barter, 2009) and Schaefer et al.(Schaefer, Santos & Asztalos, 2010)].

Table 1.6 Major HDL subclasses according to different isolation/ separation techniques [Source: (Kontush, Lindahl, Lhomme, Calabresi, Chapman & Davidson, 2015)].

Density (ultracentrifugation)
HDL2 (1.063–1.125 g.mL^{-1})
HDL3 (1.125–1.21 g.mL^{-1})
Size (GGE)
HDL2b (9.7–12.0 nm)
HDL2a (8.8–9.7 nm)
HDL3a (8.2–8.8 nm)
HDL3b (7.8–8.2 nm)
HDL3c (7.2–7.8 nm)
Size (NMR)
Large HDL (8.8–13.0 nm)
Medium HDL (8.2–8.8 nm)
Small HDL (7.3–8.2 nm)
Shape and charge (agarose gel)
α-HDL (spherical)
Preβ-HDL (discoidal)
Charge and size (2D electrophoresis)
Preβ-HDL (preβ1 and preβ2)
α-HDL (α1, α2, α3 and α4)
Preα-HDL (preα1, preα2, preα3
Protein composition (electroimmunodiffusion)
LpA-I
LpA-I:A-II

1.5 Structure of HDL

Many laboratories have worked to reveal the structure (assembly) of HDL specially when it became clear that plasma levels of HDL were inversely correlated with cardiovascular disease (Edelstein, Lim & Scanu, 1972), (Laggner, Muller, Kratky, Kostner & Holasek, 1973), (Atkinson, Davis & Leslie, 1974) (Schonfeld, Bradshaw & Chen, 1976), (Tardieu, Mateu, Sardet, Weiss & Luzzati, 1976). After the sequences of the major HDL proteins, apoA-I and apoA-II, were reported in 1970s (Brewer, Lux, Ronan & John, 1972), many researchers noted periodically repeating units that, when mapped on a helical wheel plot, indicated the presence of amphipathic alpha helices. With hydrophobic faces mediating lipid interactions and polar faces interacting with water, these structures turned out to be responsible for the detergent-like ability of these proteins to solubilize lipids into stabile lipoprotein particles (McLachlan, 1977; Segrest, Jackson, Morrisett & Gotto, 1974).

Jonas and colleagues combined purified apolipoproteins with lipids under control of detergents to produce recombinant forms of HDL (Matz & Jonas, 1982) and by electron microscopy work, it was revealed that they have a discoidal shape (Forte & Nordhausen, 1986), and these particles were referred as reconstituted (r)HDL discs (Forte & Nordhausen, 1986).

Kontush and his team has succeeded, by mass spectrometry, careful measurements of the protein/lipid components indicated a range 3-5 apoA-I molecules per particle, depending on the size. **Figure 1.13** shows one proposal for how 4 and 5 apoA-Is can be accommodated by changing the hinge bend angles of the trefoil while maintaining the same intermolecular interactions, and hence cross-linking pattern. Furthermore, it was proposed that HDL particle size is modulated via a twisting motion of the residence apoA-Is (Kontush, Lindahl, Lhomme, Calabresi, Chapman & Davidson, 2015).

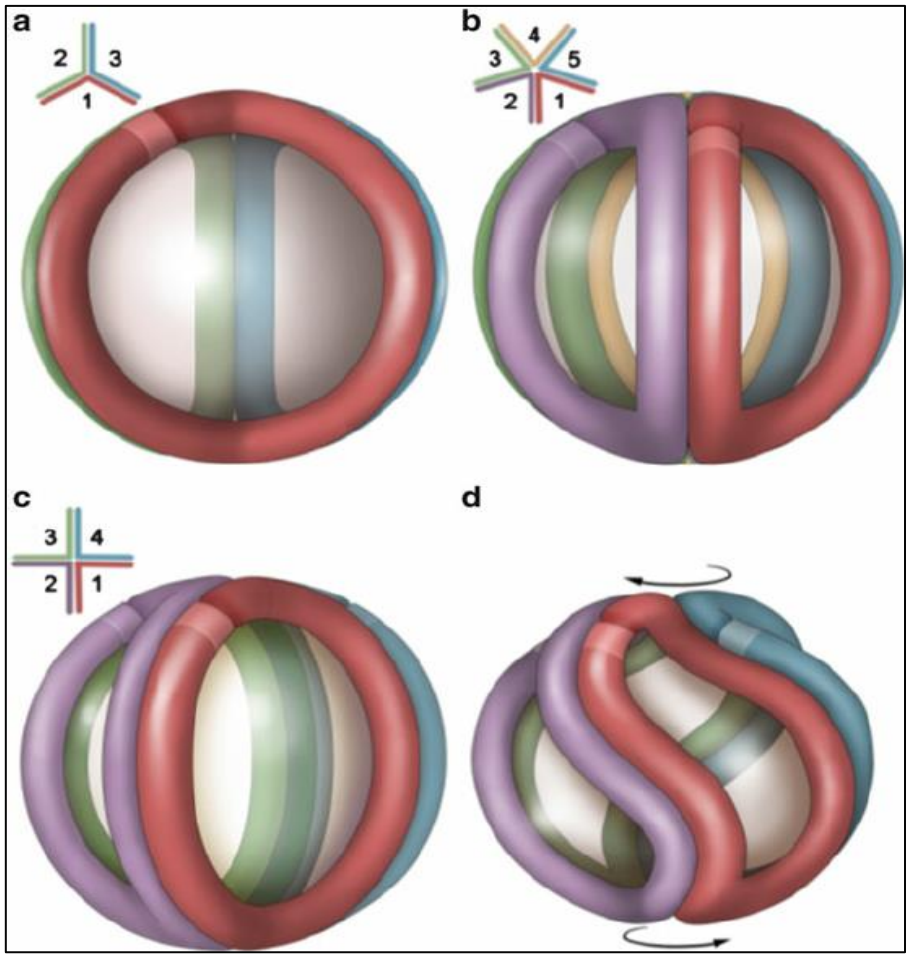


Figure 1.13 Models of apoA-I in human plasma HDL particles of various size and number of apoA-I molecules. (a) LpA-I2b contains 5 apoA-I molecules, but shows a similar cross-linking patters to reconstituted particles that have only 2 and 3 molecules of apoA-I. This figure shows that more apoA-I molecules can be added to the trefoil framework by increasing the hinge bend angle and adding more apoA-I molecules. (c) A possible model for LpA-I2a that has four apoA-I molecules on average. (d) Further reductions in HDL particle size may be accomplished by a twisting action of resident apoA-I molecules.[Source: (Kontush, Lindahl, Lhomme, Calabresi, Chapman & Davidson, 2015)]

1.6 Functions of HDL

High density lipoprotein (HDL) continually undergoes remodelling throughout its life-span and carries out various functions (Murphy, 2013). In addition to its role in reverse cholesterol transport, HDL interacts with various cell types that influence cardiovascular and metabolic health (**Figure 1.14**). HDL also inhibits lipid oxidation, restores endothelial function, exerts anti-inflammatory, cytoprotective, vasodilatory, anti-infectious, antithrombotic and antiapoptotic activities. Such properties could contribute considerably to the capacity of HDL to inhibit atherosclerosis.

1.6.1 Role of HDL in Cholesterol Homeostasis

On the one hand, cholesterol is considered as an essential substance for maintaining cell membranes, manufacturing vitamin D on the surface of the skin, producing hormones and possibly aiding cell connections in the brain, while on the other hand, high cholesterol levels in the blood increase risk of coronary disease (Daniels, Killinger, Michal, Wright & Jiang, 2009; Von Eckardstein, Nofer & Assmann, 2001). Control of cholesterol homeostasis in the body is important since cholesterol accumulation in arteries serving the heart muscle causes more death and disability than all types of cancer combined (Lloyd-Jones, 2009). The uptake of modified lipoproteins by macrophages of the vascular wall plays an important role in the pathogenesis of atherosclerosis since accumulation of lipids turns them into activated foam cells, which produce various growth factors, cytokines, and proteases and thereby influence the course of atherosclerosis (Von Eckardstein, Nofer & Assmann, 2001).

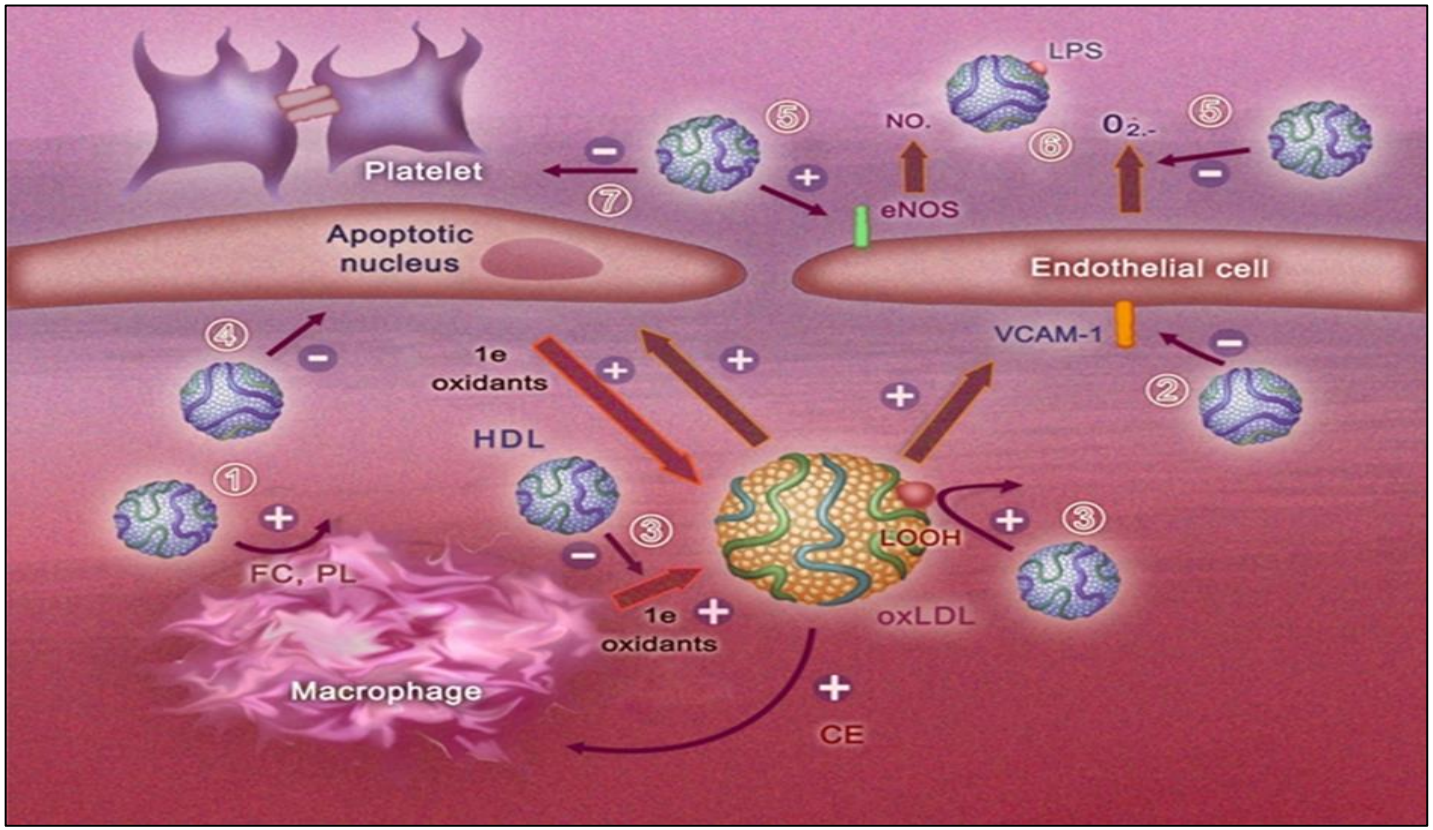


Figure 1.14 Major biologic activities of HDL. (1) Cholesterol efflux capacity (through cholesterol and PL efflux from macrophages). (2) Anti-inflammatory activity (via inhibition of VCAM-1 expression in endothelial cells induced by oxidized LDL). (3) Antioxidant potency (through inhibition of LDL oxidation by cell-derived one-electron oxidants and removal of oxidized lipids from oxidized LDL). (4) Cytoprotective activity (by inhibiting endothelial cell apoptosis induced by oxidized LDL). (5) Vasodilatory activity (as stimulation of cellular NO production and inhibition of cellular superoxide release). (6) Anti-infectious activity (via LPS-binding). (7) Antithrombotic action (via inhibiting platelet aggregation). 1e oxidants, one-electron oxidants; FC, free cholesterol; PL, phospholipid; oxLDL, oxidized LDL.[Source: Kontush and Chapman (Kontush & Chapman, 2011)]

Reverse Cholesterol Transport

In 1968, Glomset introduced the concept of reverse cholesterol transport (RCT) in which he described the pathway by which peripheral cholesterol is returned to liver by HDL for secretion into bile and excretion through the feces (Glomset, 1968). This route is thought to represent the main basis for the anti-atherogenic properties of HDL (Kontush & Chapman, 2006; Von Eckardstein, Nofer & Assmann, 2001). RCT maintains neutral cholesterol balance in the body by removing the 9 mg of cholesterol per kilogram of body weight that is synthesized by peripheral tissues every day (Von Eckardstein, Nofer & Assmann, 2001).

The efflux of cholesterol from a variety of cell types, including macrophages, to HDLs in the extracellular space is mediated by two distinct processes. One is the efflux of cholesterol induced by a specific cellular transporter (Dikkers, Freak de Boer, Annema, Groen & Tietge, 2013), and the other is passive aqueous diffusion of cholesterol from cell membranes to HDLs . Then, excess cholesterol from peripheral tissues will be transported back to the liver for excretion in the bile and ultimately the feces. Multiple steps are present in the RCT pathway as described in the following four parts (**Figure 1.15**) (Joy & Hegele, 2009).

Part One: The Formation of Nascent HDL

Lipid-free or lipid-poor apoA-I produced in the liver can mediate cellular efflux of both cholesterol and PLs from macrophages through the ABCA1 and congregate them on the surface of pre-β HDL, creating a rapid lipidation of apoA-I to generate mature α HDL, which is called nascent HDL particles (Curtiss, Valenta, Hime & Rye, 2006). The mature HDL particles can then serve as acceptors of cholesterol delivered by ABCG1 (Jiang et al., 2001) or SR-B1 (Song, Kim, Park, Kim, Choi & Cho, 2015). ABCG1 is another member of the ATP-binding cassette family that plays a critical role in the efflux of cellular PL and free cholesterol (FC) to mature HDL, but not pre-β HDL. Numerous studies have demonstrated the significance of ABCA1 and ABCG1 in several aspects of cholesterol efflux from macrophages (Freeman et al., 2014; Westerterp et al., 2013).

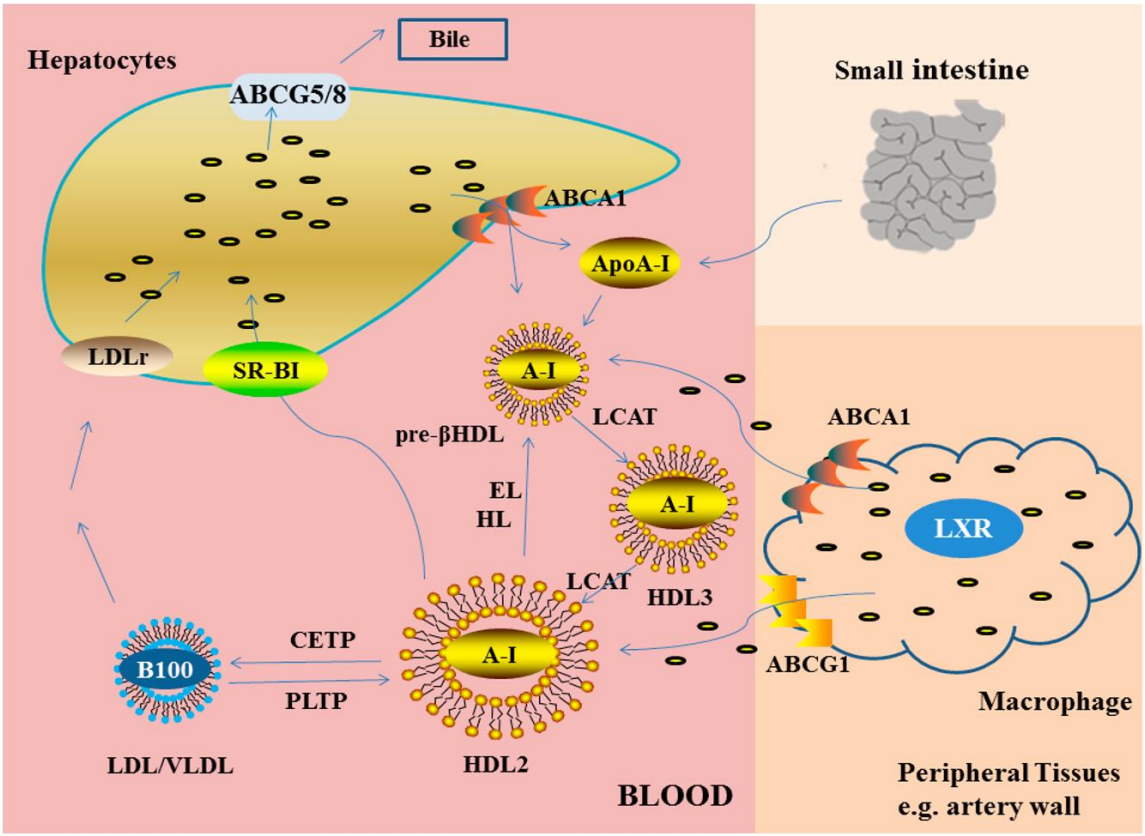


Figure 1.15 HDL in reverse cholesterol transport pathway. Lipid-poor apoA-I also promotes the efflux of free cholesterol from macrophages via ABCA1. LCAT esterifies free cholesterol to cholesteryl esters to form mature HDL, which promotes cholesterol efflux from macrophages via the ABCG1 transporter, as well as from other peripheral tissues by processes not fully defined. In macrophages, both ABCA1 and ABCG1 are regulated by Liver X receptors (LXR). Mature HDL can transfer its cholesterol to the liver directly via SR-B1 or indirectly via CETP-mediated transfer to ApoB-containing lipoproteins, with subsequent uptake by the liver via the LDLr. Hepatic cholesterol can be excreted directly into the bile as cholesterol or after conversion to bile acids and, unless reabsorbed by the intestine, is ultimately excreted in the feces. HL, EL, and PLTP, play an indispensable role in remodeling HDL, thus, the RCT pathway is dependent on interaction with them. EL, endothelial lipase; HL, hepatic lipase; SR-B1, scavenger receptor class B type 1; LDLr, low density lipoprotein receptor; LXR; Liver X receptors LXR. [Source:(Marsche, Saemann, Heinemann & Holzer, 2013)].

LXR, the members of the steroid nuclear receptor superfamily, are oxysterol-activated transcription factors that, after heterodimerization with the 9-cis-retinoic acid receptor (RXR), bind to specific LXR response element (LXRE), thus regulating the expression of target genes involved in intra-and extracellular lipid metabolism (Bultel et al., 2008). To a certain extent by modulating cholesterol efflux from macrophages to apoA-I and HDL, LXRs induce the direct target genes ABCA1 and ABCG1/ABCG4 to promote reverse cholesterol transport (Cao, Pan, Xiao, Zhou, Guo & Su, 2015). The oxidation of steroids from free cholesterol can trigger LXR and regulate gene expression of ABCA1 and ABCG1 to stimulate peripheral tissue cholesterol secretion. Meanwhile, the LXRs are also easily oxidized by PPARα. PPARα controls lipid and glucose metabolism in several tissues and cell types including liver, heart, kidneys, adipose tissue and macrophages. PPARα-activation suppresses chylomicron and increases HDL production by enterocytes (Colin et al., 2013; Hanf et al., 2014). In addition, its agonists promote secretion of macrophage cholesterol via stimulating expression of ABCA1 and LXR to increase reverse cholesterol transport (Sahebkar, Chew & Watts, 2014).

Part Two: The Process of Cholesterol Esterification by LCAT

LCAT is a key player in the RCT pathway. LCAT has two different catalytic activities that account for its ability to esterify cholesterol. One is phospholipase A2 activity, and the other is its transesterification activity. It requires apoA-I and, to a lesser extent, other apolipoproteins, which most likely stimulate LCAT by modifying the presentation of its substrates, namely, PLs and cholesterol, on the surface of lipoproteins (Rousset, Shamburek, Vaisman, Amar & Remaley, 2011). Once free cholesterol efflux to pre-β HDL (the nascent, discoidal-shaped HDL), cholesterol in HDLs may be esterified by the enzymatic activity of LCAT. The LCAT reaction occurs in two steps. After binding to a lipoprotein, LCAT splits the fatty acid in the sn-2 position of phosphotidylcholine and transfers it onto a serine residue. Next, the fatty acid is transesterified to the 3-β-hydroxyl group on the A-ring of cholesterol to form cholesterol ester. Cholesterol esters formed by LCAT, which are more hydrophobic than free cholesterol, are transferred from the surface of lipoproteins to the hydrophobic core. This route converts pre-β HDL to HDL2 and HDL3 particles, which are the main HDL species found in plasma and which represent larger, spherical-shaped α-migrating forms of HDL. LCAT is essential in the process of RCT by generating a gradient of free cholesterol from cells to HDL (Soran, Hama, Yadav & Durrington, 2012). This effect of LCAT prevents the back exchange of cholesterol by passive diffusion from HDL to peripheral cells and thus is believed to promote net removal of cholesterol from peripheral cells to HDL. Without ongoing esterification of cholesterol, the capacity of HDL to remove and bind additional cholesterol would eventually be diminished. Two lipases, endothelial lipase (EL) and HL, are the complete opposite of LCAT in HDL metabolism. HL and EL are members of the triglyceride lipase family, which also includes LPL (Chatterjee & Sparks, 2011; Olivecrona & Olivecrona, 2010). EL has high phospholipase A1 activity and remodels HDL into small particles, whereas HL is more effective in hydrolyzing TG (Yasuda, Ishida & Rader, 2010). Although HL causes a remodeling of HDL into smaller particles, it also promotes the release of lipid-poor apoA-I (Annema & Tietge, 2011). The combined function of HL and EL have a significant effect on plasma high density lipoprotein cholesterol (HDL-C) levels (Jaye et al., 1999; Ruel, Couture, Cohn, Bensadoun, Marcil & Lamarche, 2004).

Part Three: The Exchange of cholesterol Esters Mediated by CETP

CETP is a hydrophobic glycoprotein that is synthesized in several tissues but mainly in the liver. It facilitates the exchange of CEs and TGs between HDL and apoB-containing particles (LDL, IDL, VLDL) and represent a major branching point for RCT (Barter et al., 2007). Most CEs derived from LCAT do not return to the liver via the HDL SR-B1 pathway but, through more atherogenic pathways. CETP mediates the transfer of most CE from HDL to VLDL or to other more atherogenic intermediate-density lipoproteins and remnants, and the transfer of triglycerides from VLDL-1 to HDL results in larger, relatively TG-enriched HDL species (Chapman, Le Goff, Guerin & Kontush, 2010). Transfer of CE from HDL directly to LDL by CETP could also be antiatherogenic if the LDL is cleared by the liver LDL receptor. Another transfer protein in this part of RCT, PLTP, transfers PLs between VLDL and HDL (Rao, Albers, Wolfbauer & Pownall, 1997). PLTP is one of the main modulators of plasma HDL size, composition and function (Yu et al., 2014) and one of the major modulators of HDL metabolism in plasma (Albers, Vuletic & Cheung, 2012).

Part Four: Catabolism of HDL Cholesterol in Biliary Pathway

After efflux, cholesterol in HDLs may be esterified by the enzymatic activity of LCAT at which HDLs can deliver the excess cholesterol from peripheral cells back to the liver in distinct ways: HDL cholesterol esters, but not the protein components of HDLs, are selectively taken up into the liver via SR-B1. Ultimately, cholesterol is excreted from the liver into the bile, either directly as free cholesterol or after conversion into bile acids, and eliminated from the body via the feces. In humans, HDL-C can be metabolized by the liver via another pathway: CETP exchanges of HDL CE for TGs in apoB-containing lipoproteins, followed by hepatic uptake mediated by LDLR (Cao, Pan, Xiao, Zhou, Guo & Su, 2015).

By means of the classic RCT pathway, excessive cholesterol collected from peripheral tissues, which is delivered back to the liver, is followed by biliary secretion and elimination via the feces. In addition to the traditional RCT-mediated biliary pathway, in the last few years, direct trans-intestinal excretion of plasma-derived cholesterol (TICE) was shown to contribute substantially to fecal neutral sterol (FNS) excretion in mice, decreasing the transport of cholesterol from blood to the intestinal lumen directly via enterocytes. The TICE pathway was called a non-hepatobiliary-related route, which has been shown to have a high degree of correlation with the main contribution Niemann-Pick disease, type C1/2 (NPC1/2), ABCG5/G8, LDLR, and LXR (Blanchard, Moreau, Cariou & Le May, 2014). The application of PPAR δ agonist and LXR agonists, have been shown to stimulate the process of TICE (Blanchard, Moreau, Cariou & Le May, 2014). In the RCT pathway, HDL plays an important role. In contrast, there is evidence from animal experiments that HDL plays an essential role in TICE (Vrins et al., 2012).

1.6.2 Role of HDL in Vascular Physiology

Preservation of Endothelial Function and Survival

Vascular endothelial cells constitute a structurally modest but functionally important organ that regulates diverse biological processes like hemostasis, fibrinolysis, inflammation, blood pressure, lipoprotein metabolism, and angiogenesis (O'Connell & Genest, 2001). Experimental and translational studies have revealed several potential antiatherogenic effects of HDL which involve endothelium, including protective properties on endothelial cell functions. HDL has been proposed to support endothelial cell functions by inhibition of LDL oxidation and by opposing the effects of this lipoprotein on endothelium. Furthermore, HDL from healthy subjects is believed to trigger endothelial cell production of nitric oxide and anti-inflammatory, anti-apoptotic, and anti-thrombotic effects in addition to endothelial repair process (Riwanto & Landmesser, 2013). On the other hand, latest clinical trials using HDL cholesterol-raising agents, such as torcetrapib, dalcetrapib, and niacin were controversial in that no benefit relative to cardiovascular disease was found (Ali, Wonnerth, Huber & Wojta, 2012). This unexpected result is explained possibly by emerging evidence that suggests that the vascular functions of HDL can be highly diverse and vasoprotective properties of HDL are modified in patients with coronary disease (Riwanto & Landmesser, 2013). The drug trials may not induce the appropriate changes in HDL function.

Inhibition of Platelet Aggregation and Thrombosis

Platelets are deeply involved in the initiation and progression of atherosclerotic lesions (Lindemann, Krämer, Daub, Stellos & Gawaz, 2007; Weber, 2005) (Siegel-Axel, Daub, Seizer, Lindemann & Gawaz, 2008). Arterial thrombus development is determined by the equilibrium between prothrombotic mediators, such as tissue factor (TF) and PAI-1, and antithrombotic factors, such as tissue factor pathway inhibitor (TFPI) and tissue plasminogen activator (tPA) (Furie & Furie, 2008). Platelets produces proinflammatory cytokines, chemokines and growth regulatory molecules which will promote endothelial dysfunction and alter smooth muscle cells function. Furthermore, platelets direct leukocyte integration into plaque through-mediated leukocytes adhesion which results in narrowing or complete occlusion of coronary arteries (Gawaz, Neumann, Ott, Schiessler & Schömig, 1996; Ott, Neumann, Gawaz, Schmitt & Schömig, 1996).

Numerous studies support the concept that HDL particles are powerful inhibitors of platelet activation and aggregation. The antithrombotic potency of HDL is detected as inhibitory actions on platelet aggregation and activation (Nofer, Brodde & Kehrel, 2010) as well as on factors that promote blood coagulation, including tissue factor, and factors X, Va, and VIIIa (Calabresi, Gomaraschi & Franceschini, 2003; Nofer, Brodde & Kehrel, 2010; Nofer, Kehrel, Fobker, Levkau, Assmann & von Eckardstein, 2002). Moreover, in a study on mice lacking SR-B1 receptor, it was revealed that their platelets aggregate poorly which suggests that lack of ability to efflux cholesterol via SR-B1 may impact function possibly due to formation of a cholesterol laden membrane (Dole et al., 2008). In further work Nofer and colleagues revealed that HDL3 exerts a progressive regulatory effects on the Na+/H+ counter-transporter system in human platelets by attachment to glycoprotein IIb/IIIa with activation of protein kinase C and phospholipase C (Nofer et al., 1998).

1.6.3 Role of HDL in LDL Oxidation

HDL also has well-acknowledged antioxidative properties. The antioxidant property of HDL is believed to be critical in potential antiatherogenic effects. Oxidized low-density lipoprotein (oxLDL) is believed to be the main offender in endothelial dysfunction; oxLDL induces endothelial damage, monocyte adhesion, and platelet aggregation and inhibits apoptosis and endothelial nitric oxide synthase (eNOS) expression and (or) activity, all of which contribute to atherosclerotic process (Li & Mehta, 2003). HDL has been shown to prevent oxidative modification of LDL, thus reducing the production of macrophage foam cells in the vessel wall (Barter, Nicholls, Rye, Anantharamaiah, Navab & Fogelman, 2004).

The antioxidant activity of HDL is predominantly related to various types of associated apolipoproteins and antioxidant enzymes that serve to hydrolyze and/or remove oxidized lipids (Navab et al., 2004). For example, HDL-associated apoA-I, apoA-II, apoA-IV, apoE, apoJ, and apoM are known to contribute to antioxidant activity (Elsoe et al., 2012). The antioxidant activity of both apoA-I and apoM is thought to be attributable to their ability to remove oxidized PLs from both LDL and peripheral cells-including those in the arterial wall which may then be eliminated from the body through the liver (Elsoe et al., 2012).

The mechanism by which HDL performs antioxidant activity is complex and multifactorial. The lipid hydroperoxides formed on LDL will migrate to its surface as a result of their greater hydrophilicity. Consequently, this will facilitate their transfer to HDL. HDL might therefore

provide a pathway for the passage of lipid peroxides and lysophospholipids to the liver via hepatic scavenger receptors. HDL , in point of fact, actually metabolizes lipid hydroperoxides preventing their accumulation and hence impeding the atherogenic structural modification of LDL (Soran, Schofield & Durrington, 2015). Moreover, Kunitake and colleagues has demonstrated that HDL exhibit chelation properties due to the presence of proteins such as ceruloplasmin on the surface of the lipoprotein, although the clinical relevance is controversial (Kunitake, Jarvis, Hamilton & Kane, 1992).

HDL is also known to associate with enzymes possessing antioxidant activity, including paraxonase 1 (PON1), and LCAT (Navab et al., 2004). These enzymes provide antioxidant activity to HDL by hydrolysing LDL-derived oxidized PL species, thereby inhibiting the formation of oxLDL (Kontush & Chapman, 2006). HDL has also been shown to bind glutathione selenoperoxidase (GSPx), which further serves to protect against oxidative stress by reducing lipid hydroperoxidase (Chen, Liu, Greiner & Holtzman, 2000).

In addition to the antioxidant activity conferred from associated apolipoproteins and enzymes, HDL can prevent oxLDL formation by sequestering oxidizing transition metal ions (Kunitake, Jarvis, Hamilton & Kane, 1992). HDL is also a known carrier of antioxidant micronutrients, including vitamin E and carotenoids. However, the extent by which these micronutrients contribute to the antioxidant activity of HDL remains unclear (Andersen & Fernandez, 2013).

HDL with defective antioxidant activities have been identified in metabolic syndrome (Hansel et al., 2004), type 2 diabetes (Nobecourt et al., 2005), and healthy postmenopausal women (Zago et al., 2004) when compared to healthy and premenopausal women, respectively.

1.6.4 Role of HDL in Inflammation

In addition to antioxidant activity, functional HDL is known to possess anti-inflammatory properties. Anti-inflammatory activity of HDL typically refers to the ability of HDL to inhibit endothelial cell expression of adhesion molecules in response to cytokines, thereby reducing monocyte adhesion to the arterial wall in the initial stages of atherosclerosis development (Kontush & Chapman, 2006). Arterial inflammatory pathways and adhesion molecules

expression can be activated by the same accumulation of oxidized lipids that cause oxidative stress (Andersen & Fernandez, 2013).

The anti-inflammatory activity of HDL is mostly attributed to the same enzymes that exert antioxidant activity, including HDL-associated apolipoproteins (i.e. apoA-I, apoA-II, apoA-IV, apoM) and oxidized lipid-hydrolyzing enzymes (PON1, PAF-AH, and LCAT) (Elsoe et al., 2012). Bioactive PL species carried by HDL such as SIP- which binds to apoM- may also play a role in HDL's anti-inflammatory activity, in addition to antiapoptotic and immunoregulatory functions (Christoffersen et al., 2011).

Inflammation is a major element in the pathogenesis of atherosclerosis that develops in response to LDL-derived cholesterol deposition in arteries. In general, HDL has potent anti-inflammatory properties by inhibiting pro-inflammatory adhesion molecules expression and by stimulating TGFβ2 expression in endothelial cells (Barter, Nicholls, Rye, Anantharamaiah, Navab & Fogelman, 2004; Cockerill et al., 2001; Norata, Callegari, Marchesi, Chiesa, Eriksson & Catapano, 2005) . HDL also reduce the effect the pro-inflammatory activity of C-reactive protein (CRP) (Wadham et al., 2004) , inhibits pro-inflammatory prostaglandins production by monocytes (Jambou et al., 1993) and inhibit, or neutralizes ,the effects of oxidized LDL PLs on endothelium (Navab et al., 2001).

1.6.5 Role of HDL in Diabetes Mellitus

Type 2 diabetes mellitus (T2DM) is a pandemic of major public health whose importance cannot be disputed. From 1990 to 2010, T2DM has become the worldwide ninth most prevalent cause of death (von Eckardstein & Widmann, 2014). Cardiovascular disease, chronic kidney disease and cancer are the major reasons for premature mortality of diabetic patients. HDL appears to have a protective role by improving β cell secretory function and antagonizing the apoptosis of these cells (Roehrich et al., 2003).

Promotion of β-cell Survival

The main pathophysiological event in diabetes mellitus is the deterioration of pancreatic β cell function following persistent insulin resistance. At the time of T2DM diagnosis, the secretory function of β cells has dropped by approximately 50% of normal (U.K, 1995).In vitro, HDL enhanced beta-cell survival and protected them from apoptosis (Pétremand et al.,

2009; Rutti et al., 2009). HDL halts β cell death caused by a variety of stimuli including inflammatory cytokines, F.F.As like palmitate, thapsigargin, tunicamycin, protein overexpression, etc. Some of these stimuli induce endoplasmic reticulum (ER) stress which drives beta-cell dysfunction and death in the course of diabetes mellitus development (Eizirik, Cardozo & Cnop, 2008; Oyadomari, Araki & Mori, 2002; Volchuk & Ron, 2010)

Promotion of Insulin Secretion

In addition to protecting beta cells from death, HDL may also support their survival and function by expanding their proliferation or their insulin secretory capacity (Drew, Rye, Duffy, Barter & Kingwell, 2012). In UK prospective diabetes study (UKPDS), log(TG)/HDL-C ratio, as a surrogate marker of atherogenic dyslipidemia, was associated with declined insulin sensitivity and impaired β cell function in 585 male patients with T2DM (Hermans, Ahn & Rousseau, 2010). In a study for Drew and his team, infusion of reconstituted high density lipoprotein (rHDL), 80 $mg.kg^{-1}$ over 4 hours, declined plasma glucose, raised plasma insulin and improved the HOMA index compared with placebo in patients with type 2 diabetes (Drew et al., 2009). In addition, Verger and colleagues underlined the role of ABCA1 in glucose metabolism by demonstrating impaired insulin secretion in carriers of loss-of-function mutations in ABCA1, which demonstrate that ABCA1 is essential for normal beta-cell function in humans (Vergeer et al., 2010). This finding suggests that the removal of excess cholesterol from beta- cells through ABCA1 may play a role in the HDL-mediated modulation of the insulin secretory pathway and in the maintenance of cholesterol homeostasis in pancreatic beta-cells.

1.7 Genetics of HDL

In spite of the fact that HDL-C levels are inversely correlated to cardiovascular disease risk and that HDL particles possess a range of anti-atherosclerotic properties, several recent clinical trials using various strategies aimed at raising HDL-C levels have failed to yield the expected improvement in clinical outcomes. This has highlighted the need for a better understanding of HDL particle function and metabolism (Barter et al., 2007; Boden et al., 2011; Group, 2013; Keene, Price, Shun-Shin & Francis, 2014; Landray et al., 2014; Schwartz et al., 2012). The study of human genetics of HDL has provided an outstanding view of

lipoprotein biology, identifying rare genetic disorders, as well as a number of key players of HDL metabolism. Based on twin studies, heritability estimates for HDL-C suggested that 62-77% of the variance in HDL-C is due to genetic factors (Beekman et al., 2002; Goode, Cherny, Christian, Jarvik & de Andrade, 2007; Souren et al., 2007; Sung, Lee & Song, 2009).

ApoA-I

ApoA-1 is the principal protein constituent of HDL which is synthesized and secreted by the liver and intestine. Modification of apoA-1 leading to its functional impairment appears to be a critical step in the pathogenesis of atherosclerosis. Norum and colleagues was the first to describe genetic deficiency of ApoA-I in two sisters with remarkably low HDL-C levels and premature CVD whereas the concentrations of other lipoproteins like low density lipoprotein cholesterol (LDL-C) were not significantly altered. This has established that apoA-1 is essential for the biogenesis of HDL particles but not for other lipoproteins (Norum et al., 1982). Individuals who are homozygous or compound heterozygous for apoA-I gene mutation have almost a complete absence of apoA-I and HDL-C in plasma. Those individuals are reported to have increased risk for premature CVD and may develop corneal opacities or xanthomas. To-date, forty-three mutations have been reported in the APOA1 gene that cause low HDL-C or low plasma apoA-I levels (Stenson et al., 2009) . Some mutations have been related with increased CVD risk (Hovingh et al., 2004). Individuals of ApoA-I_{Milano} , carriers of one variant (P.R197C), have considerably reduced HDL-C levels but not related with CVD which gives increase to the theory that it could be a beneficial variant (Sirtori et al., 2001).

Certain mutations in apoA-I, many of which are in the amino terminus of the encoded protein, also cause hereditary amyloidosis that is inherited in an autosomal dominant manner. In contrast to loss-of-function mutations in apoA1 that lead to low HDL-C, it has been suggested that amyloidogenic mutations in apoA-I are gain-of-function variants (Obici et al., 2006). Plasma lipoproteins levels in these individuals are normal or can be reduced (Joy, Wang, Hahn & Hegele, 2003) implying that these mutations do not universally impede the assembly of HDL (Brunham & Hayden, 2015).

ABCA1

The first step in the reverse cholesterol transport pathway is the efflux of PLs and unesterified cholesterol from cell membranes to lipid-poor form of apolipoprotein A-I in a process promoted by ABCA1. In 1961, Fredrickson recognized an autosomal recessive disorder in which a mutation in the ABCA1 gene results in TD (Bodzioch et al., 1999; Brooks-Wilson et al., 1999; Lawn et al., 1999; Rust et al., 1999; Saku, Jimi, Ohta & Arakawa, 1994). Patients with TD have almost no HDL-C, reduced LDL-C, and yellow lipid-swollen tonsils (Bale, Clifton-Bligh, Benjamin & Whyte, 1971; Ferrans & Fredrickson, 1975), manifest splenomegaly and consumptive thrombocytopenia too (Brunham & Hayden, 2015). Plasma from patients with TD contains only small amounts of pre-β HDL, with no α-migrating particles (Asztalos, Brousseau, McNamara, Horvath, Roheim & Schaefer, 2001). Most reports suggest that patients with TD have increased CVD risk, although not as great as would be expected based on the extremely low levels of HDL-C (Serfaty-Lacrosniere et al., 1994) but whether heterozygous mutations in ABCA1 increase the susceptibility to atherosclerosis in humans remains the subject of debate (Clee et al., 2000).

One-hundred and seventy-seven ABCA1 variants are catalogued in the Human Gene Mutation Database (Stenson et al., 2009) and these are associated with a broad range of biochemical and clinical phenotypes (Singaraja, Brunham, Visscher, Kastelein & Hayden, 2003). Only some of these variants are pathogenic mutations. The effect of mutations in ABCA1 appears to be dominant to mutations in other HDL-related gene. For example, in patients with mutations in SCARB1 or HL who are expected to have high HDL-C, the presence of a mutation in ABCA1 results in low HDL-C (Brunham et al., 2011). This could be related to the fact that ABCA1 acts in a very proximal step in HDL biogenesis and the corresponding effect in genes that act downstream of ABCA1 will generally be masked by mutations in ABCA1 (Brunham & Hayden, 2015).

While both TD and apoA-I deficiency have near absence of plasma HDL-C and inability to generate HDL particles, there are notable clinical differences between these two disorders. For instance, the systemic manifestations of TD, including hepatosplenomegaly, peripheral neuropathy and abnormal glucose metabolism are notably absent in patients with apoA-I deficiency. This indicates that the lack of ABCA1-mediated cholesterol efflux activity is the

one responsible for the multi-organ system involvement in TD rather than the absence of HDL itself.

LCAT

LCAT is secreted principally in the liver and circulates in plasma bound to HDL and LDL, where it catalyzes the esterification of free cholesterol to cholesterol ester in HDL (Kwan, Kronenberg, Beddhu & Cheung, 2007). Two human genetic diseases are caused by mutations in LCAT; familial LCAT deficiency (FLD) in which a severe mutation in LCAT leading to complete loss of enzymatic action (Norum & Gjone, 1967) and Fish Eye Disease (FED), so-called because of the appearance of the eyes of those patients, where milder mutations in LCAT mean that enzyme activity on HDL is lost but is preserved in other lipoprotein particles (Carlson, 1982). FLD patients present with corneal opacities, lipemia, anemia, proteinuria and renal failure that can progress to end-stage renal disease (Stoekenbroek, van den Bergh Weerman, Hovingh, Potter van Loon, Siegert & Holleboom, 2013).

Both FLD and FED are rare autosomal recessive disorders, but mutations in LCAT occur frequently in patients with low HDL-C in the general population (Brunham & Hayden, 2015). While the functional significance of many of these mutations uncertain, the data indicate that, in addition to being the cause of the rare Mendelian disorders, FLD and FED, LCAT mutations are a common cause of low HDL-C in the general population, particularly in certain ethnic populations (Brunham & Hayden, 2015).

CETP

Cholesterol in HDL can be returned to the liver through two pathways, the direct reverse cholesterol transport pathway in which HDL-containing cholesterol is taken by the liver by HDL receptor, and the indirect reverse cholesterol transport pathway, in which cholesterol is first transferred to an apoB- containing lipoproteins for hepatic uptake via LDL receptor. CETP mediates the equimolar transfer of cholesterol ester from HDL for triglycerides from LDL, VLDL or chylomicrons. The first deficiency in human CETP was discovered in five Japanese families with a common splice mutation in intron 14 (Brown et al., 1989; Inazu et al., 1990). Patients with homozygous CETP mutations have significantly high HDL-C and apoA-I levels. The catabolism of HDL apolipoproteins is reduced in these patients while their production rate is unchanged (Ikewaki et al., 1993). Patients with CETP deficiency also have

~35% reductions in LDL-C (Inazu et al., 1990) with rapid LDL-C catabolism (Ikewaki et al., 1995). Those changes in LDL-C are also observed in patients receiving potent pharmacologic CETP inhibitors (Cannon et al., 2010).

Lipases: LPL, LIPC and LIPG

HDL CE depletion and TG enrichment by CETP primes HDL particles for hydrolysis by lipase enzymes. Three enzymes are known to play roles in remodeling of HDL in humans, LPLs (encoded by LPL gene), HL (encoded by LIPC) and endothelial lipase (encoded by LIPG) (Brunham & Hayden, 2015).

Patients with milky lipemic plasma were recognized as early as the late 18th century and familial chylomicronemia (Frederickson hyperlipoproteinemia type 1) was first reported in the early 20th century (Brunham & Hayden, 2015). This disorder, which manifests as severe hypertriglyceridemia, eruptive xanthomas and recurrent pancreatitis, was subsequently shown to be due to an absence of LPL activity (Havel & Gordon, 1960). The first mutations in the LPL gene in familial chylomicronemia were reported in 1989 (Langlois, Deeb, Brunzell, Kastelein & Hayden, 1989). Patients with LPL deficiency also display significantly reduced plasma LDL-C and HDL-C, in particular a near absence of HDL2 and reduced HDL3. Accordingly, these observations indicate that plasma HDL-C, and in particularly cholesterol transported by HDL2, are dependent on the activity of LPL, possibly by LPL releasing apolipoproteins from chylomicrons and VLDL particles necessary for the generation and maturation of HDL. These findings also provide a molecular underpinning for the known inverse relationship between plasma TG and HDL levels. Recently, a gene therapy product for LPL deficiency consisting of delivery of the beneficial LPL ^{S447X} gene variant (Ross et al., 2005) obtained regulatory approval, becoming the first approved gene therapy product in humans (Yla-Herttuala, 2012).

HL, encoded by the LIPC gene, is synthesized in the liver and secreted into plasma where it acts primarily to hydrolyze triglycerides in HDL and to a lesser extent PLs. Patients with deficiency of HL have increased levels of total plasma cholesterol, TG and HDL-C (Breckenridge et al., 1982). In particular, HDL-TGs tend to be increased in these patients. Genetic deficiency of LIPC is a very rare condition, with only 12 pathogenic mutations reported in this gene (Stenson et al., 2009). Patients with LIPC deficiency are generally considered to have increased CVD risk despite their elevated HDL-C (Connelly, Maguire, Lee

& Little, 1990). This finding accentuates the point that not all mechanisms leading to increased HDL-C are associated with protection from CVD.

Endothelial lipase is the most recently identified member of the lipase family and acts primarily as a phospholipase. Studies in mice have shown that overexpression of LIPG results in reduced HDL-C and apoA-1 (Jaye et al., 1999). Inhibition of LIPG activity in mice using an inhibitory antibody results in increased HDL-C and retard catabolism of HDL-PLs. Carriers of rare, loss-of function mutations in LIPG have increased HDL-C, and HDL from these patients has enhanced ability to elicit cholesterol efflux from cells (Singaraja et al., 2013). Some studies have suggested that carriers of LIPG loss-of-function mutations have reduced CVD (Singaraja et al., 2013). In contrast, a large Mendelian randomization study reported that a variant in LIPG was not associated with a decrease in incident myocardial infraction (Voight et al., 2012).

SCARB1

Scavenger receptor class B member 1 (SR-B1), encoded by the SCARB1 gene, is a critical component for the mechanism by which HDL-C is taken up by the liver for removal into bile (Brunham & Hayden, 2015). Mice that have depleted SCARB1 (SR-B1 knockout mice) have markedly elevated HDL-C levels but, paradoxically, increased atherosclerosis. The impact of SR-B1 on HDL metabolism and CHD risk in humans remains unclear (Zanoni et al., 2016). Through targeted sequencing of coding regions of lipid-modifying genes in 328 individuals with extremely high plasma HDL-C levels, Zanoni and colleagues has identified a homozygote for a loss of-function variant, in which leucine replaces proline 376 (P376L), in SCARB1. The P376L variant impairs posttranslational processing of SR-B1 and abrogates selective HDL cholesterol uptake in transfected cells, in hepatocyte-like cells derived from induced pluripotent stem cells from the homozygous subjects, and in mice. Large population-based studies revealed that subjects who are heterozygous carriers of the P376L variant have significantly increased levels of plasma HDL-C. P376 carriers have a profound HDL-related phenotype and an increased of CHD (Zanoni et al., 2016).

PLTP

PLTP is involved in the transfer of PLs from apoB-containing lipoproteins to HDL in a process that is essential for HDL maturation and maintenance. Jiang and co-workers has found that mice lacking PLTP display markedly reduced HDL-C and PLs levels and reduced plasma apoA-1 concentrations (Jiang et al., 1999). In humans, however, genetic variants in PLTP has been identified that are associated with both high and low HDL-C and some of these variants lead to reduced PLTP action (Aouizerat et al., 2006; Engler et al., 2008; Kiss et al., 2007). In genome wide association studies (GWAS), a new role for PLTP, in influencing HDL-C levels in humans, has been highlighted through the identification of PLTP as a locus for both TG and HDL-C (Willer et al., 2013). To-date, no rare loss-of-function mutations in PLTP have been established in humans that lead to highly pointing abnormalities of plasma HDL-C (Brunham & Hayden, 2015).

1.8 Epidemiology of Cardiovascular Disease.

Thrombosis and atherosclerosis of the arterial vessel wall are the main underlying causes of cardiovascular disease (CVD). CVD causes premature death as well as mass disability. Although CVD mortality has fallen considerably over recent decades in many European countries, it is still a major global problem since it is estimated that > 80% of all CVD mortality now occurs in developing countries (Perk et al., 2013).

Types of Cardiovascular Diseases

Cardiovascular diseases (CVDs) are a collective term for a wide range of conditions that involve narrowed or blocked blood vessels that can lead to a heart attack, chest pain (angina) or stroke. The term "heart disease" (CHD) is often used interchangeably with the term cardiovascular disease (CVD). The main clinical types of CVD are coronary artery disease (CAD), ischemic stroke, and peripheral arterial disease (PAD) (Catapano et al., 2011).

1.9 Cardiovascular Risk Markers

A large number of risk factors have been identified as associated with CVD. These can be divided into lifestyle factors (such as smoking, diet, exercise and alcohol intake), metabolic factors (such as diabetes) and inflammatory factors. The factors multiply together to

increase the risk of CVD as shown in **Figure 1.16** and have been used in international guidelines to develop risk prediction algorithm such as Score (Perk et al., 2013).

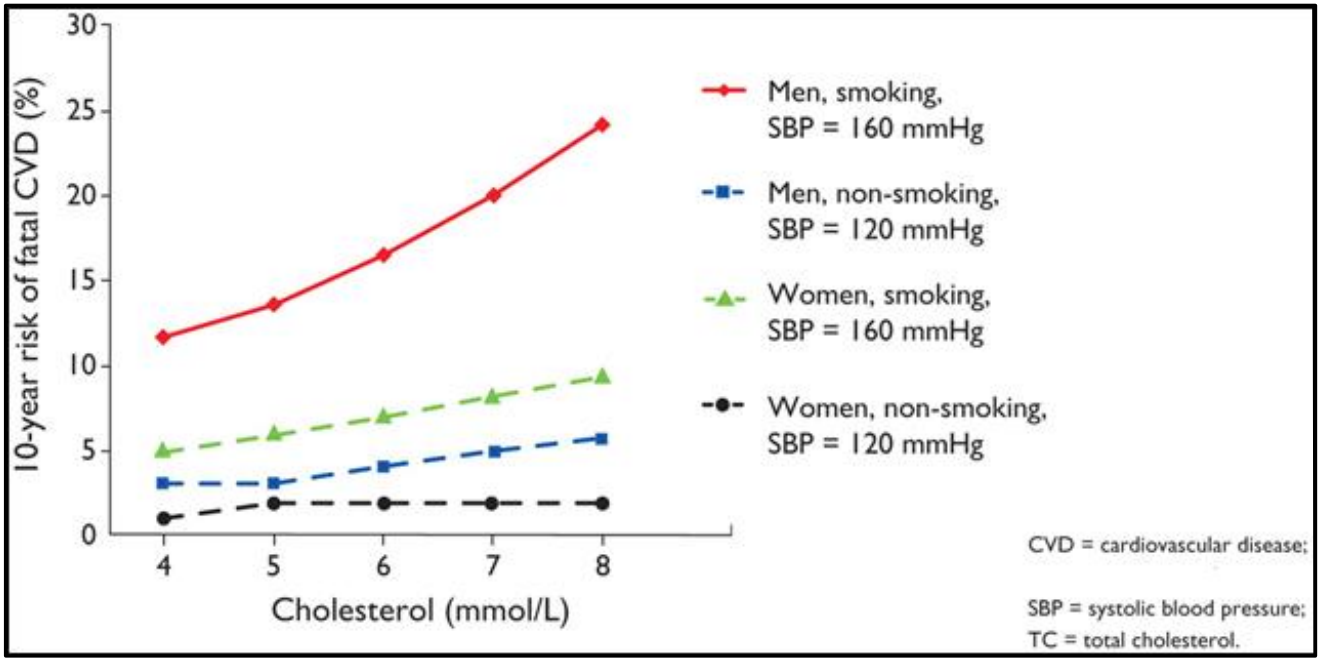


Figure 1.16 Relationship between total cholesterol and 10-year fatal CVD events in men and women aged 60 years with and without risk factors, based on a risk function derived from the SCORE project. [Source: (Perk et al., 2013)].

1.9.1 Lipid Markers of CVDs

Dyslipidaemias include a wide spectrum of lipid abnormalities, some of which are of great significance in CVD prevention. Genetic and pathological studies, as well as observational and interventional studies, have established the crucial role of dyslipidemia, especially hypercholesterolemia, in the development of CVD.

Total Cholesterol

In screening programs, total cholesterol (TC) is suggested to be used to estimate total CVD risk. In specific cases, however, TC may be misleading. This is particularly so in women who regularly have high HDL-C levels and in individuals with diabetes or metabolic syndrome who often have low- HDL-C levels (Catapano et al., 2011).

Low-Density Lipoprotein Cholesterol

Most of the cholesterol in blood plasma is generally carried in LDLs (Crowley, 2004) and, over a wide range of cholesterol concentrations, there is a strong and positive association between TC as well as LDL-C and the risk of CVD (Neaton et al., 1992) . This association applies for both women as well as men without CVD and patients with established disease (Perk et al., 2013). Meta-analysis of many trials revealed a clear dose-dependent relative reduction in CVD risk with LDL-C lowering. A 1.0 $mmol.L^{-1}$ reduction in LDL cholesterol is associated with a 20-25% reduction in CVD mortality and non-fatal myocardial infraction. In more recent trials, it was confirmed that for high-risk individuals, the target LDL-C level should be < 1.8 $mmol.L^{-1}$ (~70 $mg.dL^{-1}$) and / or a reduction of ≥ 50% from baseline LDL-C (Baigent et al., 2010).

High-Density Lipoprotein Cholesterol

Observational studies have revealed that low concentrations of HDL-C are an independent risk factor for CVD. Therefore, HDL-C is also included in new Score chart (Chapman et al., 2011). Reduced concentrations of HDL-C may even compete with LDL-C as the most evident risk factor for CHD in some people (Fruchart et al., 2008). On the other hand, the current

state of evidence is not enough for any HDL-C value to be considered as a goal of therapy, even though, it is considered that HDL-C < 1.0 mmol.L-^{1} (~40 mg.dL^{-1}) in men and < 1.2 mmol.L^{-1} (~45 mg.dL^{-1}) in women may be a marker of increased risk (Graham et al., 2007).

Triglycerides

Hypertriglyceridemia is a significant independent CVD risk factor. However, the relationship is not as strong as for hypercholesterolemia (Sarwar et al., 2007). At this time, fasting TGs > 1.7 mmol.L-^{1} (~150 mg.dL^{-1}) is considered as a marker of increased risk, but concentrations ≤ 1.7 mmol.L-^{1} are not evidence-based target levels for therapy (Perk et al., 2013).

Apolipoprotein B/Apolipoprotein A-I Ratio

Apolipoprotein A-I is the major protein component of HDL. The apoB/A-I ratio reflects the relative number of apoB-containing atherogenic particles compared with the number of 'protective' apoA-I containing particles. This ratio is superior to the standard LDL-C/HDL-C ratio in predicting myocardial infraction (MI) risk (Sniderman & Marcovina, 2006) but it is not generally recommended as a treatment goal. Moreover, because it involves protein determinations, it is considered to be more complicated technologically and more expensive than currently used markers of cholesterol (Perk et al., 2013).

1.9.2 Non Lipid Markers of CVDs

Genetics and Family History

CVD encompasses a broad range of disorders, including diseases of the vasculature, diseases of the myocardium, diseases of the heart's electrical circuit, and congenital heart disease (Roger et al., 2012). For nearly all of these disorders, inherited DNA sequence variants play a role in determining risk (Kathiresan & Srivastava, 2012). A number of genetic polymorphisms seem to have significant effects on CVD risk at the population level. On the other hand, the influence of these polymorphisms remains rather moderate. Genetic testing can identify variants associated with increased risk to individual CVD risk factor, CHD, or stroke. Alternatively, commercial testing has not yet been proved to be useful (Botkin et al., 2010).

In some cases like hypercholesterolemia, family screening can be used to identify patients at risk and allows timely treatment of affected relatives (Huijgen et al., 2010; Paynter et al., 2010).

Age and Gender

Advancing age and male sex increase CVD risk and are considered as fixed characteristics used to stratify risk assessments (Conroy et al., 2003). Age is positively related to known (and unknown possibly) CHD risk factors. Women have lower incidence of CHD in Europe (European Heart Network, 2008). This lower rate may result from the protective effect of endogenous oestrogens. CVD mortality dramatically increases in women following the menopause, indicating that women are postponing their risk rather than avoiding it altogether.

Physical Inactivity

A lack of physical activity and sedentary lifestyle is one of the major risk factors for CVD (Warren, Barry, Hooker, Sui, Church & Blair, 2010). There is substantial evidence from epidemiological studies to support an inverse relationship between regular physical activity (particularly aerobic exercise) and fatal and non-fatal coronary events in initially healthy individuals (Graham et al., 2007; Löllgen, Böckenhoff & Knapp, 2009; Nocon, Hiemann, Müller-Riemenschneider, Thalau, Roll & Willich, 2008; Talbot, Morrell, Fleg & Metter, 2007; U.S Department of Health and Human Services, 2008) as well as individuals with coronary risk factors (Richardson, Kriska, Lantz & Hayward, 2004) and cardiac patients (Piepoli, Davos, Francis, Coats & Collaborative, 2004; Taylor et al., 2004). The benefit may be due to the fact that aerobic exercise results in reduced myocardial oxygen needs for matching level of work done. Physical activity has a positive influence on various recognized risk factors of CVD, reducing blood pressure in hypertensive patients, preventing or delaying the development of hypertension in normotensive individuals, increasing HDL-C levels, helping to control body weight, and lowering the risk of developing non-insulin-dependent diabetes mellitus (Graham et al., 2007).

Unhealthy Diet

Cardiovascular risk is known to be influenced by dietary habits. Diet is believed to influence risk either by affecting major factors such as cholesterol, blood pressure, body weight and diabetes or through effects independently of these risk factors. Measurement of the 'unhealthiness' of diet is complex and an imprecise estimate of diet quality is likely to reduce the strength of the observed relationship.

Fruits and Vegetables: Observational studies have revealed that the consumption of fruit and vegetables has a protective role on CVD. Moreover, it is also known that individuals who consume a lot of fruits and vegetables differ in many other lifestyle aspects from those who eat little fruit and vegetables, for example with respect to levels of physical activity, smoking status or other dietary habits all of which might confound the association. Although individual studies have shown weak or non-significant effects of changing fruit and vegetable intake on CVD risk, results in large cohort studies have been relatively homogeneous, and several meta-analyses have revealed a statistically significant effect. Dauchet L et al. reported a 5% reduction in risk of stroke for each additional serving of fruit and vegetables in a meta-analysis of seven large prospective cohort studies (Dauchet, Amouyel, Hercberg & Dallongeville, 2006). This study was updated by He et al by two additional cohorts, and a pooled relative risk of stroke for those eating 3-5 servings of fruits and vegetables daily and for those eating >5 servings was 0.89 (95% CI 0.83 - 0.97) and 0.74 (95% CI 0.69 - 0.79) respectively (He, Nowson & MacGregor, 2006). It was also reported a decrease in CHD risk of 4% (Relative Risk 0.96, 95% CI 0.93-0.99) for each additional serving of fruits and vegetables per day (Dauchet, Amouyel, Hercberg & Dallongeville, 2006). One serving is equivalent to ~80 g.

Fish: Stone et al reviewed three prospective epidemiological studies within population and reported a lower coronary heart disease mortality rate for men who eat at least some fish weekly compared to those who eat none (Kris-Etherton, Harris & Appel, 2002). The protective effect of fish on CVD may relate to the omega-3 (or n-3) fatty acid content. He et al has also showed that eating fish at least once a week results in a 15% reduction in risk of CHD (Relative risk of 0.85, 95% CI 0.76-0.96) (He et al., 2004). Therefore, the public health impact of a small increase in fish consumption in the general population is potentially large.

A modest increase in fish consumption of 1-2 servings a week would reduce CHD mortality by 36% and all-cause mortality by 17% (He et al., 2004).

Alcoholic Beverages

While the harmful effects of alcohol on conditions such as liver cirrhosis, injuries, and cancers of the liver, colorectum, breast, and upper aerodigestive tract have been firmly established, uncertainity remains concerning the potential protective effects of light to moderate alcohol consumption on risk of coronary heart disease and stroke. Observational studies have consistently reported that compared with non-drinkers, light to moderate drinking exhibits a reduced cardiovascular risk, with the lower risk found at approximately 12-25 British units per week, while heavier and more hazardous drinking is associated with an increased risk, resulting in the well-established U shaped association (Holmes et al., 2014). This possibly causal link was evident in analysis of over 60 ecological, case control, and cohort studies. Moreover, it was concluded from previous reviews that both men and women who drink one to two drinks a day have lower risk of coronary heart disease (Klatsky, Armstrong & Friedman, 1992; Maclure, 1993; Moore & Pearson, 1986). The most widely proposed mechanism for this purported cardioprotective effect of alcohol is an increase in HDL-C (Brien, Ronksley, Turner, Mukamal & Ghali, 2011).

In 2014, Mendelian randomization analysis based on individual participant data has, however, abolished this association and suggested that reduction of alcohol consumption, even for light to moderate drinkers, is beneficial for cardiovascular health (Holmes et al., 2014).

Smoking

Smoking has been recognized to account for 14% of deaths from CVD (Health and Social Care Information Centre (HSCIC, 2012). The risk is significantly reduced within two years of smoking cessation (Salonen, 1980). Unlike non-smokers, smokers have a 2 to 4 times increased risk of heart disease and of stroke (U.S. Department of Health and Human Services, 2004) and in fact smoking is associated with increased risk of all types of CVD-CHD, ischemic stroke, peripheral artery disease, and abdominal aortic aneurysm (Perk et al., 2013).

The exact mechanisms by which smoking increases the risk of atherosclerosis are not fully understood. It is proven that smoking boosts both the progress of atherosclerosis and the incidence of thrombotic phenomena. Reactive oxygen species-free radicals- present in inhaled smoke may cause oxidation of plasma LDL and oxidized LDL activates the inflammatory process in the intima of the arteries by stimulation of monocyte adhesion to the vessel wall, resulting in increased atherosclerosis (Weber, Erl, Weber & Weber, 1996; Yamaguchi, Haginaka, Morimoto, Fujioka & Kunitomo, 2005; Yamaguchi, Matsuno, Kagota, Haginaka & Kunitomo, 2001).

Overweight and Obesity

Obesity is an excess of body fat and is most often estimated by the ratio of weight over height. Body mass index (BMI) [weight (kg)/length $(m)^2$] is the most commonly used anthropometric index to define categories of body weight. Adults whom their BMI ranges from 25 to 29.9 kg/m^2 are considered overweight, and those where BMI ≥ 30 kg/m^2, are considered obese. Increasing BMI is highly associated with risk of CVD. However, regional distribution of adipose tissue – abdominal versus gluteal - was hypothesized to be more important in determining cardiovascular risk than total body weight. This has led to increased interest in waist to hip ratio as a measure of rather than total body fat (Perk et al., 2013).

Blood Pressure

High blood pressure is well established as a major risk factor for CHD, heart failure, cerebrovascular disease, PAD, renal failure, and more recently, arterial fibrillation (AF) (MacMahon et al., 1990; Wattigney, Mensah & Croft, 2003). Observational data involving > 1 million individuals have indicated that death from both CHD and stroke increases progressively and linearly from blood pressure levels as low as 115 mmHg systolic and 75 mmHg diastolic upwards (Lewington, Clarke, Qizilbash, Peto, Collins & Collaboration, 2002).

Raised Blood Glucose (Diabetes and Pre-Diabetes Mellitus)

There is conclusive evidence that improving glycemic control significantly reduces the risk of developing diabetic microvascular complications (retinopathy, nephropathy, and neuropathy). Despite existing data indicate a relationship between increased levels of glycemic and cardiovascular events, there has been little suggestion that specifically targeting glycemic control can reduce the frequency of cardiovascular endpoints (Perk et al., 2013).

Socio-Economic Background

Low socio-economic status, lack of social support, stress at work and in family life, depression, anxiety, hostility, and personality type have all been reported to contribute both to the risk of developing CVD and the worsening of clinical course and prognosis of CVD. These factors act as barriers to treatment adherence and efforts to improve lifestyle, as well as to promoting health and wellbeing in patients and populations. In addition, distinct psychobiological mechanisms have been identified, which are directly involved in the pathogenesis of CVD. Multiple prospective studies have shown that men and women with low socio-economic status, defined as low educational level, low income, holding a low-status job, or living in a poor residential area, have an increased all-cause as well as CVD mortality risk [relative risk (RR) ~ 1.3 - 2.0] (Stringhini et al., 2010; Tonne, Schwartz, Mittleman, Melly, Suh & Goldberg, 2005; Woodward, Brindle, Tunstall-Pedoe & estimation, 2007).

1.9.3 Other Biomarkers of CVD Risk

Despite the fact that the number of potential novel risk markers is growing yearly, in reality the number of important factors is constantly scaled once the possible candidates have passed through the grading of clinical evidence.

Inflammatory Markers

A role for inflammation in atherosclerosis has become well established over the previous decades (Ross, 1999; Tracy, 1998). From a pathology perspective, the stages of

atherosclerotic plaque formation including initiation, growth, and complication might be considered to be an inflammatory response to injury (Libby & Ridker, 1999; Plutzky, 2001). The conventional injurious factors which stimulate atherogenesis like hypertension, atherogenic lipoproteins, cigarette smoking and hyperglycemia give rise to a variety of noxious stimuli that prompt secretion of both leukocyte soluble adhesion molecules which will consequently facilitate the attachment of monocytes to endothelial cells, and chemotactic factors, which encourage the monocytes migration into the subintimal space. Fatty streak formation is then commenced by the transformation of monocytes into macrophages and by the accumulation of cholesterol from lipoproteins. Within the growing lesion, additional stimuli may continue the attraction and accumulation of macrophages, mast cells, and activated T cells inside the developing atherosclerotic lesion. One of the several factors which lead to weakening of the atherosclerotic cap is oxidized LDL as this will contribute to the loss of smooth muscle cells through apoptosis and through the secretion of metalloproteinases and other connective tissue enzymes by activated macrophages. Consequently, disrupted atherosclerotic plaque exposes the atherosclerotic core to arterial blood, which prompts thrombosis. Therefore, almost every step in atherogenesis involves cytokines, other bioactive molecules, and cells that are components of the inflammation process (Pearson et al., 2003).

Systematic biomarkers of both inflammation have been proposed as potentially useful for the early detection of the risk of cardiovascular incidents in both apparently healthy individuals and patients with CVD (Ridker, Buring, Rifai & Cook, 2007; Vidula et al., 2008). However, controversial results have been obtained using such biomarkers. An example is CRP, where some reports indicated predictable risks while other authors have obtained little information through CRP determination (Folsom et al., 2006; Melander et al., 2009).

1.9.4 Other Markers

Homocysteine: An elevated plasma level of homocysteine has long been known as an independent predictor of CVD (Refsum, Ueland, Nygard & Vollset, 1998; Wilcken & Wilcken, 1976). However, risk associated with this marker is modest, and regularly lacks consistency. This inconsistency might be due to nutritional, metabolic, like renal disease, and lifestyle confounders. Furthermore, altering homocysteine has shown to be ineffective in reducing

CVD risk in intervention studies in which vitamin B has been used to reduce plasma homocysteine. Homocysteine remains a second-line marker for CVD risk assessment (Clarke et al., 2010).

Lipoprotein-associated phospholipase A2: Lipoprotein-associated phospholipaseA2 (LpPLA2) is a 50-kd calcium-independent enzyme highly expressed by macrophages in atherosclerotic lesions (Häkkinen et al., 1999; Kolodgie et al., 2006). LpPLA2 has lately been suggested as an independent risk factor for plaque rupture and atherothrombotic events. Nevertheless, at the level of the general population, the extent of effect on risk remains moderate; study limitations or bias are present. LpPLA2 remains a second line marker for CVD risk estimation (Garza, Montori, McConnell, Somers, Kullo & Lopez-Jimenez, 2007).

1.10 CIMT and Plaque Presence

Some guidelines advocate the measurement of subclinical atherosclerosis as a risk marker since it is likely to be a precursor of clinically evident disease. A suitable candidate to assess early atherosclerosis noninvasively is the measurement of cIMT using ultrasound.

Carotid intima-media thickness (cIMT) is a well-established surrogate marker for cardiovascular disease where increased cIMT has been related to prevalent and incident CHD and stroke (Chambless et al., 1997; O'Leary et al., 1999). CIMT corresponds to the intima-media complex, which comprises endothelial cells, connective tissue, and smooth muscle and is the site of lipid deposition in plaque formation (Belcaro et al., 1996; Veller et al., 1993). In healthy adults, IMT ranges from 0.25 to 1.5 mm (Veller et al., 1993) and values of > 0.9 mm are considered to indicate high risk. Individuals without recognized CVD but with increased IMT are at bigger risk for cardiac events and stroke. This risk remains high even after correction for the presence of traditional risk factors (O'Leary et al., 1999).

Plaque is a term used for the central structure of the inner vessel wall at least ≥ 0.5 mm. Plaques can be characterized by their number, size, abnormality, and echodensity (echolucent vs. calcified). Plaques are correlated to both coronary obstructive disease and the risk of cerebrovascular events. Echolucent plaques imply an increased risk of cerebrovascular events as compared with calcified plaques. Patients with echolucent

stenotic plaques had a much greater hazard of cerebrovascular events than subjects with other means of assessing subclinical atherosclerosis.

The extent of cIMT is an independent predictor of cerebral and coronary events, but seems to be more prognostic in women than in men. Accordingly, carotid ultrasound can complement information beyond assessment of traditional risk factors that may help to sort decisions about the necessity to introduce medical treatment for primary prevention (Perk et al., 2013).

1.11 Therapeutic Effects of Elevating HDL

Worldwide, coronary artery disease (CAD) is one of the major causes of death (Yusuf, Reddy, Ounpuu & Anand, 2001) accounting for 17.3 million deaths per year, a number that is expected to grow to more than 23.6 million by 2030 (American Heart Association, 2014). One of the leading risk factors for development and progression of atherosclerosis in CAD is dyslipidemia which is characterized by change in lipoprotein spectrum particularly elevated LDL-C and decreased HDL-C (Arca et al., 2007). Therapeutic strategies are directed to lowering LDL-C, mostly by the use of statin. Despite the fact that aggressive strategies are now used to reduce LDL-C, the risk of cardiovascular events in patients with coronary artery disease remains significant. In recent years, however, there has been an inclination towards raising HDL-C as an additional target. The interest in raising HDL-C arose initially from the Framingham study which was the first large-scale study giving the evidence that a low level of HDL-C is a major risk factor for CAD.

The incidence of CAD with HDL-C levels revealed a stronger association than that with LDL levels (Gordon, Castelli, Hjortland, Kannel & Dawber, 1977). Wilson and his colleagues have re-evaluated the same study and found that low HDL-C levels were even associated with increased mortality (Wilson, Abbott & Castelli, 1988). Jenkins and colleagues have also found a significant association between HDL-C levels and the severity of atherosclerosis (Jenkins, Harper & Nestel, 1978). In 1989, Gordon et al remarked a decrease of 2-3% in CAD risk with each increase by 10 $mg.L^{-1}$ in HDL-C (Gordon et al., 1989). Moreover, a new meta-analysis for 302,430 individuals from 68 long-term prospective studies supported the importance of HDL-C measurement in the risk assessment for CAD (Di Angelantonio et al., 2009).

1.11.1 Established Treatments for Raising HDL-C

Statins: Statin, 3-hydroxymethyl-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, belong to a category of drugs that can inhibit a key enzyme in the pathway of cholesterol synthesis (Corsini et al., 1998; Vaughan, Gotto & Basson, 2000). Statins are used to lower LDL in primary (i.e., those without known CHD) and secondary cardiovascular disease prevention. A meta-analysis of all major statin trials has revealed that for each 1.0 mmol.L^{-1} fall in LDL there is a 22% decrease in CVD risk.

In recent times, a meta-analysis of 37 randomized studies, comprising 32,258 dyslipidemic patients revealed that all statins significantly raise HDL-C levels. Some studies revealed data in favor of HDL-C and its role in atherosclerosis. For example, the ASTEROID study with rosuvastatin therapy had an increase of 14.7% in HDL-C, a decrease of 53.2% in LDL-C and showed regression in atherosclerosis. However, there was no evidence whether LDL-C or HDL-C had an independent effect (Nissen et al., 2006) . Contrariwise, the JUPITER study revealed no prognostic influence of HDL-C for the end points of first non-fatal myocardial infarction (MI) and stroke in patients already on a high-dose statin treatment (Ridker et al., 2010) . However, the observation made in JUPITER could lead to false conclusion since HDL-C levels was high at baseline (Barter, Brandrup-Wognsen, Palmer & Nicholls, 2010).

Niacin: Niacin (nicotinic acid) is the oldest agent used to increase HDL-C (Altschul, Hoffer & D., 1955). Niacin has multiple beneficial effects on serum lipids and lipoproteins (Kamanna & Kashyap, 2008). It reduces TGs and LDL-C and increases HDL-C and apoA-1 by its action on apo B and apoA-containing protein respectively (Catapano et al., 2011). Numerous clinical trials have assessed the benefit of niacin on atherosclerotic disease, in monotherapy or in combination with other drugs. In monotherapy, niacin decreased the occurrence of myocardial infraction (MI) at six years along with reducing mortality at 15 years significantly (Canner et al., 1986). In combination with colestipol, a bile-acid sequestrant, the drug gave a 37% increase in HDL-C levels, a 43% reduction in LDL-C and a 26% reduction in TC. A significant difference in atherosclerosis regression was revealed as it was higher in drug treated individuals compared to placebo treated individuals (Blankenhorn, Nessim, Johnson, Sanmarco, Azen & Cashin-Hemphill, 1987). The combination of niacin with statin therapy was evaluated in two other studies; HATS and ARBITER. In both studies there was an increase in HDL-C (26% and 21% respectively) as well as evidence of regression in coronary

stenosis; for the HATS study, CIMT in niacin treated individuals was not changed while it was decreased significantly in ARBITER study (Ali, Wonnerth, Huber & Wojta, 2012). The largest trial conducted to date of niacin therapy was the HPS2-THRIVE study. Here, addition of niacin to statin therapy did not show any extra benefit over statin therapy alone and indeed there were significant side-effects (Kent et al., 2016). Accordingly, use of niacin as a lipid-regulating drug has largely ceased.

Fibrates: Fibrates (fibric acid derivatives), such as bezafibrate, ciprofibrate, fenofibrate and gemfibrozil are peroxisome proliferator-activated receptor alpha (PPAR-α). Fibrate therapy has been proven to reduce plasma TGs levels by 30-50% along with modest increase of HDL-C levels by 5-15% (Goldenberg, Benderly & Goldbourt, 2008). The clinical benefits of fibrates in monotherapy are mainly illustrated by four prospective, randomized, placebo-controlled, clinical trials: Helsinki Heart Study (HHS), Veterans Affairs High-density lipoprotein Intervention Trial (VA-HIT), Bezafibrate Infarction Prevention study (BIP), and FIELD (Frick et al., 1987; Keech et al., 2005; Rubins et al., 1999; study, 2000). Although all fibrates have been revealed to rise HDL-C significantly, their effect on all-cause mortality and cardiac mortality remains debatable (Saha, Kizhakepunnur, Bahekar & Arora, 2007).

1.11.2 Future Lipid Lowering Therapeutic Options

In almost 30 years since the introduction of HMG-CoA reductase inhibitors (statins), no other class of lipid modulators have entered the market. Raising HDL-C through inhibiting CETP is a novel approach for potentially reducing the risk of cardiovascular events (Mohammadpour & Akhlaghi, 2013).

To date, a number of CETP inhibitors have been tested in clinical trials. These are torcetrapib, dalcetrapib, anacetrapib and evacetrapib. The first CETP inhibitor to be tested in human was torcetrapib. Torcetrapib is an irreversible and potent inhibitor of CETP and it was revealed to raise HDL-C value by 70-85% (Brousseau et al., 2004; McKenney, Davidson, Shear & Revkin, 2006). Despite hopeful results in phase I and II studies, in the large-scale phase III study, ILLUMINATE, torcetrapib treatment led to an increase in all-cause mortality and cardiovascular events (Barter et al., 2007). Dalcetrapib was the second CETP inhibitor to be assessed in phase III randomized clinical trials. Despite the increase of 30-35% in HDL-C in early phase II studies, the current data with this class of CETP inhibitors do not suggest any

benefit from HDL-C modulation (Wright, 2013). Anacetrapib is a strong inhibitor that increases HDL-C and lowers LDL-C. It is the third CETP inhibitor to go through evaluation in randomized clinical trials. In DEFINE trial, anacetrapib reduced LDL-C by nearly 40% and raised HDL-C by 138% ($P < 0.001$) but there was no confirmation of safety issues or changes in blood pressure (Cannon et al., 2010). Anacetrapib is currently being evaluated in a large, phase III outcomes trials, the REVEAL study, which includes about 30,000 patients with a history of CVD. The outcome data may be available by second quarter of 2017 (Wright, 2013). The fourth CETP inhibitor to be evaluated in Phase II and Phase III clinical trials is evacetrapib. Evacetrapib raises HDL-C by 79-89% ($P < 0.05$) and lowered LDL-C by 10-15% ($P < 0.05$). The ACCELERATE trial with this drug was stopped recently for reasons of futility. Thus, CETP inhibition so far has been a disappointment as a therapeutic target.

Currently, the newest drug class is PCSK9 inhibitors. These agents mainly lower LDL and are under assessment in large trials in subject with elevated risk, such as the FOURIER study (Rallidis & Lekakis, 2016).

1.12 Aims and Objectives of Current Work

As explained above, the role of HDL in CVD is complex. It has potentially many roles in cholesterol transport and the maintenance of healthy vascular wall. There have been a number of studies over recent years indicating that HDL can fail to function effectively in subjects at risk for coronary diseases (Esteve, Ricart & Fernández-Real, 2005; Khovidhunkit et al., 2004).

It is important therefore to attempt to understand if abnormalities in HDL structure, function and metabolism are associated with variation in CVD risk. This is especially true in light of the failure of trials that raise total HDL-C to reduce risk of MI and CVD.

The overall objective of the present work was to examine in detail the relationship of HDL oxidation, the ability of HDL to protect LDL oxidation and the abundance of the major HDL-associated antioxidant enzyme, PON1, to atherosclerosis in a cohort of subjects recruited across the social economic spectrum in the West of Scotland (the pSoBid study). PSoBid is valuable as a means of testing these properties of HDL because of its mix of males and females, wide age range, and the fact that it focused in recruiting people at extremes of social deprivation.

The population has been well characterized in terms of conventional risk factors and this thesis takes the investigation to a new level of details with respect to HDL.

The major questions that were addressed:

1. **If HDL oxidation, measured by three factors; time at half maximum (T1/2max), maximum velocity of oxidation (Vmax), or maximum amount of oxidized HDL measured by optical density is related to atherosclerotic marker?**
2. **If HDL antioxidant potency to protect LDL from oxidation is related to its protective effect in atherosclerosis risk?**
3. **If HDL PON1 activity is related to atherosclerotic marker?**
4. **If some HDL subclasses, rather than HDL-C, are particularly related to atherosclerosis?**

2 General Methods

2.1 Introduction

This chapter describes the equipment and general methods implemented in this thesis. All of the analyses were carried out in the laboratory facilities of the Institute of Cardiovascular and Medical Sciences at University of Glasgow by the researcher unless otherwise acknowledged.

2.2 PSoBid Study

A total of 616 lithium heparin (Li-Hep) blood samples from the pSoBid study (which had 666 participants), stored at -80 °C, were available for this project. The clinical study was done by Dr. Kevin Deans (Department of Vascular Biochemistry, Institute of Cardiovascular and Medical Sciences, University of Glasgow) (Velupillai et al., 2008). Based on Scottish Index of Multiple Deprivation (SIMD), participants were recruited at random from areas known to be at the extremes of the socioeconomic range in Glasgow (approximately half from the bottom 5% of the SIMD (most deprived) and half from the top 20% (least deprived) of the SIMD distribution). Within the categories of least deprived and most deprived, recruitment was stratified by sex and age to achieve an overall sample containing approximately equal numbers of males and females and an even distribution across the age categories 35-44, 45-54 and 55-64 years. Due to the nature of the psychological questionnaires and cognitive assessmement, only those who understood and spoke English were invited to participate in the pSoBid study. Participants were invited to come for the first visit at their General Practice's clinic. The first visit involved completion of lifestyle and psychology questionnaires as shown in **appendix 1** , assessment of health status and measurement of blood pressure, pulse rate and indexes of obesity (height, weight, hip, waist and mid-thigh circumferences). At the second visit, at Glasgow Royal Infirmary, a fasting blood sample was taken to measure total plasma cholesterol, TGs, the cholesterol in very low density lipoprotein (VLDL-C), LDL-C and HDL-C and a range of other biomarkers for diabetes, inflammation and clotting. In addition, participants underwent ultrasound assessment of carotid intima media thickness (cIMT). Furthermore, participants completed lifestyle questionnaire which had 13 sections including basic demographic data, past and present health status, current medications, oral health, smoking history, alcohol intake, diet, physical activity level, childhood situation, birth

weight and place of birth, their parent's age and father's occupation, education levels, employment history and income levels.

2.3 PSoBid Samples

There was almost a full set of Li-Hep plasma samples available from pSoBid. The samples had been immediately stored in -80°C freezers and kept for about 5 years. These samples had not been previously thawed and were considered the best material available for the work of the present thesis.

2.4 Plasma Pool for LDL Controls used in Oxidation Assay

About 5 X 9 mL K_3EDTA fasting blood was collected from 8 donors. All plasma donors were non-smokers with no personal history of heart disease, high cholesterol, high TGs, high blood pressure, diabetes or other diseases and were not taking any medications. Bloods were spun at 2060g for 15 minutes at 4°C in a Beckman centrifuge (GS-6KR Beckman Instruments, Inc., California, US) and plasma was separated and immediately pooled in a bottle, mixed by gentle inversion and then used for LDL separation (**Section 2.6.1**). All density solution contained EDTA (1 $g.L^{-1}$).

2.5 Plasma Pool for Plasma QC used in PON1 Assay and for HDL Separation for HDL QC used in PON1 and Oxidation Assays

About 5 X 6 mL Li-Hep fasting blood samples were collected from 9 donors. All plasma donors were non-smokers with no personal history of heart disease, high cholesterol, high TGs, high blood pressure, diabetes or other diseases and were not taking any medications. Bloods were spun at 2060g for 15 minutes at 4°C in Beckman centrifuge (GS-6KR Beckman Instruments, Inc., California, US) and plasma was separated. 250 µL from each sample was taken for cholesterol (Chol), TGs, HDL-C and LDL-C and immediately pooled in a bottle, gently mixed by inversion and then divided into two parts; One used for the plasma quality control, 0.5 mL aliquots, nitrogen purged and stored directly at -80°C. The other part was used for HDL QC separation (**Section 2.6.2**). All density solutions used for lipoprotein preparation were EDTA free and were checked with densitometers.

2.6 Lipoprotein Preparation for Oxidation and PON1 Assays

The high content of lipid in lipoprotein particles, especially TG, and CE, gives them the property of having a density substantially less than that of most of the other constituents in plasma (Caslake & Packard, 1997). Therefore, they can be prepared virtually pure by ultracentrifugation where lipoproteins float by spinning the serum in solutions of increasing solvent density. This process is known as sequential ultracentrifugation in which lipoproteins are separated using a modification of the method described by Havel (Havel, Eder & Bragdon, 1955). Different starting volumes of the plasma were used according to the lipoprotein fraction needed.

2.6.1 LDL Preparation for LDL Quality Control used in Oxidation Assay

VLDL/IDL (d<1.019 g.mL^{-1})

The density of 20 mL EDTA plasma was adjusted to 1.019 g.mL^{-1} by the addition of 1.6 mL of 1.182 g.mL^{-1} density solution in polycarbonate centrifuge bottles (Beckman 355654, Beckman Coulter, Inc. USA) overlayered with 3.4 mL of 1.019 g.mL^{-1} density solution using a peristaltic pump and centrifuged at 4°C for 24 hours at 1400 g in a Beckman OPTIMAxL-100K ultracentrifuge in a 50.4 rotor (Beckman instruments Inc., UK). The top 10 mL was carefully removed from each tube by using a drawn out glass pipette and discarded.

LDL (1.019-1.063 g.mL^{-1})

The density of 15 mL of the infranatant, from the previous step, was adjusted to 1.063 g.mL^{-1} by the addition of 5.5 mL of 1.182 g.mL^{-1} density solution, overlayered with 4.5 mL of 1.063 g.mL^{-1} density solution and centrifuged at 4°C for 24 hours at 1400 g in a Beckman OPTIMAxL-100 K ultracentrifuge in 50.2 rotors (Beckman 337901, Beckman coulter, Inc. USA). The top 10 mL containing LDL was carefully harvested, pooled, gently mixed and divided into 1.2 mL aliquots, nitrogen purged and stored at -80°C until needed for oxidation (**Section 2.13**).

LDL Dialysis

In order to prepare LDL for oxidation, EDTA must be removed and this was done by dialysis. 1mL of LDL, prepared from the previous step was defrosted at room temperature for about 30 minutes and added to about 10 cm of MWCO: 12-14 kD dialysis membrane (Spectra/Por®2 132676, Spectrum Laboratories, Inc. CA, USA). Membrane containing the sample was put in a beaker with approximately 666 mL of freshly prepared cold phosphate buffer saline (PBS) (Sigma Aldrich) and stirred for 24 hours, changing the buffer twice, at 4°C. Dialysed LDL was then taken and stored at 4°C and was immediately used for oxidation.

2.6.2 HDL Separation for HDL Quality Controls used in PON1 and Oxidation Assays

VLDL/LDL (d< 1.063 $g.mL^{-1}$) Preparation

The density of 15 mL Li-Hep plasma was adjusted to 1.063 $g.mL^{-1}$ by the addition of 7.5 mL of 1.182 $g.mL^{-1}$ density solution in polycarbonate centrifuge bottles (Beckman 355654, Beckman coulter, Inc. USA), overlayered with 2.5 mL of 1.063 $g.mL^{-1}$ density solution and centrifuged at 15°C for 18 hours at 1400 g in a Beckman OPTIMAxL-100 K ultracentrifuge in 50.2 rotors (Beckman 337901, Beckman coulter, Inc. USA). The top 10 mL of the supernatant was removed carefully and discarded.

HDL (d 1.063-1.21 $g.mL^{-1}$)

The density of 15 mL of the infranatant from the previous step was adjusted to 1.21 $g.mL^{-1}$ by the addition of 7.5 mL of 1.478 $g.mL^{-1}$ density solution, overlayered with 2.5 mL of 1.21 21 $g.mL^{-1}$ density solution and centrifuged at 4°C for 60 hours at 15000 g in a Beckman OPTIMAxL-100K ultracentrifuge in a 50.2 rotors (Beckman 337901, Beckman coulter, Inc. USA). The top 7.5 mL containing HDL was carefully removed, pooled, gently mixed, aliquoted into 0.5mL amounts, nitrogen purged and stored immediately at -80°C until needed.

2.6.3 HDL Separation for PON1 and Oxidation Assays (The Rapid Method)

VLDL/LDL (d< 1.063 g.mL-1) Preparation

To remove VLDL to LDL in one spin, the plasma had to be adjusted to density 1.063 $g.mL^{-1}$. Plasma (Li-Hep) samples were thawed at room temperature for approximately 30 minutes. 400µL of the plasma were then transferred to 11 X 34 mm thick wall polycarbonate centrifuge tubes (Beckman 343778 Beckman coulter, Inc. USA). Adjusted to 500 µL with 1.006 $g.mL^{-1}$ density solution then mixed again with 250 µL of 1.182 $g.mL^{-1}$ density solution and finally overlayered with 250 µL of 1.063 $g.mL^{-1}$ density solution. Tubes were then transferred to TLA100.2 rotor (Beckman coulter, Inc. USA), and centrifuged in a TLA100 Table-Top Ultracentrifuge (Beckman coulter, Inc. USA) at 27400 g and 23°C for 2.5 hours. The supernatant (500 µL) was carefully isolated and discarded; the infranatant of the samples was used to isolate HDL.

HDL (d 1.063-1.21 $g.mL^{-1}$) Preparation

Two hundred fifty µL of 1.478 $g.mL^{-1}$ density solution was mixed with the infranatant, from the previous step, and then another 250 µL of 1.21 $g.mL^{-1}$ density solution was carefully overlayered. The samples were then centrifuged for 18 hours at 13800 g and 15°C in a TLA100.2 rotor in a TLA100 Table-Top Ultracentrifuge (Beckman coulter, Inc. USA) .The supernatant containing HDL (400 µl) was isolated carefully and adjusted to 500 µL by 100 µL PBS (Sigma Aldrich) containing 1mM $CaCl_2$ (Sigma Aldrich).

HDL Desalting for Oxidation and PON1 Assays

In order to remove the salts used in HDL isolation, columns containing Sephadex G-25 were used for this step in which PD Minitrap G-25 (Healthcare Bio-Sciences, 751 84 Uppsala Sweden) was put into a 15 mL polystyrene clear tube (Evergreen Scientific 214-2415-01K, California, USA) by using the column adaptor and equilibrated with 3x volume (approximately 8 mL) of PBS (Sigma Aldrich) contacting 1 mM $CaCl_2$. The columns were then put, along with the polystyrene tubes, in 50.4 rotors (Beckman 337901, Beckman coulter, Inc. USA), spun down at 112 g at 15°C for 5 minutes in a Beckman OPTIMAxL-100K

ultracentrifuge. 400 µL of HDL + 100 µL of PBS (Sigma Aldrich) containing 1mM $CaCl_2$ were then added slowly in the middle of the packed bed. PD Minitrap G-25 was then placed into new 15 mL polystyrene clear tubes. Samples were then eluted by centrifugation at 112 g at 15°C for 5 minutes in the same rotor. The eluate of HDL was then adjusted to 500 µL with PBS (Sigma Aldrich) +1 mM $CaCl_2$.

2.6.4 HDL Preparation for One Dimensional Gel Electrophoresis (1DGE)

VLDL/LDL (d< 1.063 g.mL-1) Preparation

In order to isolate HDL fractions, VLDL/LDL was firstly prepared. Plasma (Li-Hep) samples were thawed at room temperature for approximately 30 minutes. 160µL of the plasma were then transferred to 7x20 mm cellulose propionate centrifuge tube (Beckman 342303; Beckman coulter, Inc. USA), mixed with 80 µL of 1.182 $g.mL^{-1}$ density solution to adjust density to 1.063 $g.mL^{-1}$. Tubes were then transferred to Ti 42.2 rotor (Beckman 337901, Beckman coulter, Inc. USA) and centrifuged in a Beckman OPTIMAxL-100K ultracentrifuge at 20500 g and 10°C for 18 hours. The supernatant of (80 µL) was carefully removed and discarded. The infranatant of the samples was used to isolate HDL.

HDL (d 1.063-1.21 g.mL-1) preparation

Eighty µL of 1.478 $g.mL^{-1}$ density solution was mixed with the infranatant, from the previous step, to adjust density to 1.21 $g.mL^{-1}$. The samples were centrifuged at 20500 g and 10°C in a Ti 42.2 rotor in a Beckman OptimaXL-100K ultracentrifuge. The supernatant containing HDL was stored at 4°C, for less than 24 hours, until used for gradient gel electrophoresis analysis (**Section 2.11**).

2.7 Assays on Clinical Chemistry Analyser

All analyses, HDL-cholesterol, cholesterol, total TGs, apolipoprotein A-I, apolipoprotein B and albumin, described in this section were carried out using commercially available enzymatic colorimetric kits, calibrators, and controls (as outlined in the relevant sections below). The degree of turbidity or absorbance was measured optically using a clinically validated autoanalyser (ILab™ 600, Clinical Chemistry System, Instrumentation Laboratory, USA).

2.7.1 HDL-Cholesterol (HDL-C) Measurement

The cholesterol concentration of HDL is determined enzymatically by a kit based on cholesteryl ester esterase (HDL-C plus 3rd generation, Roche Diagnostics, Burgess Hill, UK) and cholesterol oxidase coupled to polyethylene glycol (PEG) to the amino groups (approximately 40%).

$$\text{HDL-cholesteryl esters} + H_2O \xrightarrow{\text{PEG-cholesteryl ester esterase}} \text{HDL-cholesterol} + \text{RCOOH}$$

Cholesteryl esters are broken down quantitatively into free cholesterol and fatty acids by cholesteryl ester esterase.

$$\text{HDL-cholesterol} + O_2 \xrightarrow{\text{PEG-cholesterol oxidase}} \Delta^4\text{-cholestenone} + H_2O_2$$

In the presence of oxygen, cholesterol is oxidized by cholesterol oxidase to Δ^4-cholesenone and hydrogen peroxide.

$$2H_2O_2 + \text{4-Amino-antipyrine} + \text{HSDA} + H^+ + H_2O \xrightarrow{\text{Peroxidase}} \text{purple-blue pigment} + 5\ H_2O$$

HSDA=Sodium N-(2-hydroxy-3-sulfopropyl)-3, 5-dimethoxyaniline

In the presence of peroxidase, the hydrogen peroxide generated reacts with 4-amino-antipyrine and HSDA to form a purple-blue dye. The colour intensity of this dye is directly proportional to the cholesterol concentration and is measured photometrically. Coefficients of variance (CV) for higher and for lower level quality controls was 6.28% and 6.52% respectively.

2.7.2 Cholesterol Measurement

Cholesterol was determined after enzymatic hydrolysis and oxidation with enzymatic colorimetric kits (Randox CH 200, Randox Laboratories Limited, Crumlin, County Antrim, UK). The indicator quinoneimine is formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase. CV for higher and lower level quality control was 2.88% and 3.09% respectively.

Cholesteryl ester esterase

$$\text{Cholesteryl ester} + H_2O \longrightarrow \text{Cholesterol} + \text{Fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Cholesterol-3-one} + H_2O_2$$

$$2H_2O_2 + \text{Phenol} + \text{4-Aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + 4H_2O$$

2.7.3 Total Triglycerides (TGs) Measurement

Triglycerides were determined after enzymatic hydrolysis with lipases (Randox TR 210, Randox Laboratories Limited, Crumlin, County Antrim, UK). The indicator, quinoneimine formed from hydrogen-peroxide, 4-aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase. CV for higher and lower quality control was 4.30% and 5.40% respectively.

$$\text{Triglycerides} + H_2O \xrightarrow{\text{Lipases}} \text{Glycerol} + \text{Fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{Glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + \text{O2} \xrightarrow{\text{GPO}} \text{Dihydroxyacetone phosphate} + H_2O_2$$

$$2H_2O + \text{4-Aminophenazone} + \text{4-Cholrophenol} \xrightarrow{\text{POD}} \text{Quinoneimine} + \text{HCL} + 4H_2O$$

2.7.4 Apolipoprotein A-I (ApoA-I) Measurement

The kit (Randox LP 3838, Randox Laboratories Limited, Crumlin, County Antrim, UK) is based on the reaction of a sample containing human apoA-1 and specific antiserum to form an insoluble complex which can be measured turbidimetrically at 340 nm. By constructing a standard curve from the absorbances of standards the concentration of apo A-1 can be determined. CV for higher and lower level quality controls were 4.62% and 4.42% respectively.

2.7.5 ApoB Measurement

The kit (Randox LP 3839, Randox Laboratories Limited, Crumlin, County Antrim, UK) is based on samples containing human apo B and a specific antiserum form an insoluble complex which can be measured turbidmetrically at 340 nm. By constructing a standard curve from the absorbances of standards concentration of apo B can be determined. CV for higher and lower levels quality control was 2.74% and 4.98% respectively.

2.7.6 Albumin Measurement

The measurement of albumin is based on its quantitative binding to the indicator 3,3',5,5'-tetrabromo-m cresol sulphonephthalein (bromocresol green, BCG). The albumin-BCG-complex absorbs maximally at 578 nm, the absorbance being directly proportional to the concentration of albumin in the sample. CV for the quality control was 4.25%.

2.8 Total LDL- Protein Measurement by Lowry Method

Total protein content in LDL fractions was measured using a modified Lowry method (Lowry, Rosebrough, Farr & Randall, 1951) which involved the addition of 2 mL of Biuret reagent (100 mL of a solution containing 2% Na_2CO_3 in 0.1 M NaOH)+ (1 mL of solution containing 2% NaK Tartrate) + (1 mL of solution containing 1% $CuSO_4$) to 400 mL containing samples, standards and quality controls of known concentrations. After 10 minutes, 200 µL of freshly diluted Folin and Ciocalteu's phenol reagent (Sigma Aldrich F9252, Sigma-Aldrich Company Ltd. Gillingham, Dorset, UK) (1:1 with deionized water) was then added and mixed immediately. After 30 minutes, the optical density was read at 750nm within 2 hours using Beckman DU 640 Spectrophotometer (Beckman Coulter Ltd, CA, USA). Protein concentration was calculated from the standard curve of known concentrations. Two levels of human and bovine quality control (150 $\mu g.mL^{-1}$ and 300 $\mu g.mL^{-1}$) were used to check the inter-assay precision. The CV for low quality control was (human: 7.5%, bovine: 4.8%) and 2.6% for bovine high quality control**.**

2.9 Total Protein Measurement by Bradford Method

Protein measurement was performed by Bradford (Bradford, 1976) method which involves the binding of Coomassie Brilliant Blue G-250 dye to proteins. 10 µl of the sample, standard or quality control was pipetted into a 96 flat bottom well microplate (Fisher 10288521,

Fisher Scientific Ireland Ltd, Ballycoolin, Dublin 15, Ireland). 250 µL of the dye reagent Quick start Bradford™ 1X Dye Reagent (Bio-Rad 500-0205, California 94547, USA) was then added and mixed for 30 seconds on plate shaker (Labline Instrument) set at 600 rpm. After 10 minutes, the optical density at 595 nm was then read at room temperature. Two levels of human and bovine quality control (150 µg.mL^{-1}and 300 µg.mL^{-1}) were used to check the inter-assay precision. The CV for low quality controls was (human: 11.97%, bovine: 11.44%) and (human: 7.89%, bovine: 10.50%) for the high quality control.

2.10 Preβeta-1 HDL ELISA

Preβeta-1 HDL was measured in 540 Li-Hep samples. Stabilizer was added immediately after the samples were thawed. 1:20 diluted plasma with ELISA stabilizer was done using commercially available ELISA kits (Pre-β1-HDL ELISA 289194, American Diagnosta GmbH, Pfungstadt, Germany). The method is a quantitative enzyme-linked immunosorbent assay. The Inter-assay CV was 14.2% and intra-assay CV was 9.2%.

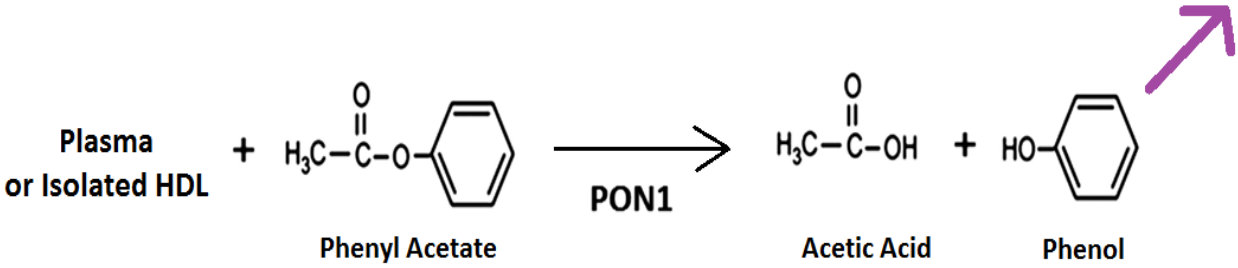
2.11 One Dimensional Gel Electrophoresis

Percentage distribution of HDL subfractions was determined by gel electrophoresis as described by Nichols (Nichols A.V. et al 1986). The method was carried out on 4-30% polyacrylamide gels (C.B.S. Scientific Company. Inc., California, USA) in Tris buffer (90 mM Tris Base (Sigma Life Science T1503, MO, USA), 92.8 mM Orthoboric acid (VWR International 100583R, Leuven, Belgium) and 2.5 mM Na_2EDTA (VWR International 100935V, Leuven, Belgium) in distilled H_2O, adjusted to PH 8.3) and prechilled at 5°C. Gel tank (Bio-Rad Mini Gel Apparatus, Hertfordshire, UK) was partly filled with Tris buffer. Gels were removed from their packaging, dried and well spacer was carefully placed. The gels were then pre-equilibrated for 20 minutes at 70 V (Bio-Rad power pack). Prior to gel loading, 40µL of each experimental sample (obtained from **Section 2.6.5**) was mixed with 20 µL Bromophenol Blue dye solution (0.149 mM Bromophenol Blue (VWR international 443053A, Leuven, Belgium), and 1.168 M sucrose (VWR international 102745C, Leuven, Belgium) in Tris Buffer). HDL standards, high molecular weight (HMW) (thyroglobulin 669KD, ferritin 440 KD, catalase 232 KD, lactase dehydrogenase 140 KD and bovine serum albumin 67KD) (Amersham™, GE Healthcare UK Limited Little Chalfont, Buckinghamshire UK) were mixed. After equilibration, 15µL of dyed sample and 8µL of HMW standard was loaded onto the gels. Electrophoresis was performed at 20 volts for 20 minutes, 70 volts for 30 minutes and 120 volts for 24 hours.

Gels were then fixed with 10% sulphosalicylic acid for 30 minutes, stained with coomassie blue (1.168 mM Coomassie Blue (Bio-Rad R250, Bio-Rad Laboratories, Inc. UK) in 4:1:5 methanol: acetic acid: H_2O). After one hour, they were destained with acetic acid: methanol: water (1.5:1:2) until the bands were visible. Gels were scanned using G750 scanning densitometer (Bio-Rad) and analysed by software Bio-Rad's Image Analysis System (Bio-Rad) to give relative concentrations of HDL2b, HDL2a, HDL3a, HDL3b and HDL3c.

2.12 Paraxonase-1 (PON1) Assay

PON1 activity was measured in an arylesterase assay using phenyl acetate as a substrate in a method adapted from Richter R.J. 2008 (Richter, Jarvik & Furlong, 2008) in which measurement of arylesterase activity relies on the ability of PON1 arylesterase to hydrolyse phenyl1 acetate into acetic acid and phenol. The production of phenol is measured at a wavelength of 270nm (wavelength of phenol).



The initial rate of hydrolysis (first 2 minutes) was recorded at 270 nm on Spectra-Max 190 plate reader (Molecular Devices. CA, USA). Briefly, plasma QC (**Section 2.5**), plasma samples, HDL QC (**Section 2.6.2**) and HDL samples (**Section 2.6.3**) were diluted (1:20) with PON1 assay buffer containing 20 mM Tris base, 0.1 mM $CaCl_2$. H_2O at PH 8.0 20 µL of diluted samples were then mixed and pipetted into each well and the reaction is initiated by adding 200 µL of freshly prepared 1 $g.mL^{-1}$ phenyl acetate (25 mL buffer + 11 µL Phenyl acetate).

2.13 Antioxidant Potency of HDL

LDL (**Section 2.6.1**) was standardized to 50 $\mu g.mL^{-1}$ of protein (with PBS), desalted HDLs from the pSoBid samples (**Section 2.6.3**) and HDL QC (**Section 2.6.2**) were standardized to 100 $\mu g.mL^{-1}$ of protein with PBS, and a stock of 5 µM of $CuCl_2$ (Sigma-Aldrich Company Ltd. Gillingham, Dorset, UK) was freshly prepared for each run. Wells were pipetted with PBS

(Sigma Aldrich), depending on the quantity needed for each well, then HDLs (80 µL), followed by LDL (100 µL), and finally $CuCl_2$ solution (20 µL) was added. Oxidation was monitored at 234 nm in a Spectra max 190 plate reader (Molecular Devices, CA, USA) and UV Microplate corning (Fisher 3635 10288521) at 37˚C, Automix 60 seconds, for 13 hours and 2 minutes interval. Note that $CuCl_2$ solution was added within 1 hour of taking LDL- from dialysis to initiate oxidation.

2.14 Statistics

Statistical analyses were carried out using IBM SPSS Statistics Data Editor (version 22) software. As a first step, all parameters were tested for normality using the Shapiro-Wilk test. Variables with normal distributions were described as mean (± standard deviation), while median (with inter-quartile range) was used to describe non-normally distributed variables, and number (%) to describe categorical variables. Data were log-transformed when they did not approximate normality. Independent- two tailed T-test was used to compare means of different groups , like genders and areas, using data on a normal scale, or log transformed, or square root transformed depending on the test for normality. One-Way ANOVA was used to compare variable means between age groups. Associations between variables were determined using Pearson correlation. Significance was accepted at $P < 0.05$ level.

3 Method Development to Assess the Antioxidant Function of HDL and Measure HDL-PON1 Enzyme Activity

3.1 Introduction

3.1.1 Assessing the Oxidation Status of LDL

Considerable attention over the last decades has been focused on the oxidative modification of LDL and its potential role in the formation of atherosclerotic lesions (Steinberg, 1997). A variety of indirect assays have been developed in the past few years to assess LDL oxidation in vitro. One simple and widely used method is to measure lipid peroxides formed during LDL oxidation by spectrophotometric assessment of the conversion of iodide to iodine (el-Saadani, Esterbauer, el-Sayed, Goher, Nassar & Jürgens, 1989). Although it is considered easy to carry out, that assay lacks sensitivity as it measures other peroxides as well. Improving the iodometric assay by determining lipid peroxides after hydrolysing esterified lipids and extracting them with acetate (el-Saadani, Esterbauer, el-Sayed, Goher, Nassar & Jürgens, 1989; Gorog, Kotak & Kovacs, 1991) or by replacing iodide with ferrous ion in the presence of xylenol orange (Jiang, Hunt & Wolff, 1992) still limits specificity in measuring lipid peroxides. The most accurate way for measuring lipid peroxides in plasma is by HPLC which detects isoluminol chemiluminescence (Frei, Yamamoto, Niclas & Ames, 1988) but this method is prolonged and not suitable for clinical laboratory settings. Another commonly used assay for LDL oxidation is the thiobarbituric acid - reactive substances (TBAR) assay which measures the change in TBARS spectrophotometrically at 532 nm (Janero, 1990). The main disadvantage for the TBAR assay is that it is non-specific and reaction conditions have a significant effect on colour development (Antolovich, Prenzler, Patsalides, McDonald & Robards, 2002). In addition, other compounds such as sugars, amino acids, aldehydes, bilirubin, DNA, prostaglandins and thromboxanes may also interfere in the assay (Jialal & Devaraj, 1996). Measuring the electrophoretic mobility of LDL was also applied to quantify LDL oxidation in vitro but this assay is overwhelmed by other aldehydic modification which will also alter electrophoretic mobility of LDL. Other assays such as using apoB fluorescence are also a potential route to evaluate the modifications arising during lipoprotein oxidation

(Cominacini et al., 1991). Monitoring the disappearance of the three main fatty acids in polyunsaturated fatty acids (PUFA) found in LDL by gas chromatography (GC), after extraction and methylation, could also be a good indication of the oxidative susceptibility of LDL particles (Lepage & Roy, 1988) but it is time consuming and not all laboratories are equipped with GC. Other techniques for measuring LDL oxidation are by measuring aldehydic lipid peroxidation products where aldehydes , such as malondialdehyde and hydroxynonenal, developed during lipid peroxidation from polyunsaturated fatty acids modify lysine residues of apoB and these can be extracted with thin layer chromatography (TLC) then scanned by HPLC with ODS columns (Esterbauer, Jurgens, Quehenberger & Koller, 1987). Although HPLC is considered as a specific and sensitive technique for LDL oxidation, using HPLC after TLC is hard to adapt to the routine laboratory or large numbers of samples. Oxysterols, which are a product of cholesterol oxidation, can be measured by GC (Jialal, Freeman & Grundy, 1991), and used as a marker of LDL oxidation but this method is not widely used. The characterization of specific hydroperoxy and hydroxyl fatty acids was also measured by gas chromatography- mass spectrophotometry (GC-MS) but is also too hard to undertake for large scale work. Measuring F2-Isoprostanes was also used as an indication or LDL oxidation by solid phase extraction followed by GC-MS (Gopaul, Nourooz-Zadeh, Mallet & Anggård, 1994) but it should be noted that this parameter reflects whole body oxidation rather than just that occurring in LDL.

A more objective and easy method for LDL oxidation was developed originally by Esterbauer et al (Esterbauer, Striegl, Puhl & Rotheneder, 1989) by continuously measuring the in vitro oxidation of LDL. The method is based on monitoring the change of the conjugated dienes absorbance at 234 nm where the typical time course starts with a lag phase, in which diene absorption shows only a slight increase followed by a propagation phase in which absorption rapidly increases and then decreases (**Figure 3.1**). Consequently, the typical oxidation curve is demonstrated by S-shaped graph and the lag phase is lengthened by preventive or chain breaking antioxidants, which scavenge the initiation reaction. Hence the lag phase reflects the antioxidant status of lipoproteins and their resistance to oxidation (Cadenas & Sies, 1998).

The simplicity and reproducibility of the copper induced oxidation assay make it attractive for both routine and research assays(Ahmed, Ozbak & Hemeg, 2015), (Jin et al., 2014),

(Guermouche, Soulimane-Mokhtari, Bouanane, Merzouk, Merzouk & Narce, 2014),(Lee, Kim & Min, 2013) (Aoki, Abe, Yamada, Matsuto & Okada, 2012).

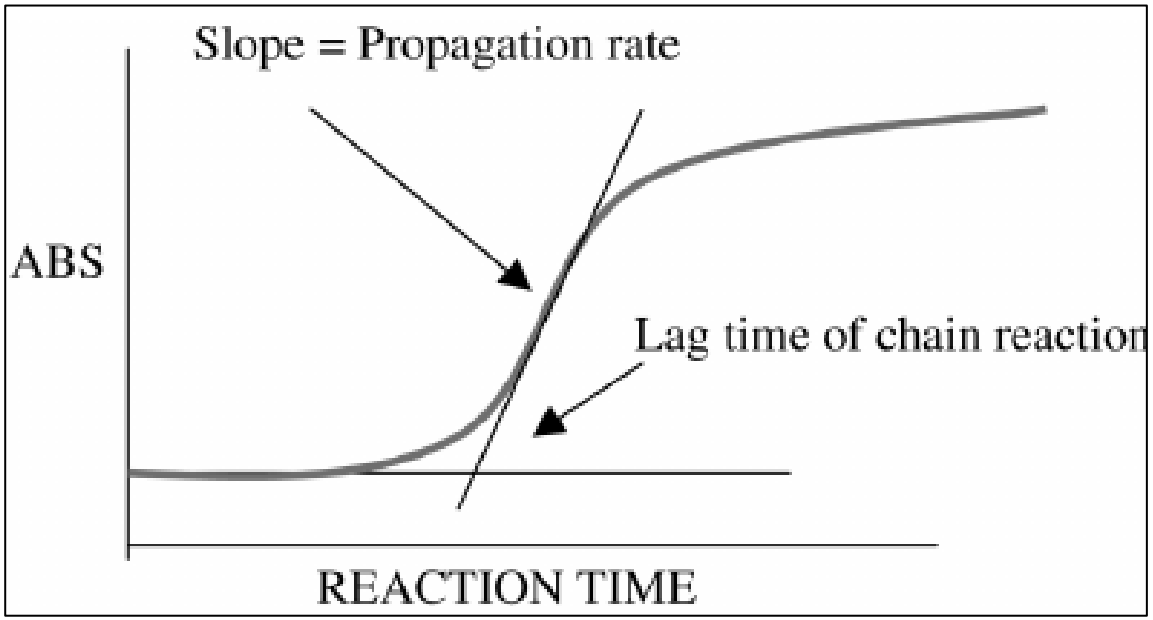


Figure 3.1 Determination of lag time and propagation rate in LDL oxidation. [Source: (Kgomotso, Chiu & Ng, 2008)].

3.1.2 Assessing the Antioxidant Potency of HDL

Despite the fact that HDL can themselves undergo oxidative changes, it has been shown that HDL provides an antioxidant effect that reduces the extent of LDL oxidation (Kontush et al., 2004). Integrated cell-free assays of HDL antioxidant capacity have been developed in a number of laboratories. The use of reverse oxidation of LDL by HDL was developed by Navab and co-workers (Navab, Hama, Hough, Subbanagounder, Reddy & Fogelman, 2001). In this assay, 2, 7, 7- dichlorofluorescein diacetate and oxidized PLs preparation produces dichlorofluorescein, which is an indication of lipid oxidation, is measured before and after incubation with HDL. But this assay still needs calibration and validation. Other assays assess HDL antioxidant effect by the gradient of lipid hydroperoxides concentration after 3h of copper-catalysed oxidation of LDL in the absence and the presence of HDL or HDL subclass (Boemi, Leviev, Sirolla, Pieri, Marra & James, 2001; Mackness, Arrol, Abbott & Durrington, 1993; Sampaio et al., 2013) but this method lacks specificity as it measures other peroxides as well. Measuring the absorbance increase at 234 nm due to conjugated diene formation in LDL (control) and study of the effect of adding HDL has been used by many researchers. Initiation of oxidation is usually with copper (de Juan-Franco et al., 2009; McEneny et al., 2013; Morena, Cristol, Dantoine, Carbonneau, Descomps & Canaud, 2000) or with azo-initiator 2, 2'-azobis-(2-amidinopropane) (AAPH) (Hansel et al., 2004; Pankhurst et al., 2003) . This method has the advantage in that lipoprotein fraction(s), whether it is LDL, HDL or HDL+LDL dissolve completely in buffer and there is no need to extract the lipoprotein fraction before the assay. The main disadvantage is that after the decrease phase of 234 nm absorption will increase again in the decomposition phase because aldehydess formed also absorb in the 210-240-nm region.

3.1.3 Paraxonase as an HDL- Associated Antioxidant Enzyme

PON1 enzyme circulates in blood associated with the surface of HDL (Kotani K., Yamada T. et al 2013 and (Macharia, Hassan, Blackhurst, Erasmus & Matsha, 2012). Several studies have demonstrated its protective role against vascular disease (Draganov & La Du, 2004; Movva & Rader, 2009). Two techniques are primarily used for measuring PON1; direct quantifying the enzyme by immunological methods using specific antibodies; or measuring its activity by spectrophotometric assays. Using antibodies is time consuming and costly compared to spectrophotometric assays (Costa, Cole, Vitalone & Furlong, 2005). In addition, HDL is

required to be isolated by fixed angle rotors which limits throughput to a maximum of 10 samples per day and hence is not suitable for 96-well ELISA kits and storing HDL samples after isolation might affect the PON1 enzyme stability. Many assays involve spectrophotometric methods to measure the rates of hydrolysis of specific PON1 substrates such as paraxon, phenyl acetate, 4- nitrophenyl acetate, 5-thiobutil butyrolactone (TBBL) and dihydrocoumarin (Ceron, Tecles & Tvarijonaviciute, 2014).

3.1.4 Aim

The aim of the work described in this chapter was to set up a suitable protocol for isolating HDL from pSoBid samples (**Chapter 2, Section 2.2** and **2.3**), measuring the inherent antioxidant potency of each HDL to protect a standard LDL preparation from oxidation, and to measure PON1 enzyme activity of the same isolated HDLs with a workload of 10 samples per day.

3.2 Samples for Lipoprotein Isolation

As our project aimed to test the antioxidant functional quality of pSoBids' HDLs and as there was almost a full set of Li-Hep plasma samples, stored at -80°C, left from the study, we decided to use these samples to isolate HDL for antioxidant and PON1 assays. On the other hand, in the oxidation assay, it was important to measure each HDL's antioxidant potency against the same LDL preparation. Since EDTA protects from oxidation and because LDL takes about 3 days to be isolated, it was important to use EDTA plasma for as the source of LDL and to remove EDTA just before the oxidation assay. So, for method development, depending on the lipoprotein needed, plasma was obtained from healthy donors in either K_3EDTA (Fisher Scientific UK Ltd, Loughborough, UK) or Li-Hep (Fisher Scientific UK Ltd, Loughborough, UK). Plasma samples, K_3EDTA or Li-Hep, were isolated immediately after spinning at 2060 g and 4°C for 10 minutes.

3.3 Lipoprotein Preparation- LDL

Lipoproteins were separated using a modification of the method described by Havel (Havel, Eder & Bragdon, 1955) Different starting volumes of the plasma were used according to the lipoprotein fraction needed. LDL was isolated as described in **Chapter 2** (**Section 2.6.1**).

3.3.1 LDL Protein Measurement

It was important to add a consistent amount of LDL particles to each oxidation assay and it was decided to base this on the protein concentration in LDL. To measure LDL-protein content, we initially followed the Bradford method since the Lowry method was excluded in our pilot studies. The Bradford method had been used successfully for LDL by McEneny (McEneny et al., 2013), and in addition, it is very quick, takes less than 15 minutes, and needs only 10 µL of LDL fraction. (On the other hand, the Lowry method takes about 50 minutes which was not suitable as the oxidation run should be started within 1 hour after taking LDL from dialysis). However, our first pilot measurement of LDL protein using the Bradford assay were not reproducible. In addition, it is known that predicted value from LDL cholesterol measurements protein/cholesterol ratio is about 0.7 to 0.8 (Vega, F. & Grundy, 1985) and this was not our finding in the results from the Bradford assay.

As an alternative, we considered that the best approach to assess the LDL particle concentration was to measure LDL cholesterol which could be done rapidly (10 minutes) and accurately using a commercial kit and convert it to protein equivalent concentration using a fixed ratio.

To estimate the best ratio to use we isolated LDL and measured its cholesterol content and protein in different ways. 45 mL of EDTA blood was taken from 5 healthy donors. Samples were centrifuged for 10 minutes at 4°C and 2060g. Plasma was isolated, pooled and mixed, gently by inversion, then LDL was isolated from the plasma pool by ultracentrifugation as described in **Chapter 2** (**Section 2.6.1**). LDL fractions were pooled and mixed gently by inversion, and protein was measured by Lowry method as described in **Chapter 2** (**Section 2.8**), Apo-B was measured immunoturbidimetrically as described in **Chapter 2** (**Section 2.7.5**) and LDL-Cholesterol was measured using kits as described in **Chapter 2** (**Section 2.7.2**). Results for cholesterol ($mmol.L^{-1}$) were multiplied by 38.6 to convert to $mg.dl^{-1}$, then converted to $\mu g.mL^{-1}$ by multiplying by 10 and finally converted to protein concentration ($\mu g.mL^{-1}$) by multiplying with 0.8 as shown in the following equation:

Cholesterol ($mmol.L^{-1}$) X 10 X 38.6 X 0.8= Protein ($\mu g.mL^{-1}$)

The factor of 0.8 was used in calculations of LDL protein concentration since it gave the closest results to the apoB concentrations.

The average LDL-protein for the plasma pool measured by 3 methods was 1358 µg.ml^{-1} (±210), %CV 15.47 from Lowry, 1083µg.mL^{-1} (±188), %CV 17.3 from Apo-B and 1186 µg.mL^{-1} (±180), %CV 15.2 from cholesterol (n= 14 for all methods).

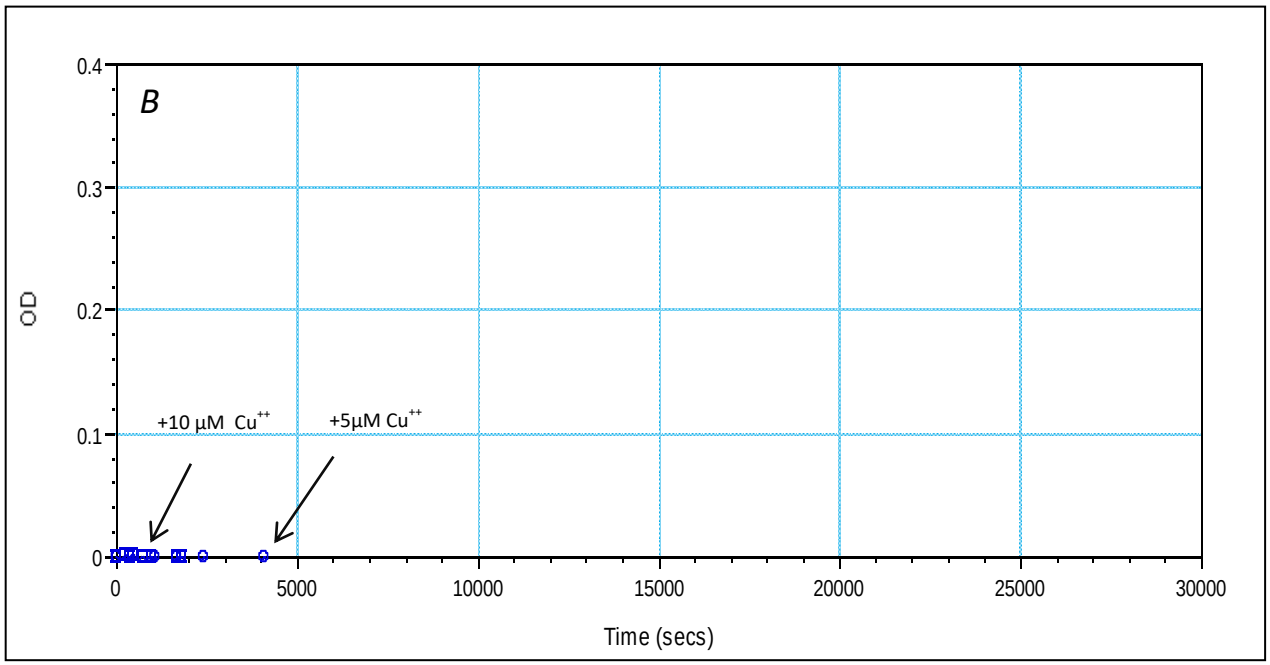
3.3.2 LDL Desalting and Removal of EDTA

As EDTA protects LDL from oxidation, it was important to remove EDTA from LDL to make it usable in the in vitro oxidation test. Three methods for removing EDTA from LDL were assessed; desalting once, or desalting twice by gel filtration chromatography, or dialysis.

Forty-five mL of EDTA blood was taken from one healthy donor. Tubes were centrifuged for 10 minutes at 4°C and 2060 g. Plasma was isolated and mixed. LDL was isolated from plasma by ultracentrifugation (**Section 2.6.1**). For LDL desalted one time, PD Minitrap G-25 Columns containing Sephadex G-25 Medium (GE Healthcare Bio-Sciences AB, Uppsala Sweden) was prepared by washing with PBS, the collector tube of the column was replaced with a new one, 0.5 ml of LDL fraction was mixed and loaded on the columns, column(s) were then transferred to 50.4 rotor (Beckman Coulter Ltd, High Wycombe, UK), spun in Optima XL-100K Beckman Ultracentrifuge (Beckman Coulter Ltd, High Wycombe, UK), Zonal centrifugation at 2060 g for 5 minutes at 15°C. LDL was collected after the spin stopped. For LDL desalted twice, a new 0.5 mL of LDL fraction was desalted in the same way as in desalting once then PBS was added again to the desalted fraction to make the volume up to 500 µL. The fraction was desalted again using a new washed desalting column (PD Minitrap G-25 columns). For dialysis; a fresh 0.5 mL of LDL fraction was put in Spectral/Por2 dialysis membrane (Spectrum Laboratories, Inc., Ca, USA). The tube was then put in a beaker containing 333 ml of PBS (Sigma Aldrich). Dialysis was carried out for 24 hours at 4C, in the cold room, changing the buffer two more times. All LDL preparations were standardized to 50 µg. mL^{-1} protein after protein measurement (**Section 3.3.1**) and tested for oxidation using 5 and 10 µM $CuCl_2.2H_2O$ (Sigma Aldrich) and with 1, 2 mM 2,2'-Azobis (2-methylpropionamidine) dihydrochloride (AAPH) (Sigma Aldrich) as described in **Chapter 2** (**Section 2.13**).

Results

Neither LDL desalted for one time nor LDL desalted for two times showed any response to oxidation with either 5µM or 10 µM $CuCl_2$ but they responded to oxidation with 1 and 2 mM AAPH. On the other hand, dialysed LDL showed an S-shaped graph with 5 and 10 µM of $CuCl_2$. This means that dialysis is capable of eliminating more EDTA than desalting columns, and hence was the method of choice for the assay.



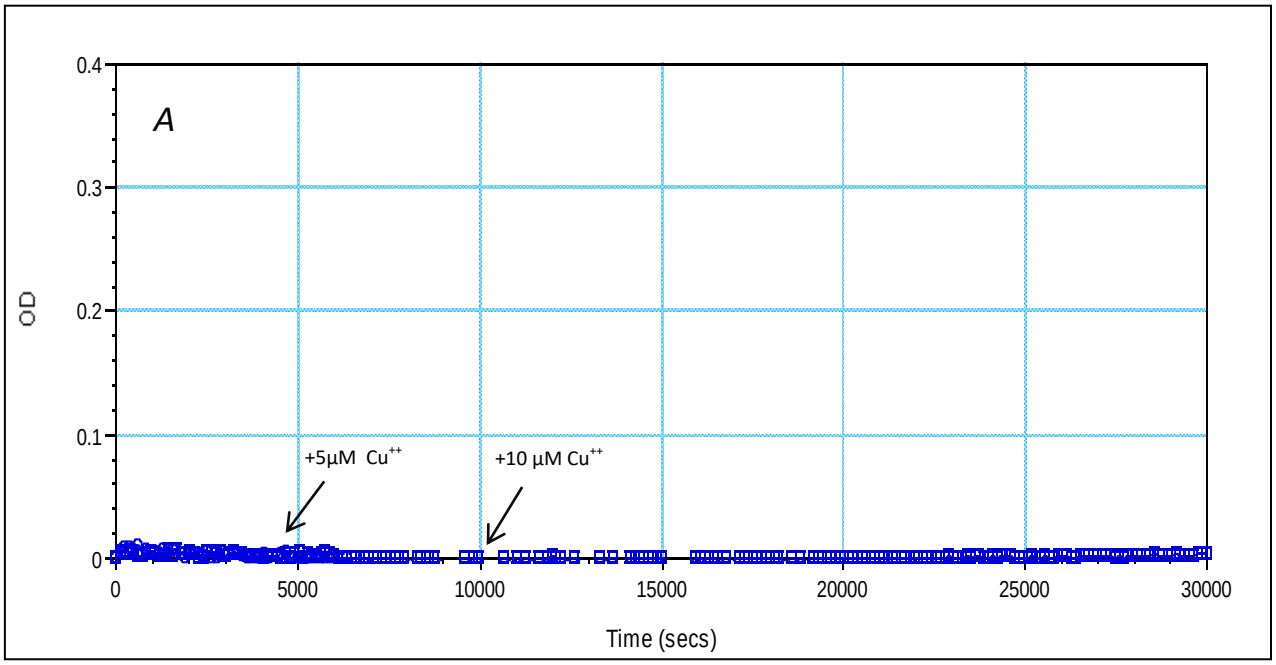


Figure 3.2 Oxidation graph of 50 µg.ml^{-1} LDL protein (desalted once) + 5 and 10 µM Cu^{++} (A); Oxidation graph of 50 µg.ml^{-1} LDL protein (desalted twice) + 5 and 10 µM Cu^{++}(B). Cu^{++}, $CuCl_2$; OD, optical density; secs, seconds.

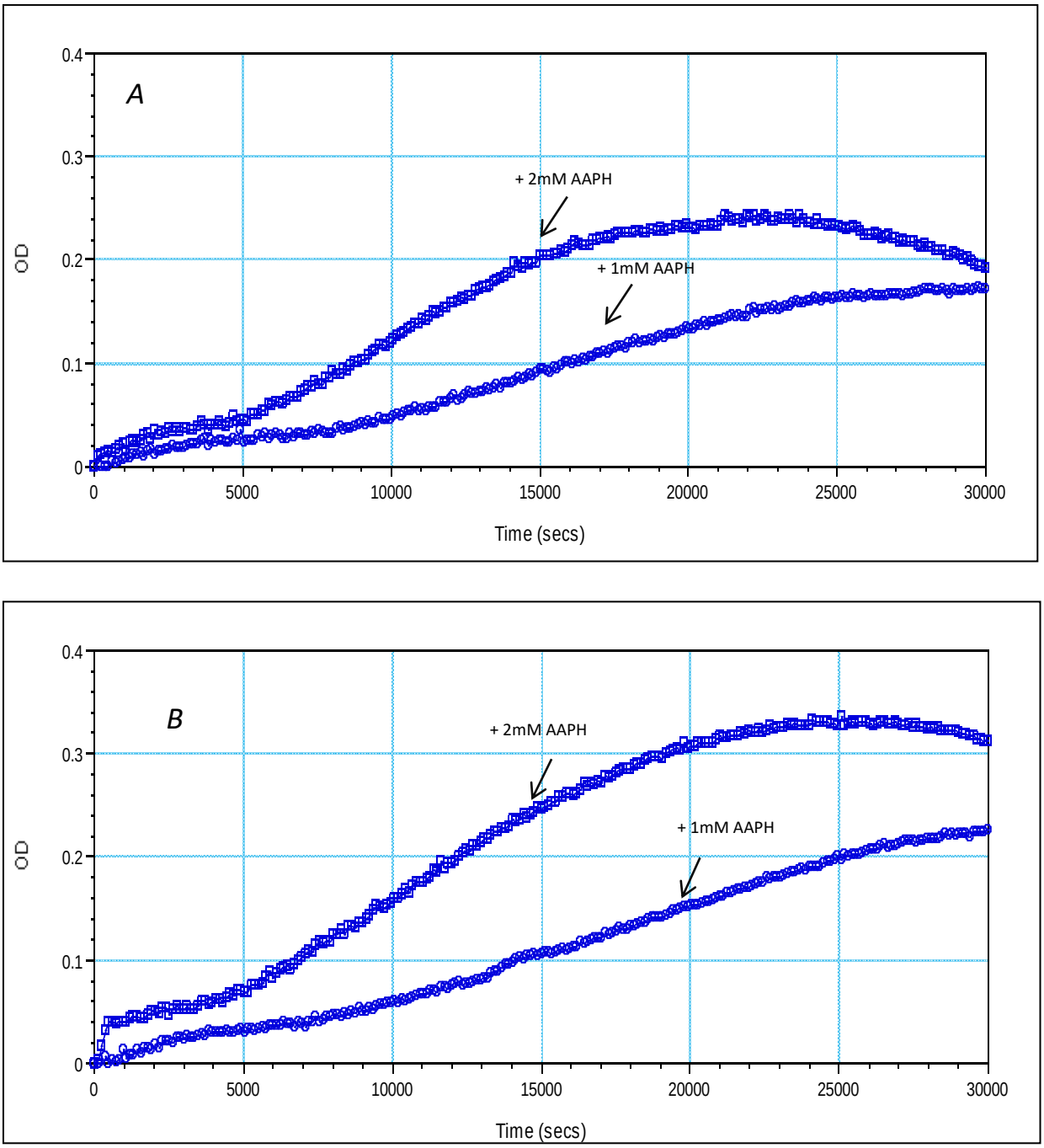


Figure 3.3 Oxidation graph of 50 µg.mL^{-1} LDL protein (desalted once) + 1 and 2 mM AAPH (A); Oxidation graph of 50 µg.mL^{-1} LDL protein (desalted twice) + 1 and 2 mM AAPH (B). AAPH, 1, 2 mM 2,2'-Azobis (2-methylpropionamidine) dihydrochloride; OD, optical density; secs, seconds

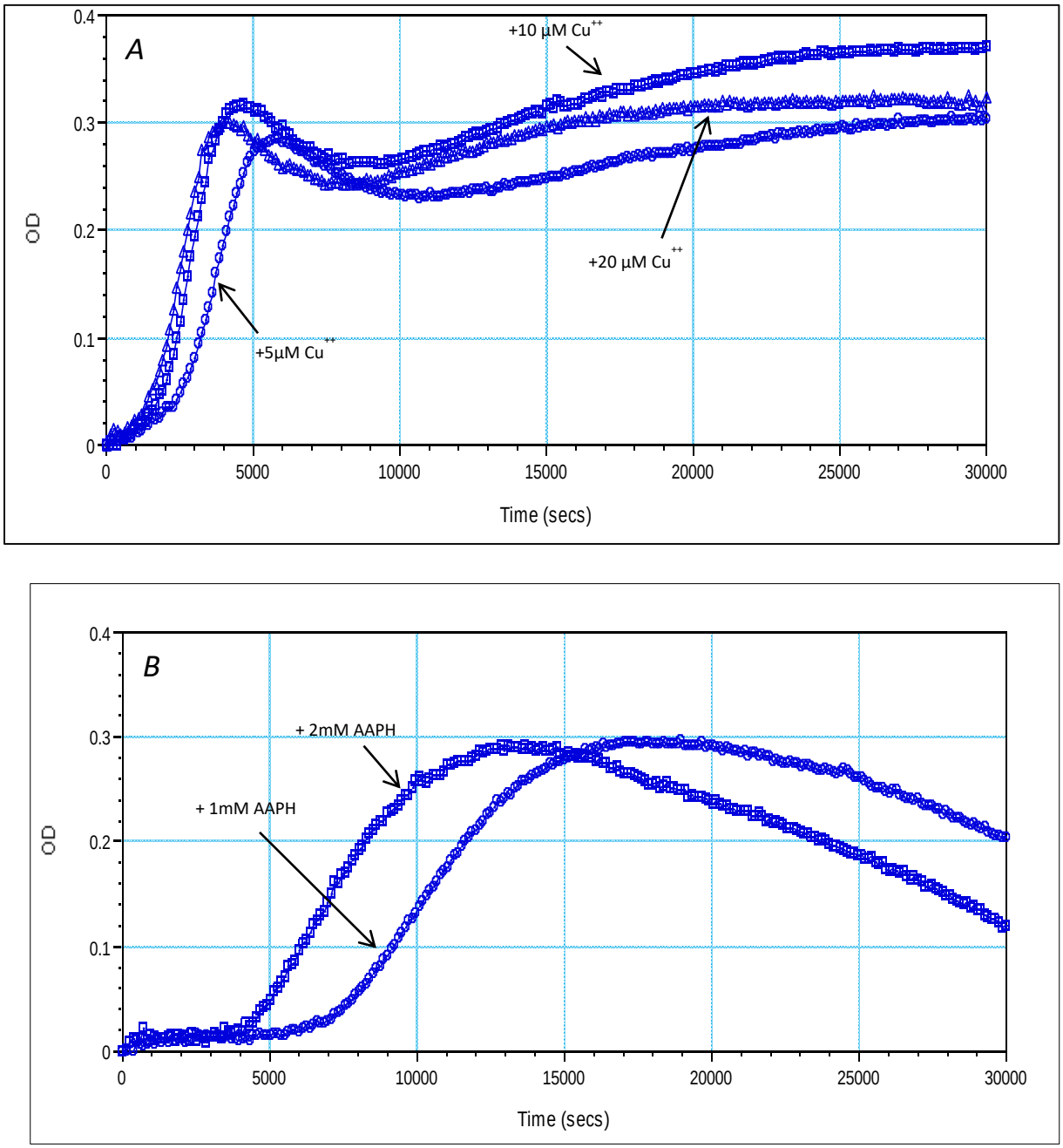


Figure 3.4 Oxidation graph of 50 µg.mL^{-1} LDL protein (dialysed) + 5, 10 & 20 µM Cu^{++} (A); Oxidation graph of 50 µg.mL^{-1} LDL protein (dialysed) + 1 and 2 mM AAPH (B). Cu^{++}, $CuCl_2$; AAPH, 2,2'-Azobis (2-methylpropionamidine) dihydrochloride (B); OD, optical density; secs, seconds; OD, optical density.

3.3.3 Optimizing Conditions for LDL Oxidation Assay

Copper Chloride Concentration

Previous publications have demonstrated that the influence of HDL on LDL oxidation depends on the concentration of copper; at high copper concentrations, addition of HDL to LDL promotes LDL oxidation (Raveh, Pinchuk, Fainaru & Lichtenberg, 2001) .

We aimed to find out whether $CuCl_2$ with concentration lower than 5 µM would oxidize 50 $\mu g.mL^{-1}$ dialysed LDL protein.

The same procedure was done as in previous trial for isolating and dialysing LDL (**Section 3.3.2**). LDL was standardized to 50 $\mu g.ml^{-1}$ proteins with PBS and oxidation was initiated with 0.5, 1 and 5 µM $CuCl_2$.

Conclusion

As shown in **Figure 3.5-A**, 0.5 µM Cu was capable of initiating oxidation on 50 $\mu g.mL^{-1}$ dialysed LDL protein and the T1/2max was inversely proportional to the copper chloride concentration. Therefore, we have decided to precede our oxidation trials with 0.5 µM $CuCl_2$ for oxidation assays.

3.3.4 LDL-Protein Concentration (LDL Standardisation)

Since we have studied the effect of different copper concentration on LDL oxidation, it was also important to understand the impact of using different LDL protein concentrations.

To find out the best LDL concentration to use in oxidation assays with 0.5 µM $CuCl_2$, the same procedure was done as in previous trial for isolating and dialysing LDL (**Section 3.3.2**). LDL was standardized as 50, 100 and 150 $\mu g.mL^{-1}$ protein and oxidation was initiated with 0.5 µM $CuCl_2$.

Conclusion

While oxidation showed a clear S-shaped graph for 50 µg.mL^{-1} LDL protein, mean of 143.9 (±3.33) min for T1/2max, CV% of 2.31 (n=3), oxidation did not show the typical time course for oxidation , with lag phase, propagation phase and steady phase, for 100 µg.mL^{-1} LDL protein (n=3). Finally no oxidation was detected for any of the wells for LDL 150 µg.mL^{-1} LDL protein (**Figure 3.5-B**).

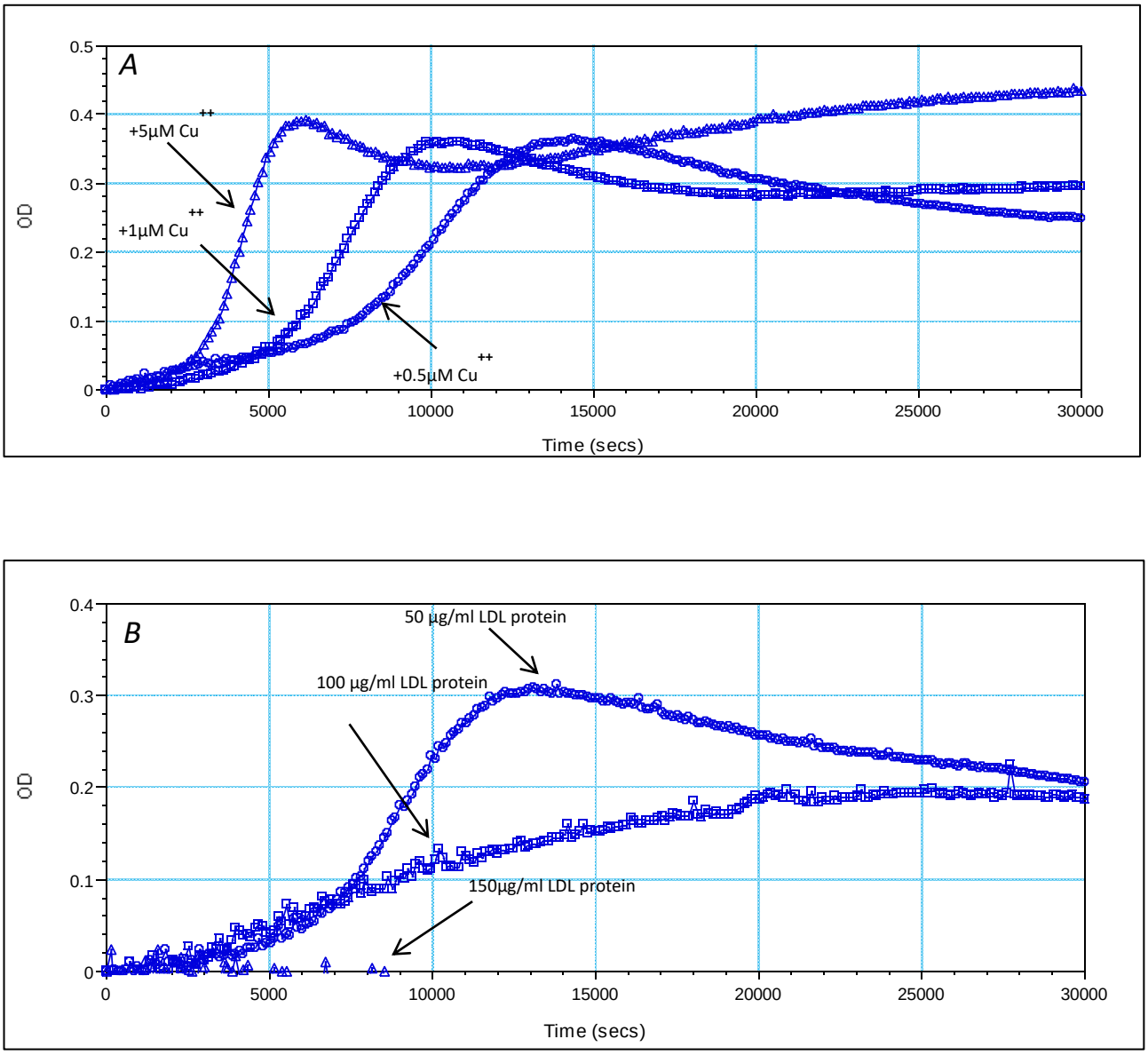


Figure 3.5 Oxidation graph of 50 µg.mL^{-1} LDL protein+ 0.5 , 1 and 5 µM Cu^{++} (A); Oxidation graph of 50 , 100 and 150 µg.mL^{-1} LDL protein (B); + 0.5 µM Cu^{++}. Cu^{++}, $CuCl_2$; OD, optical density; secs, seconds.

3.3.5 Formation of LDL Pool for Oxidation Assay

To measure HDL antioxidant potency, a standard LDL pool was needed that would be constant in each assay. And because we only could prepare 10 HDL per day, and because storing isolated LDL in the dark at 4°C, even with nitrogen purging, was not giving consisting results for oxidation kinetics, It was important to review multiple ways of preparing and storing LDL pools to find out the most stable preparation for use in the oxidation inhibition assay.

To compare the consistency of oxidation kinetics for four different LDL pools; (a) LDL which was freshly isolated from frozen plasma pool (LDL1), (b) LDL that has been prepared by ultracentrifugation, aliquoted and stored, at -80°C without dialysis and when required, (c) LDL was defrosted and dialysed against PBS rapidly for 4 hours (LDL2i), or dialysed against PBS at 4°C for 24 hours (LDL2ii) and (d) LDL that was prepared by ultracentrifugation and dialysed against PBS at 4°C for 24 hours space, aliquoted, and stored (LDL3). For oxidation, each LDL concentration was adjusted to 50 $\mu g.ml^{-1}$ protein as described previously in **Section 3.3.1** and oxidation was run within one hour with 0.5 µM $CuCl_2$.

Thirty-five mL of EDTA blood was drawn from 3 healthy donors. Samples were then centrifuged for 10 minutes, at 4°C and 2060 g. Plasma was isolated, pooled and mixed gently by inversion. About 20 ml of the pool was aliquoted as 1.2 mL portions in 1.5 mL tubes, stored at -80°C, after purging with nitrogen and labelled as plasma pool for LDL1. When required for oxidation, aliquots were defrosted, spun for LDL isolation as described in **Chapter2** (**Section 2.6.1**), dialyzed against PBS in the dark at 4°C for 24 hours, changing the buffer system 2 more times. The rest of plasma was used to isolate LDL as described in **Chapter 2** (**Section 2.6.1**) for LDL2i, LDL2ii and LDL3. When the spin stopped, LDL was isolated carefully, pooled and mixed gently by inversion. About 9 mL of LDL was aliquoted, 1.2 mL each, labelled as LDL2i, and another 9 mL of LDL was labelled as LDL2ii. Both LDL2i and LDL2ii aliquots were nitrogen purged and stored at -80°C. When required, they were defrosted and dialyzed against PBS at 4°C for 24 hours, changing the buffer system 3 times. Another portion of LDL, about 5 mL, was dialysed with PBS rapidly for 4 hours in the dark, changing the buffer +-system 7 more times. LDL was standardized to 50 $\mu g.ml^{-1}$ proteins and oxidation was run within one hour with 0.5 µM $CuCl_2$ as described in **Chapter 2** (**Section 2.13**).

Conclusion

While LDL2 (i) did not show any response to oxidation, LDL2(ii) was the most stable LDL for oxidation with mean of t1/2 max 127.55 (±11.56) minutes & maximum amount of conjugated dienes formed was 0.32 (±0.02) units. CVs. were 9.07 & 5.04 for t1/2 max and maximum conjugated dienes formed respectively. LDL3 was the second best as the mean for t1/2 max was 139.85 (±14.03) minutes and for maximum conjugated dienes formed was 0.32 (±0.04) units with CVs. of 10.03 & 11.47 for T1/2 max and maximum conjugated dienes formed respectively. LDL1 mean for T1/2 max was 151.88 (±35.39) minutes and maximum conjugated dienes was 0.33 (±0.03) units and CVs were 23.30 & 8.59 for T1/2 max and OD respectively. Therefore, we have decided to prepare our LDL pool as we did for LDL2 (ii): collecting blood then mixing and isolate plasma. LDL is then isolated as described in **Chapter 2** (**Section 2.6.1**), then stored, after aliquoting, at -80°C freezers (without dialysis) and when required, LDL is defrosted and dialysed against PBS at 4°C for 24 hours.

Table 3.1 %CV for T1/2max and maximum conjugated dienes formed for 4 different LDL pools

LDL Number	LDL preparation	Dialysis	%CV	
			T1/2max	Max. C.D.
LDL1 (n=5)	LDL freshly isolated from frozen plasma pool	Dialyzed against PBS at 4°C for 24 hours (changing the buffer system 3 times)	23.30	8.59
LDL2(i) (n=2)	LDL prepared by ultracentrifugation and stored at -80°C after N_2 purging without dialysis. , LDL was defrosted when required	Dialyzed against PBS rapidly for 4 hours (changing the buffer system 8 times)	No Oxidation	No Oxidation
LDL2(ii) (n=5)		Dialyzed against PBS at 4°C for 24 hours (changing the buffer system 3 times)	9.07	5.04
LDL3 (n=7)	LDL isolated by ultracentrifugation and dialyzed against PBS at 4°C for 24 hours, N_2 purged and stored at -80°C		10.03	11.47

LDL, low density lipoprotein; T1/2max, time at half maximum; Max. C.D.; maximum conjugated dienes formed; N_2, nitrogen.

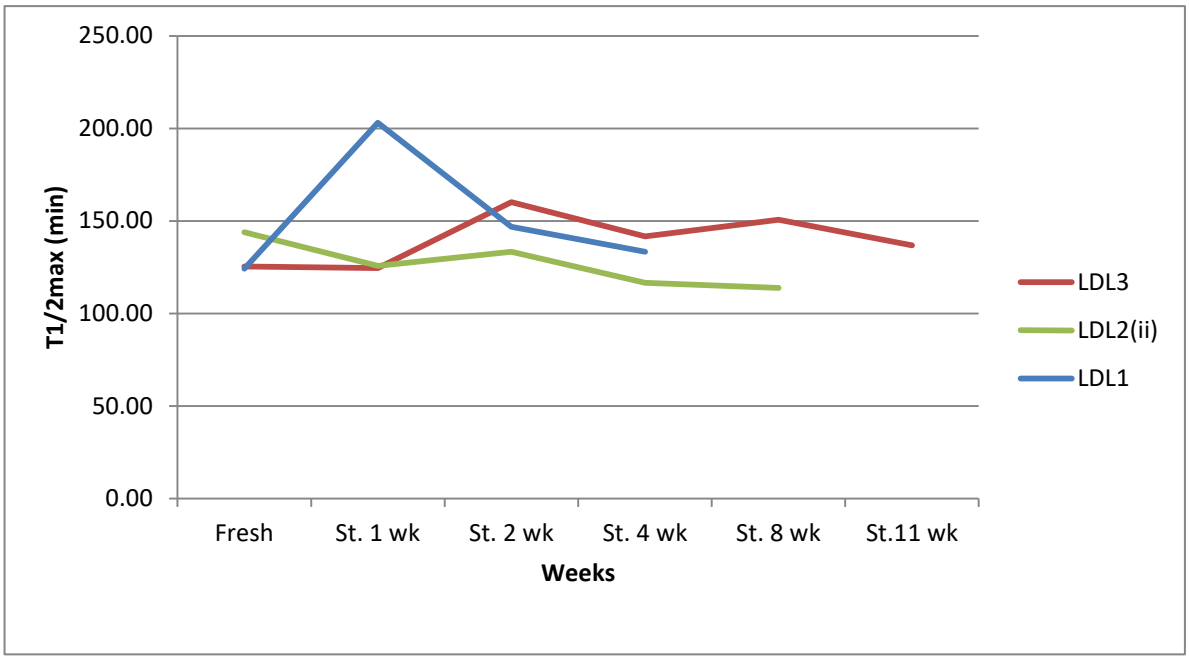


Figure 3.6 T1/2max for 3 different LDL pools +0.5 µM $CuCl_2$; freshly isolated from frozen plasma and directly used for oxidation (LDL1) (Blue); freshly isolated LDL from plasma then stored at -80° C without dialysis and when needed, defrosted and dialysed against PBS for 24 hours (LDL2(ii)) (green); LDL prepared by ultracentrifugation then directly dialysed against PBS at 4°C for 24 hours space, aliquoted, stored at -80°C and defrosted when needed (LDL3) (red). St, stored; wk, week; T1/2, time at half maximum

3.4 Lipoprotein Preparation-HDL

3.4.1 HDL Isolation

As limited volumes of Li-Hep samples were left from pSoBid, and because the classical HDL isolation method takes about 60 hours which was considered unlikely to keep the antioxidant potency of HDL fractions or to retain the activity of Paraxonase 1 (PON1) enzyme, a new method for HDL isolation which took one working day using a small volume of sample was necessary to be developed for the project.

A number of possible techniques were explored in an attempt to invent a quick method starting with 400-500 µL of plasma. All density solutions used were made up of NaCl/KBr (EDTA free). All analysis were made by automated clinical chemistry analyser (ILab^{Tm} 600, Instrumentation Laboratory, USA).

Comparison of the traditional method using 50.4 rotor and a new methods using TLA100.2

Since we were intending to design a new method for isolating HDL , it was important to prepare HDL sample using the current method used in the laboratory to gain insight into the expected numerical outcomes and also to become aware of the problems that such a lengthy spin causes and to compare with the new method.

Twelve mL of K_3EDTA blood was drawn from 12 donors. Samples were then centrifuged for 10 minutes, at 4°C and 2060 g**.** Plasma was removed and transferred into 5 mL bottles and mixed gently. 250 µL was taken from each sample to analyse. For the traditional method, 2 mL from 8 different plasma were transferred into 6.4 ml centrifuge tubes (344088 Beckman Coulter Ltd, High Wycombe, UK). 1 mL of 1.182 g.mL^{-1} density solution was added and mixed, to adjust the density to 1.063, resulting in a 2:1 ratio of plasma to density solution respectively. A further 3 mL of 1.063 g.mL^{-1} density solution was over layered using automated pump. Samples were transferred into Beckman 50.4 rotors (Beckman Coulter Ltd, High Wycombe, UK) and centrifuged in Beckman Optima XL-100 K ultracentrifuge (Beckman Coulter Ltd, High Wycombe, UK) at 11000 g and 15°C for 20 hours. Once the spin stopped, the top 2 mL was carefully isolated. To the remaining 4 mL, 2 mL of 1.478 g.mL^{-1}

density solution was added and mixed, to adjust the density to 1.21 $g.mL^{-1}$, and a further 1.21 $g.mL^{-1}$ density solution was over layered using automated pump. Samples were then centrifuged again in Beckman 50.4 rotors in Beckman centrifuge for 36 hours at 11000 g and 15°C in Optima XL-100 K ultracentrifuge (Beckman Coulter Ltd, High Wycombe, UK). When the spin stopped, HDL was then isolated at 1 mL and stored for analysis.

For the new method, 400 µL of 4 different plasma were transferred into 11X34 mm thickwall polycarbonate centrifuge tubes (343778 Beckman Coulter Ltd, High Wycombe, UK) and 100 µL of 1.006 $g.mL^{-1}$ density solution was added and mixed to complete to 500 µL then 250 µL of 1.182 $g.mL^{-1}$ was added and mixed, to adjust the density to 1.063 $g.mL^{-1}$, then over layered with 250 µL of 1.063 $g.mL^{-1}$ density solution. Tubes were then transferred to TLA100.2 rotor (Beckman Coulter Ltd, High Wycombe, UK) and centrifuged for 16 hours at 4300 g and 23°C in a TL-100 Table-Top Ultracentrifuge (Beckman coulter, Inc. USA). Once the spin stopped, the top 500 µL, containing VLDL/LDL was removed. For the remaining 500 µL plasma, 250 µL of density solution 1.478 $g.mL^{-1}$ was added and mixed, to adjust the density to 1.21 $g.mL^{-1}$, and then 250 µL of 1.21 $g.mL^{-1}$ density solution was over layered. Samples were then placed again in TLA100.2 rotor (Beckman coulter, Inc. USA) and centrifuged for 5 hours at 27400 g and 23°C in and centrifuged in a TLA100 Table-Top Ultracentrifuge (Beckman coulter, Inc. USA). When the spin stopped, 250 µL of the top fraction containing HDL was removed and analysed.

Analysis was done for plasma, HDL fractions from each the old and the new spin for HDL-cholesterol, cholesterol, ApoA-1, Apo-B and Albumin as described in **Chapter 2** (**Section 2.7).**

Conclusion

Albumin amounts in HDL fractions resulted from both spin were negligible. Recoveries for HDL-C and ApoA-1 using the traditional method (long spin times) was 39.35% and 32.88% respectively while these were 59.11% and 57.39% respectively from the new method. On the other hand, the apo-B amount contaminant in the new method was 0.31 $mg.dL^{-1}$ compared to the traditional method which was 1.41 $mg.dL^{-1}$**.** This means that the new method, using TLA 100.2 rotor, is promising in producing HDL samples with substantially better recoveries for HDL-C and Apo-A1.

3.4.2 HDL Protein Measurement

To find out the quickest and most reliable way for measuring HDL protein concentration, 12 ml of EDTA blood was drawn from 7 healthy donors. Samples were then centrifuged for 10 minutes, at 4°C and 2060 g. HDL was isolated from each of the 9 samples by ultracentrifugation as described in the new method, using TLA100.2 rotor (**Section 3.4.1**). Each HDL fraction was desalted by loading 250 µL HDL + 250 µL PBS as described in **Chapter 2** (**Section 2.6.3**). HDL protein for each sample was measured by two ways; Lowry method as described in **Chapter 2** (**Section 2.8**) and Bradford method as described in **Chapter 2** (**Section 2.9**) and ApoA-1 as described in **Chapter 2** (**Section 2.7.4**).

Conclusion

Average percentage of HDL-Apo-A1, measured by the analyser, to HDL-total protein, measured by Bradford was 57.87 (±5.41), %CV= 9.35 while average percentage of HDL-ApoA-1, measured by the analyser, to HDL- total protein measured by Lowry method was 47.61(±4.12), %CV= 8.65. Comparing Bradford: Lowry measures of total proteins, it was found that they gave a constant difference (83.17% (±2.76), %CV= 3.32) and because Bradford was more rapid and more convenient for adaptation to a plate reader, we have decided to use the Bradford method for HDL-total protein measurement for oxidation and PON1 assays.

3.4.3 Selecting Buffer for Desalting HDL samples

Some publications indicate that calcium inclusion in density solutions used for HDL isolation or during HDL dialysis protects paraxonase enzyme (PON1) (Kontush et al., 2004; Lynch, Lorenz & Klotz, 2014). This has been applied because PON1 activity is known to be calcium dependent (Kuo & La Du, 1998). Accordingly, we set about trying to detect if 1 mM of calcium inclusion in PBS used in HDL desalting would make any significant difference in HDL antioxidant potency or in measuring PON1 assay.

One EDTA blood sample was taken from 1 healthy donor and 5 Lithium Heparin, blood samples were taken from 5 other healthy donors. Samples were then centrifuged for 10 minutes, at 4°C and 2060 g**.** Plasma was removed and transferred into 1.5 mL bottles for

EDTA samples and 5 mL bottles for Li-Hep samples. 1 EDTA aliquot was used to prepare LDL fraction by 50.4 rotors as described in **Chapter 2** (**Section 2.6.1**) then dialysed as described in **Chapter 2** (**Section 2.6.1**) and protein measured as described previously (**Section 3.3.1**). Four Li-Hep aliquots, two for each donor, were used to isolate HDL as explained the new method in **Section 3.4.1** using TLA100.2 rotor. For HDL desalting, two HDL fractions, one for each donor, was desalted as explained in **Chapter 2** (**Section 2.6.3**) with PBS and the other two were desalted with PBS+ 1 mM $CaCl_2$. All HDL samples had protein measured by the Bradford method as described in **Chapter 2** (**Section 2.9**) and concentrations were standardized to 50 $\mu g.ml^{-1}$ of HDL-protein, for 1:1 LDL: HDL wells, or 100 $\mu g.mL^{-1}$ of HDL-protein for 2:1 HDL: LDL wells. LDL was standardized to 50 $\mu g.mL^{-1}$ of LDL-protein and oxidation was run as described in **Chapter 2** (**Section 2.13**). PON1 assay was also run for measuring HDL PON1 activity as described in **Chapter 2** (**Section 2.12**).

Conclusion

It was found that adding 1 mM $CaCl_2$ to the PBS used for HDL desalting resulted in a longer T1/2max (**Figure 3.7**). This observation is consistent with preservation of the antioxidant capability of HDL.

Although the benefits of adding $CaCl_2$ were borderline significant, (P=0.051), there was a clear trend**,** as shown in **Figure 3.8,** of higher PON1 activity results and that PON1 enzyme activity appeared to be better preserved with $CaCl_2$.

Hence, the decision was made to use PBS + 1mM$CaCl_2$ rather than PBS alone in desalting HDL samples.

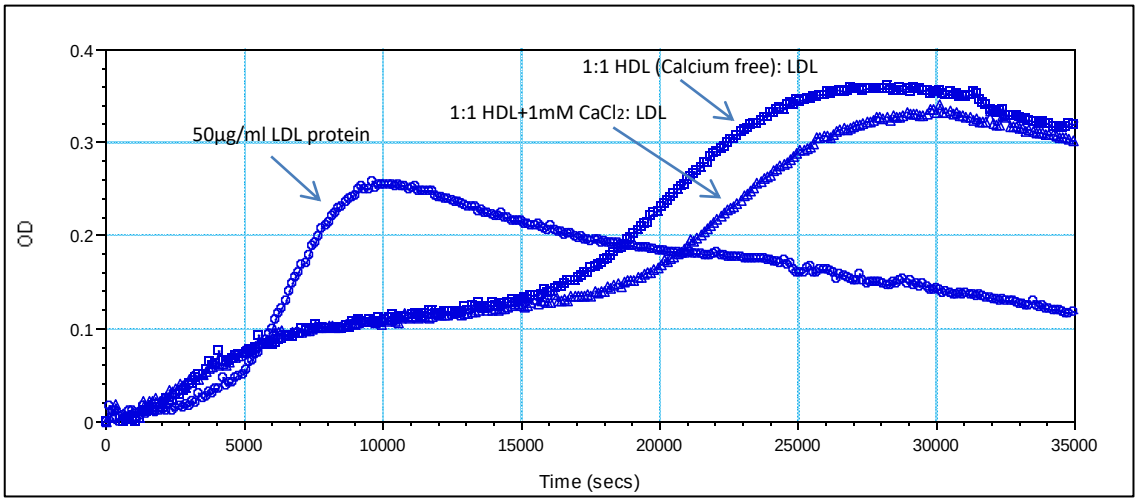


Figure 3.7 Oxidation graph of 50 µg.mL^{-1} LDL protein, 1: 1 HDL: LDL or 1:1 HDL (+1 mM $CaCl_2$): LDL oxidation graphs + 0.5 µM $CuCl_2$.

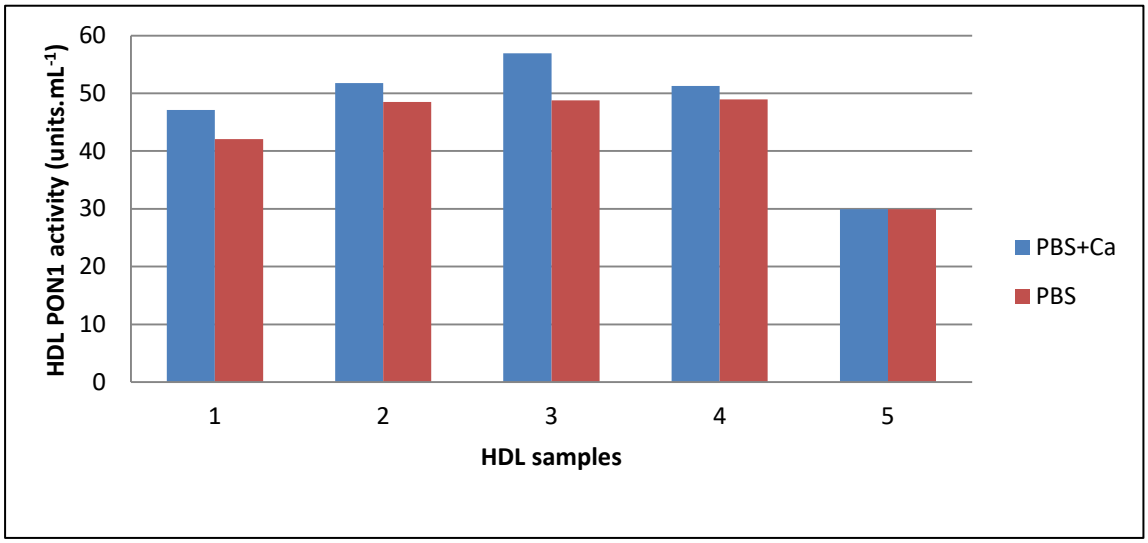


Figure 3.8 PON1 assay activity for five HDLs samples desalted with or without $CaCl_2$. PON1, paraxonase1 enzyme; Ca, $CaCl_2$; PBS, phosphate buffer saline.

3.4.4 Selection of Optimal Centrifugation conditions

One Day Spin Versus Two Separate Days Spin

To see the outcome for HDL recoveries, HDL antioxidant potency as well as PON1 assay result after isolating HDL in 2.5/5 hours in either 1 day or in 2 separate days.

Thirty-five ml of Li-Hep samples were collected from 5 healthy donors. Samples were then centrifuged for 10 minutes, at 4°C and 2060 g**.** Plasma was removed and transferred into 5ml bottle. One aliquot for each of the donor was stored, at 4°C for next day spin. Another aliquot was transferred as 400 µL into five 11 X 34 mm thickwall polycarbonate centrifuge tubes (343778 Beckman Coulter Ltd, High Wycombe, UK), and 100 µL of 1.006 $g.ml^{-1}$ density solution was added on the top of each sample, to make the volume up to 500µL, followed by 250 µL of 1.182 $g.mL^{-1}$ density solution and mixed, to adjust the density to 1.063 $g.mL^{-1}$, then over layered with 250 µL of 1.063 $g.mL^{-1}$ density solution. Tubes were then transferred to TLA100.2 rotor (Beckman Coulter Ltd, High Wycombe, UK) and centrifuged for 2.5 hours at (27400 g) and 23°C in a TL-100 Table-Top Ultracentrifuge (Beckman coulter, Inc. USA). Once the spin has stopped, the top 500 µL, containing VLDL/LDL was removed from all tubes. The infranatant was stored overnight at 4°C in the dark. Next day, stored plasma aliquots, from previous day, were taken from the fridge, prepared and spun for VLDL/LDL in the same way as for the samples in the previous day. When the spin stopped, VLDL/LDL was isolated as 500 µL. Tubes containing the infranatant from previous day spin was taken and completed, along with the same day samples for HDL spin; 250 µL of 1.487 $g.mL^{-1}$ density solution was added on the top of all samples and mixed, to adjust the density to 1.21 $g.mL^{-1}$, then overlayered with 250 µL of 1.21 $g.mL^{-1}$ density solution and transferred again to TLA 100.2 rotor and spun for 5 hours 27400 g on the same ultracentrifuge. When the spin stopped, the top fraction, 500 µL, containing HDL was isolated, analysed for recoveries, protein measured by Bradford as described in **Chapter 2** (**Section 2.9**) and run for oxidation as described in **Chapter 2** (**Section 2.13**) with 0.5 µM $CuCl_2$ and PON1 assay as described in **Chapter 2** (**Section 2.12**).

Conclusion

The one day spin showed a better HDL recoveries than the two separate days spin; an average of 59.94% (±9.85), %CV= 16.44 and 56.06% (±3.49), %CV= 6.22 versus 49.85% (±4.80), and 52.68% (±3.09), % CV=5.87 for HDL-C and Apo-A1, respectively (n=5). Albumin was more unfavourable as it was less than 1 $g.dL^{-1}$ in the one day spin and more than 2 $g.dL^{-1}$ in the two separate days' spin. On the other hand, Bradford measurement for HDL protein for those samples prepared in 2 days were very high compared to the same samples prepared in one day (an average difference of 48.51 ± 5.55, %CV = 11.4) which indicates possibly that the 2 day spin is contaminated with albumin or even Apo-B proteins and that was not expected as the change in spin length would not alter LDL separation from HDL.

For oxidation, HDL prepared over two separate days showed a very weak oxidation activity while for HDL isolated within a day showed a clear oxidation results (**Figure 3.9**).

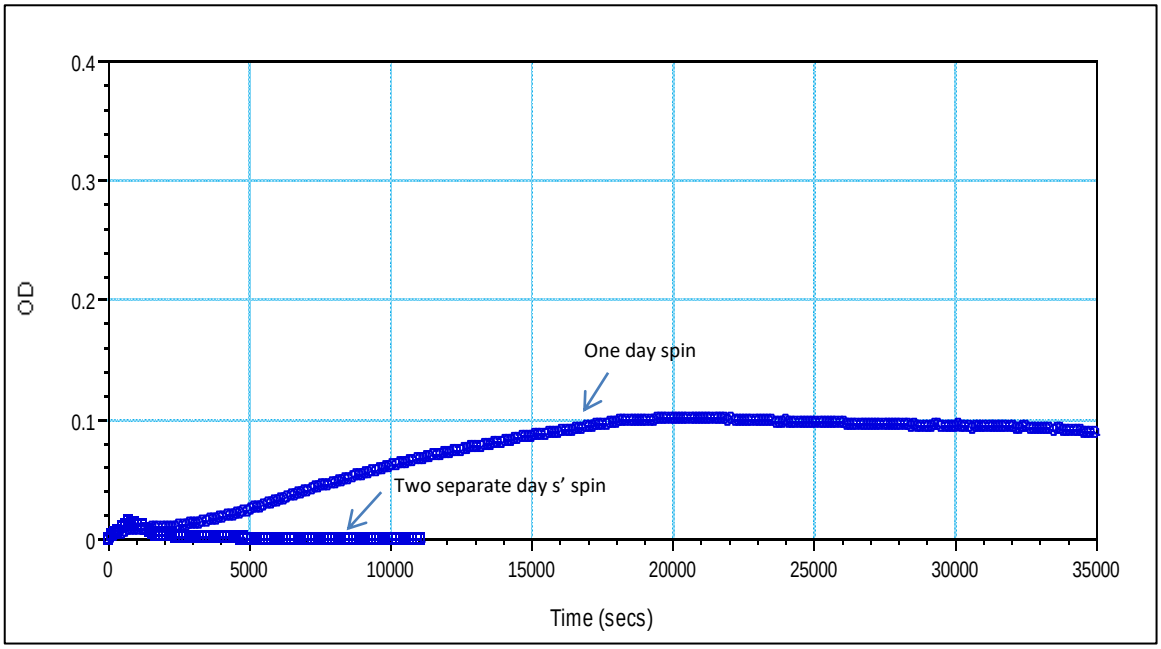


Figure 3.9 Oxidation graph for HDL (isolated in one day) and HDL (isolated in over days) + 0.5 µM $CuCl_2$. Sec, seconds; OD, optical density.

Studying 2.5/18 hours Spin with a Comparison for 250 and 500 µL Isolation Volume of HDL

As the one day spin results were better for HDL recoveries as well as oxidation results than the two separate days' spin and as it was not practical to do the one day spin every day. We wanted to find a more convenient way of HDL isolation with 2.5/18 hours spin times.

To study HDL, isolated in 2.5/18 hours, recovery and to compare the results of isolating HDL in either 250 µL or 500 µL. 35 mL of Li-Hep blood was drawn from 2 healthy donors. Samples were then centrifuged for 10 minutes, at 4°C and 2060 g**.** Plasma was removed and transferred into 5mL bottle. 400 µL of plasma sample, 2 for each donor, were transferred into 11X34 mm thickwall polycarbonate centrifuge tubes (343778 Beckman Coulter Ltd, High Wycombe, UK). 100 µL of 1.006 $g.mL^{-1}$ density solution was added, to complete to 500 µL, followed by 250 µL of 1.182 $g.mL^{-1}$ density solution and mixed, to adjust the density to 1.063 $g.mL^{-1}$, then over layered with 250 µL of 1.063 $g.mL^{-1}$ density solution. Tubes were then transferred to TLA100.2 rotor (Beckman Coulter Ltd, High Wycombe, UK) and centrifuged for 2.5 hours at 27400 g and 23°C in a TL-100 Table-Top Ultracentrifuge (Beckman coulter, Inc. USA). Once the spin has stopped, the top 500 µL, containing VLDL/LDL was removed for all the tubes. 250 µL of 1.487 $g.mL^{-1}$ density solution was added on the top of each tube, to adjust the density to 1.21 $g.mL^{-1}$, then overlayered with 250 µL of 1. $g.mL^{-1}$ density solution and transferred again to the same rotor and spun for 18 hours at 13800 g and 15° C On the same ultracentrifuge. When the spin stopped, the top 250 µL was isolated carefully from each of the two donors' samples and 500 µL was isolated from the other two tubes. Each HDL fraction was desalted with PBS+ 1 mM $CaCl_2$ as explained in **Chapter 2** (**Section 2.6.3**) and measured for recoveries of ApoA-1, HDL-C and Albumin by the chemical chemistry analyser as explained in **Chapter 2** (**Section 2.7**) and protein by Bradford (**Section 2.9**).

Conclusion

Although the recoveries for HDL-C and Apo-A1 for the 500 µL fractions was higher; 63.31% (±2.30), %CV= 3.63 and 79.61% (±2.10), %CV= 2.64 for the 500 µL HDL fractions versus 32.45% (±7.99), %CV= 24.62 and 48.70% (±12.59), %CV= 25.84 respectively for the 250 µL HDLs fractions, it was noticed that HDL protein measurement by Bradford was abnormal;

very high > 4000 μg.mL^{-1} for all fractions, 250 μL and 500 μL HDLs, We can also conclude that isolating HDL in 250 μL is not reproducible (%CV > 24).

Studying 2.5 /18 hours and Isolating HDL in 400 μL and Completing to 500 μL with PBS+$CaCl_2$ and Completing again with PBS+$CaCl_2$ after Desalting.

As the previous trial has shown an inconsistency for isolating HDLs in 250 μL and as all HDL fractions, 250 μL or 500 μL HDL fractions, were highly contaminated with protein, we tried isolating HDL between the two volumes; 400 μL and to make up the volume of the fraction up to 500 μL with PBS before and after desalting and study the outcomes.

To repeat the previous trial (2.5/18 hour spin) in isolating HDL with the same spin conditions and only with one change; isolate HDL in 400 μL and to complete to 500 μL with PBS+1mM $CaCl_2$ before and after desalting.

Thirty-five mL of Li-Hep blood was drawn from 2 healthy donors. Samples were then centrifuged for 10 minutes, at 4°C and 2060 g**.** Plasma was removed and transferred into 5 mL bottle. 400 μL of plasma sample, 2 for each donor, were transferred into 11 X 34 mm thickwall polycarbonate centrifuge tubes (343778 Beckman Coulter Ltd, High Wycombe, UK). 100 μL of 1.006 g.mL^{-1} density solution was added, to complete to 500 μL, followed by 250 μL of 1.182 g.mL^{-1} density solution and mixed, to adjust the density to 1.063 g.mL^{-1}, then over layered with 250 μL of 1.063 g.mL^{-1} density solution. Tubes were then transferred to TLA100.2 rotor (Beckman Coulter Ltd, High Wycombe, UK) and centrifuged for 2.5 hours at 27400 g and 23°C in a TL-100 Table-Top Ultracentrifuge (Beckman coulter, Inc. USA). Once the spin has stopped, the top 500 μL, containing VLDL/LDL was removed from all the tubes. 250 μL of 1.487 g.mL^{-1} density solution was added on the top of each tube, to adjust the density to 1.21 g.mL^{-1}, then over layered with 250 μL of 1.21 g.mL^{-1} density solution and transferred again to the same rotor and spun for 18 hours 13800 g and 15°C on the same ultracentrifuge. When the spin stopped, the top 400 μL containing HDL fractions were isolated carefully from each sample. Each HDL fraction was completed to 500 μL by 100 PBS +1 mM$CaCl_2$, desalted (as described in **Section 2.6.3**) then the amount of the sample coming out from the desalting columns was checked again and if it was less than 500 μL , it

was completed again to 500 µL by adding PBS + 1 mM $CaCl_2$. Recoveries for HDL-C and APoA-1 and albumin contamination was studied. Estimate proteins By Bradford method and run oxidation trial for HDL samples with 0.5 µM $CuCl_2$ as described in **Chapter 2** (**Section 2.13**).

Conclusion

Using 400 µL of plasma, and completing to 500 µL with PBS+$CaCl_2$, as a starting volume for HDL separation was giving reasonable measurement for HDL-protein by Bradford method; 918.0 (±125.9) $mg.mL^{-1}$, %CV= 13.71, Recoveries for HDL-C was 60.94% (±11.05), %CV= 18.14 (n=2) and Apo-A1 was 62.7% (±4.88), %CV= 7.79 (n=2). Albumin was ≤1.95 $g.dl^{-1}$. This means that the above method is superior in isolating HDL.

3.5 Optimizing Conditions for Oxidation Assay

The assay conditions had to meet two other criteria; HDL: LDL ratio and $CuCl_2$ concentration. Trials were done to find the best HDL: LDL ratios which show the clearest HDL antioxidant potency along with the most suitable $CuCl_2$ concentration. The trial was run with 0.5 and 5 µM $CuCl_2$ along with 1:1 and 2:1 HDL: LDL ratios.

Conclusion

0.5 µM Cu was the best concentration to use in oxidation run as it was enough to initiate LDL oxidation for both HDL: LDL ratios. 5 µ M Cu is not suitable for the oxidation runs as it showed a negative effect of HDL. For HDL: LDL ratios, it is better to use 2:1 than 1:1 HDL: LDL as 2:1 ratio showed a clearer inhibitory effect of HDL antioxidant potency on LDL.

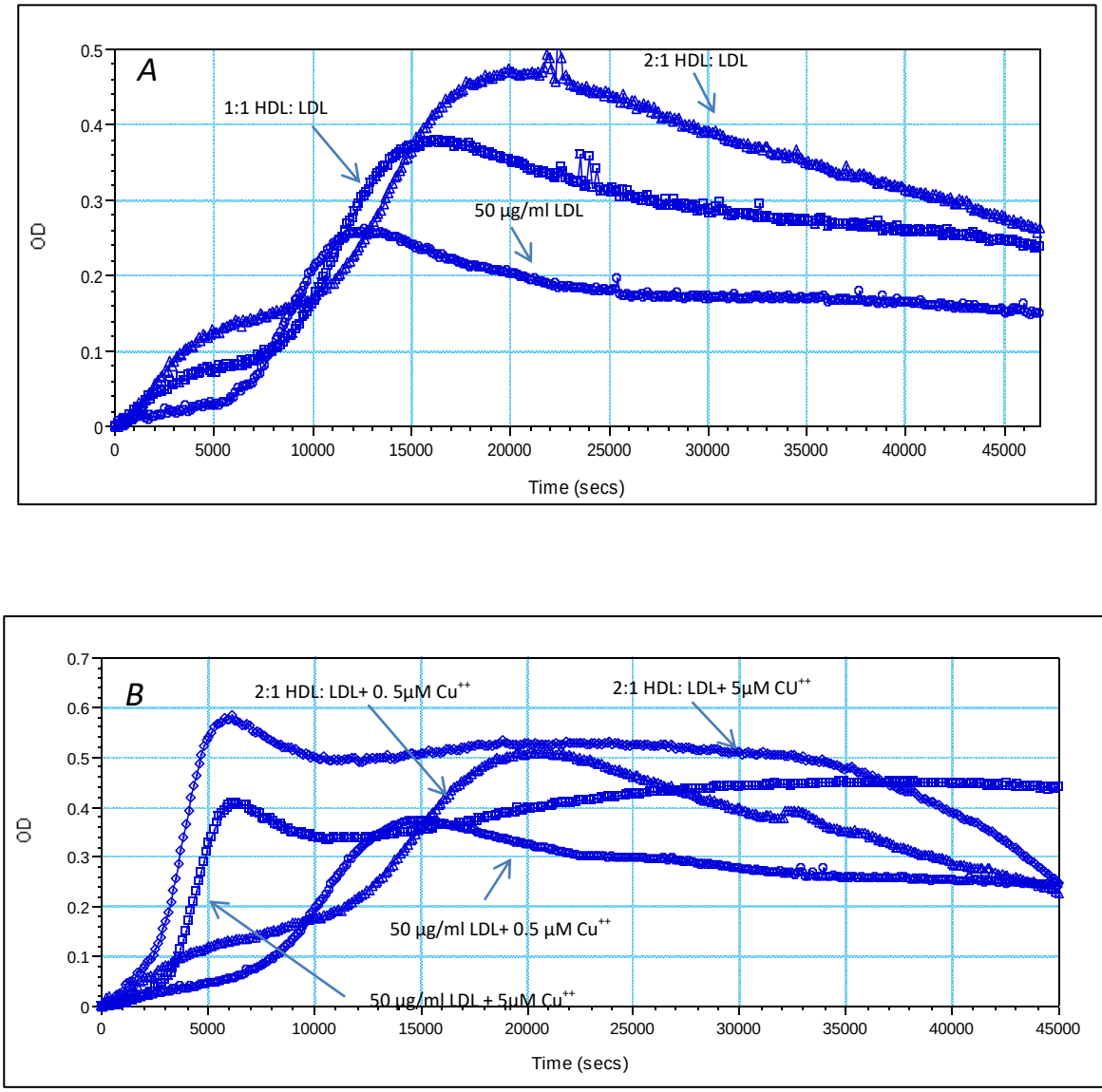


Figure 3.10 Oxidation graph of 50 µg.mL^{-1} LDL protein, 1: 1 HDL: LDL & 2:1 HDL (+0.5 µm $CuCl_2$) (A). Oxidation graph of 50 µg.mL^{-1} LDL protein, 1: 1 HDL: LDL & 2:1 HDL (+0.5 & + 5 µm $CuCl_2$) (B).

3.6 Final Oxidation Assay Protocol

The final oxidation assay was applied by collecting EDTA blood samples from healthy donors for LDL pool and Li-Hep samples from healthy donors for HDL pool for quality control (QC.). LDL was isolated by 50.4 rotor using density solutions containing EDTA as explained in **Chapter 2** (**Section 2.6.1**), then aliquoted and stored in -80°C. HDL (QC.) was isolated by 50.2 rotor using density solutions free of EDTA, then aliquoted and stored at -80°C. One night before oxidation assay, LDL aliquot was defrosted and taken for dialysis. 10 Li-Hep samples from pSoBid (stored in freezers) was also defrosted and 400 µL of plasma was used for HDL spin, for 2.5 hours and then for 18 hours, in TLA100.2 rotor using EDTA free density solutions. Next day, after 24 hours of dialysis, LDL fraction is taken and cholesterol was measured by the clinical analyser and then converted to protein amount as explained in **Section 3.3.1**. When HDL spin stopped, HDL was isolated by 400 µL. HDL QC was also defrosted (400 µL) and all HDL was made up to 500 µL with PBS + (1 mM) $CaCl_2$, desalted by NAP-5 columns as explained in **Chapter 2** (**Section 2.6.3**) and made up again to 500 µL with PBS + (1 mM) $CaCl_2$. All HDL samples protein was measured by Bradford method and then standardized to 100 $\mu g.mL^{-1}$ protein with PBS. LDL protein was also standardized to 50 $\mu g.mL^{-1}$ with PBS. Oxidation was run for LDL, HDL QC and pSoBid HDLs in triplicate within 1 hour of taking LDL from dialysis and of HDL desalting. Oxidation was monitored for 13 hours at 37°C.

Calculations of LDL oxidation Inhibition were as follows;

The percentage of HDL inhibition for LDL oxidation was calculated using an equation proposed by Hillstrom and co-workers (Hillstrom, Yacapin-Ammons & Lynch, 2003);

For maximum propagation rate (Vmax):

$$\%\ \text{Inhibition} = \frac{(\text{AvgVmaxLDL}+\text{AvgVmaxHDL})-(\text{AvgVmaxHDL}+\text{LDL})}{(\ \text{AvgVmaxLDL}+\text{AvgVmaxHDL})}\ \text{X}\ 100$$

For maximum conjugated dienes produced (max-min OD):

$$\% \text{ Inhibition} = \frac{(\text{Avg(OD)LDL+Avg(OD)HDL})-(\text{Avg(OD)HDL+LDL})}{(\text{ Avg(OD)LDL+Avg(OD)HDL})} \text{ X 100}$$

For Time at half maximum (T1/2max), however, another equation was used:

$$\% \text{ Inhibition} = [\frac{(\text{Avg T1/2maxLDL+HDL})}{(\text{ AvgT1/2maxLDL})} \text{ -1] X 100}$$

Those results were further corrected to the reading of the LDLQC, HDLQC and HDLQC+LDLQC on the same run as follows:

%inhibition for LDLQC (from HDLQC) X %Inhibition of LDLQC (from pSoBid HDL)

$$\text{Corrected Result} = \frac{\%inhibition\ for\ LDLQC\ (from\ HDLQC)}{Mean\ \%\ Inhibition\ (of\ LDLQC\ by\ HDLQC)\ for\ all\ Runs}\ X\ \text{\%Inhibition of LDLQC (from pSoBid HDL) ...}$$

The above equation was used to correct the calculated inhibition for all T1/2max, maximum propagation rate and maximum conjugated dienes formed.

3.6.1 HDL Oxidation Potential Assay

HDL samples themselves, from the pSoBid study, were assessed for their oxidation potential using the same assay conditions as for the LDL oxidation assay. The measures derived were T1/2max, Vmax and maximum diene formation.

3.7 Final Paraxonase Assay

PON1 assay for pSoBid's HDLs was completed as explained in **Chapter 2: Section 2.5** for preparing plasma pool and HDL pool for plasma QCs and HDLQCs; **Section 2.6.2** for HDL separation for HDL QCs; **Section 2.6.3** for HDL separation from pSoBids; **Section 2.9** for HDL-protein measurement by Bradford method; and **Section 2.12** for paraxonase assay.

Calculations for plasma and HDL PON1 activity (Units.mL^{-1}):

$$\text{PON1 activity (Units.mL}^{-1}) = \frac{\text{absorbance (mM/Minute)x total volume of solution in well (220 µL)}}{\text{Pathlength(0.66) X extension coefficient of phenol (1.31) X 1000}}$$

PON1 activity for each HDL sample were standardized to HDL protein

$$\text{HDL PON1 standardised activity (Units.mL}^{-1}) = \frac{\text{HDL PON1 activity}}{\text{HDL protein µg/mL}} \text{ x Av. protein for all HDL samples}$$

3.8 Discussion

The purpose of the work described in this chapter was to establish the best methods for measuring HDL antioxidant potency and HDL PON1 enzyme activity in relatively small volumes of Li-Hep samples stored at -80°C from 2008 (stored for about 7 years). Although most studies have used EDTA-plasma for isolating HDL like Kontush and his colleagues (Kontush et al., 2004), some studies have either used ''cleaned'' tubes for collecting blood like Brite and colleagues (Brites, Zago, Verona, Muzzio, Wikinski & Schreier, 2006) or Na-Heparin tubes like Hillstrom and colleagues (Hillstrom, Yacapin-Ammons & Lynch, 2003).

EDTA-LDL removal was one of the main obstacles where oxidation was not detected unless using high $CuCl_2$ concentrations (≥ 25 μM). Various publications reported using desalting columns for EDTA removal from LDL solutions, (Halevy et al., 1997; López-Alarcón, Speisky & Lissi, 2007; Seccia, Albano & Bellomo, 1997). Furthermore, G-25 beads fractionation range is > 5000 M_r which is higher than EDTA molar mass (292.24 $g.mol^{-1}$) and this made us initially favour this technique in eliminating EDTA. However, in our own studies we did not find desalting columns useful and turned to dialysis as the method of choice.

Using AAPH as an oxidizing agent to promote our oxidation assay was initially considered a good option especially since AAPH gave promising results with different oxidation conditions. However, there is a paucity of literature using AAPH which made $CuCl_2$ a better choice for LDL oxidation assays and it is a well-established agent in LDL oxidation studies (Esterbauer, Striegl, Puhl & Rotheneder, 1989; Lynch, Lorenz & Klotz, 2014; Morena, Cristol, Dantoine, Carbonneau, Descomps & Canaud, 2000).

Although HDL is known to undergo oxidation by itself, some previous studies have not considered the amount of HDL's own oxidation in combined oxidation assay of HDL and LDL like Brite and his team (Brites, Zago, Verona, Muzzio, Wikinski & Schreier, 2006). The Hillstrom equation is a simple proportion calculation based on two assumptions; first, if HDL had no effect on LDL oxidation, when incubated together, then both lipoproteins would become fully oxidized and the absorbance measured would be equal to the total amount of conjugated dienes formed (absorbance) when LDL and HDL are incubated separately. Second, when both LDL and HDL are incubated together, if the final absorbance is less than LDL alone, then this would be due to HDL action in reducing the amount of lipid oxidation in

LDL. The result of the calculation is therefore by definition is a positive number between 0 and 100%. On the other hand, this is not likely be the case for T1/2max measurements which may not be additive for HDL and LDL. For example, when incubating HDL alone may give a T1/2max of 10 minutes (slow oxidation) and LDL alone may give a T1/2 max of 5 minutes (more rapid oxidation) but when incubated together the T1/2max will not be 15 minutes (the sum of the two) but another value e.g. 7 minutes which is the weighted mean of the two. Therefore, for the percentage of inhibition of HDL to LDL calculated by T1/2max, we invented a new parameter to describe the decrease or delay in oxidation as a result of HDL inhibition.

Our assay, for HDL antioxidant potency on LDL, might be the first to include HDL samples isolated from stored frozen samples (at -80°C) for about 5 years. Those samples are for individuals from different deprivation areas in the city of Glasgow, males, and females from different age bands. For each participant, there is a record of lipid measurements along with carotid intima media thickness which is a surrogate marker of CVD. Furthermore, many details are also available for lifestyle which includes basic demographic data, smoking history, alcohol intake and physical activity level. That information could also be used to interpret the lifestyle factors interfering with HDL oxidation/antioxidant state. We selected stored samples from the freezer in a random fashion, so any one assay run would include people with high or low HDL's oxidation potential or different individuals from most or least deprived areas, so day to day assay variation did not influence interfere any associations seen. Moreover, HDL isolated samples will also be measured for PON1 activity for each of the pSoBid. And because of many measurements had to be conducted on freshly isolated materials, it was important to schedule carefully the sequence of preparations and assay steps during the working day. A typical example is shown in **appendix 7.**

4 HDL Functional Assays

4.1 Introduction

4.1.1 HDL Antioxidative Activity

Over the last 25 years, oxidative modification of low density lipoproteins (LDLs) has emerged as a potentially critical pathway in atherogenesis (Quinn, Parthasarathy, Fong & Steinberg, 1987; Steinberg, Parthasarathy, Carew, Khoo & Witztum, 1989; Steinbrecher, Parthasarathy, Leake, Witztum & Steinberg, 1984). LDL trapped in the arterial intima is oxidized by free radicals generated by surrounding cells, leading to the formation of oxLDL. LDL oxidation is a complex process during which both the protein and the lipids undergo oxidative changes and form complex products which promote inflammatory process and lead to atherosclerotic lesions (Parthasarathy, Raghavamenon, Garelnabi & Santanam, 2010). A less well recognised process is that high density lipoprotein (HDL) is also subject to oxidative modification, and is actually oxidized more rapidly than LDL during in vitro oxidation (Hurtado, Fiol, Gracia & Caldú, 1996; Nakajima et al., 1995; Ohmura et al., 1999). This may be viewed as one of the protective or buffering factors against LDL oxidation. Evidence for this is based on the observations that oxidative changes occur more slowly in LDL-HDL mixtures than LDL alone (Kunitake, Jarvis, Hamilton & Kane, 1992; Mackness, Arrol, Abbott & Durrington, 1993; Mackness, Abbott, Arrol & Durrington, 1993; Ohta, Takata, Horiuchi, Morino & Matsuda, 1989; Parthasarathy, Barnett & Fong, 1990). HDL antioxidant potency could be related to HDL chemical composition; apolipoproteins (especially apo-A1), the content of liposoluble antioxidants (primarily, tocopherols), and the presence of associated enzymes like paraxonase 1 (PON1) enzyme, platelet-activating factor acetylhydrolase (PAF-AH) and LCAT (Kontush & Chapman, 2010). Other physiochemical characteristics of HDL, such as lipid composition, size and density, could also be involved in HDL antioxidant capacity.

4.1.2 Paraxonase Enzyme

Paraxonase1 (PON1) plays a protective role against poisoning by organophosphate derivatives such as insecticide paraxon (Litvinov, Mahini & Garelnabi, 2012) . PON1 has recently emerged as the component of HDL most likely to explain its ability to metabolize lipid peroxides and to protect against their accumulation on LDL (Durrington, Mackness & Mackness, 2001). It has also been postulated as a member of the plasma antioxidant system. Decreased PON1 activity has been associated with atherosclerosis in persons with diabetes mellitus, familial hypercholesterolemia, and renal disease (Mackness, Durrington & Mackness, 2004; Mackness & Mackness, 2004). PON1 can be measured based on its activity by spectrophotometric assays and also can be directly quantified by immunological techniques using specific antibodies (Costa, Cole, Vitalone & Furlong, 2005). The spectrophotometric assays based on the ability of PON1 to hydrolyse substrates are currently more commonly used, most likely due to their low cost and availability (Ceron, Tecles & Tvarijonaviciute, 2014). In addition to PON1 enzyme ability to hydrolyse paraxon, the toxic oxon metabolite of parathion, hydrolyses many other substrates such as other organophosphorous compounds, non-phosphorous arylesters as well as lactones, which have been considered as its primary substrates. Measurement of arylesterase activity relies on the ability of PON1 to hydrolyse phenyl1 acetate into acetic acid and phenol and is not affected by PON1 genotype (Cao, Girard-Globa, Berthezene & Moulin, 1999; La Du, Piko, Eckerson, Vincent-Viry & Siest, 1986).

4.1.3 pSoBid Study

PSoBid is a cross sectional study carried out in 2007 in Glasgow. 666 Participants aged 35-66, equal number of males & females from the most and least deprived areas (Deans et al., 2009). Deprivation was associated with increased carotid plaque score and carotid intima-media thickness (cIMT) which is a surrogate measure of atherosclerosis. Systolic blood pressure (SBP) was associated with cIMT ($p<0.001$). HDL cholesterol had a negative association with cIMT ($p<0.001$) but the association of LDL cholesterol with cIMT was of borderline significance ($p=0.055$).

4.2 Aim

The first aim of this part of the project was to investigate the relationships of the physiochemical properties of HDL with the antioxidant capacity of the lipoprotein in vitro using stored plasma samples from the pSoBid study. The hypothesis was to see, if new factors: HDL oxidation potential and or HDL inhibition to LDL oxidation related to cIMT.

The second aim of this part of the project was to explore if PON1 activity, which is an HDL associated enzyme, is associated with cIMT.

4.3 Participants

308 Lithium- Heparin samples left from pSoBid study were used in this part of the project as explained in **Chapter 2** (**Section 2.2** and **2.3**). The subject's ' demographic details and lipid risk factors used are shown in **Table 4.1**. and subject's risk factors and socioeconomic status are shown in **appendix 2**

4.4 Materials and Method

All materials and methods are explained in **Chapter 2 (Section 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 2.12 and 2.13).**

4.5 Blood Collection for Controls

Blood samples collected for LDL controls is explained in **Chapter 2** (**Section 2.4**) and for and HDL controls for oxidation and for PON1 assays is explained in **Chapter 2** (**Section 2.5** and **2.6.2**). Plasma for plasma controls used in PON1 assay is collected from the same pool which has been used for HDL control (**Section 2.5**).

4.6 HDL Antioxidant Assay

The assay is explained in **Chapter 2** (**Section 2.13**). Results are expressed either as time (minutes) at half maximum (T1/2max) which is an equivalent of lag time; Vmax (milli.U.min^{-1}) or by the maximum amount of conjugated diene produced which is measured by the difference in optical density (max-min OD) (units).

4.7 HDL Paraxonase Activity Assay

The assay is explained in **Chapter 2** (**Section 2.12**). Results are expressed in unit.mL^{-1}, 1 unit of arylesterase hydrolyses 1 mmol of phenyl acetate (substrate) per minute. PON1 measurement was done for plasma samples and HDL of the same samples. Further calculations were made for ratio of plasma PON1 activity to HDL PON1 activity, PON1 for proteins standardized HDL and ratio of plasma PON1 activity to PON1 of protein standardized HDL.

4.8 Calculations and Statistical Analysis

Calculations for percentages of HDL protection to LDL oxidation were done as explained in **Chapter 3** (**Section 3.6**) and for PON1 activity as explained in **Chapter 3** (**Section 3.7**). Statistical analyses were performed using IBM SPSS Statistics Data Editor (version 22). Normality was checked for all the data using the Shapiro-Wilk test. Data were log-transformed when they did not approximate normality. Specifically, TGs, very low density lipoprotein cholesterol (VLDL-C) and %Inhibition of LDL Oxidation by HDL (max-min). For genders and areas, independent sample two-tailed T Test was used to compare means of different groups using data on a normal scale, or log transformed, depending on normality. One-Way ANOVA was used to compare variable means between age groups. Association between variables were determined using Pearson correlation. Significance was accepted at $P < 0.05$ level. Data are presented as means (±standard deviation) unless otherwise stated.

Table 4.1 Subjects' demographic details and lipid risk factors used in present chapter

Variable	Total Cohort (n=308)	Gender		Area		Age band			P Value		
		Male n=146	Female n=161	L.D n=167	M.D n=140	35-44 n=80	45-54 n=111	55-64 n=116	Gender	Area	Age
Cholesterol ($mmol.L^{-1}$)	5.08 (1.03)	4.93 (1.01)	5.22 (1.03)	5.21 (1.00)	4.93 (1.05)	4.94 (0.85)	5.09 (0.96)	5.18 (1.20)	0.012	0.017	0.301
LDL-C ($mmol.L^{-1}$)	3.01 (0.87)	2.95 (0.86)	3.06 (0.87)	3.14 (0.84)	2.85 (0.87)	2.94 (0.72)	3.02 (0.81)	3.04 (1.00)	0.288	0.004	0.739
TGs ($mmol.L^{-1}$)	1.25 (0.95-1.70)	1.30 (1.00-1.71)	1.15 (0.88-1.73)	1.15 (0.85-1.45)	1.45 (1.00-2.14)	1.05 (0.75-1.59)	1.30 (0.95-1.80)	1.30 (1.00-1.89)	0.108	<0.001	0.002
VLDL-C ($mmol.L^{-1}$)	0.65 (0.5-0.85)	0.65 (0.54-0.90)	0.65 (0.45-0.85)	0.60 (0.45-0.80)	7.00 (0.55-1.03)	0.60 (0.40-0.75)	0.65 (0.55-0.90)	0.70 (0.50-0.90)	0.403	0.001	0.207
HDL-C ($mmol.L^{-1}$)	1.35 (0.37)	1.22 (0.32)	1.47 (0.37)	1.43 (0.36)	1.26 (0.36)	1.35 (0.37)	1.32 (0.30)	1.39 (0.42)	<0.001	<0.001	0.418
ApoA-I ($g.L^{-1}$)	1.41 (0.28)	1.33 (0.27)	1.49 (0.27)	1.45 (0.27)	1.37 (0.28)	1.39 (0.29)	1.39 (0.26)	1.45 (0.29)	<0.001	0.012	0.197
Apo-B ($g.L^{-1}$)	0.91 (0.23)	0.92 (0.22)	0.91 (0.23)	0.92 (0.22)	0.91 (0.23)	0.88 (0.21)	0.93 (0.21)	0.93 (0.24)	0.721	0.508	0.237
ApoB/ApoA-I (mol:mol)	0.67 (0.20)	0.71 (0.21)	0.63 (0.18)	0.65 (0.19)	0.68 (0.22)	0.65 (0.20)	0.68 (0.19)	0.66 (0.21)	<0.001	0.239	0.527
cIMT- (mm)	0.70 (0.15)	0.72 (0.17)	0.68 (0.13)	0.68 (0.12)	0.72 (0.17)	0.62 (0.09)	0.70 (0.13)	0.76 (0.17)	0.047	0.023	<0.001

Descriptive statistics are presented as mean (standard deviation) for normally distributed variables and median (inter-quartile range) for not normal distributed variables. LDL-C, low density lipoprotein cholesterol; TGs, Triglyceride; VLDL-C, very low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; Apo-A, Apolipoprotein A-I; ApoB, apolipoprotein B; cIMT, Carotid intima media thickness; mm, millimetre; L.D, least deprived; M.D, most deprived

4.9 Results

4.9.1 HDL Oxidation and % Inhibition of LDL Oxidation by HDL

HDL samples underwent oxidation with a wide range of responses in the oxidation assay. The mean T1/2max was 82.4 minutes and the range was 58 to 122 minutes. Likewise, mean for Vmax was 1.43 milli U.min^{-1} with a range from 0.42 to 3.66 milli.U.min^{-1} and the mean of maximum diene produced was 0.15 units and varied from 0.07 to 0.21 units (**Figure 4.1)**. From **Table 4.2**, it can be clearly seen that the extent of HDL oxidation varied by sex and by deprivation area but not by age. Females had generally higher HDL oxidation potential (measured by the three factors: T1/2max , maximum propagation rate and maximum conjugated dienes produced) than males (P≤ 0.02) and those who lived in more affluent areas, had higher HDL oxidation potential than those who lived in deprived areas (P≤ 0.015) for the three oxidation factors.

From **Figure 4.2,** adding HDL of pSoBid participants to the LDL oxidation assay inhibited LDL oxidation, although for a few HDL samples an increase in LDL oxidation was noted measured by T1/2max, most of the time. The T1/2max was extended by an average of 25.95% with a range of -11.47 to 126.70% (**Table 4.2 and Figure 4.2-A**). Maximum propagation rate was inhibited by an average of 39.5% with a range of 0.00 to 95.53% (**Table 4.2 and Figure 4.2-B**)and the maximum amount of conjugated diene produced was reduced by 11.97% with a range of 0.00 to 59.0% (**Table 4.2 and Figure 4.2-C**). In **Table 4.2**, it can be seen that the percentage of inhibition as measured by the increase in T1/2max differed significantly by the two genders as it was higher in males than in females (95% CI, 2.30 to 15.0; P= 0.008) and between the two areas as it was higher in most deprived than least deprived areas (95% CI, 16.05 to 3.34; P= 0.003) but the other two measures of LDL inhibition, showed no significant difference by gender (P= 0.366) when measured by maximum propagation rate and (P= 0.454) when calculated by the maximum amount of conjugated diene produced. Furthermore, HDL inhibition was not different between the two deprivation areas when measured by maximum propagation rate (P= 0.506) or maximum dienes produced (P= 0.932).

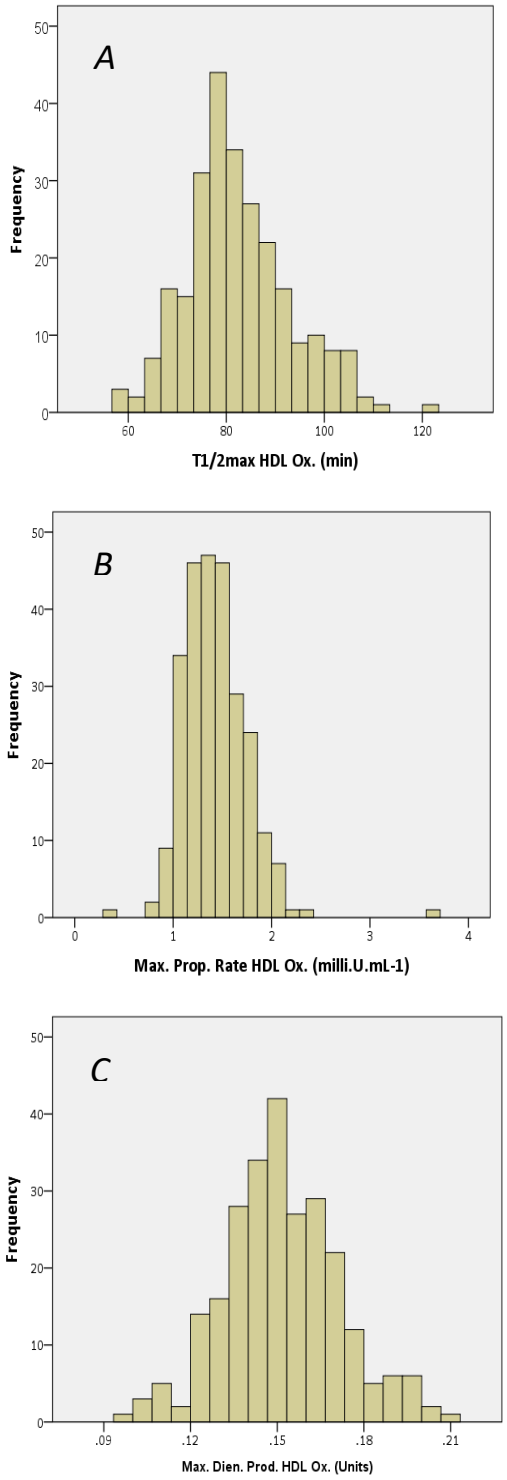


Figure 4.1 Oxidation results for pSoBid HDLs measured by: time at half maximum (minutes) (A); Maximum Propagation rate (milli U.min^{-1}) (B); maximum diene produced (units) (C). T1/2max, Time at half maximum; Max. Prop. Rate HDL Ox. , maximum propagation rate of HDL oxidation; Max. Diene Prod. HDL Ox., maximum conjugated diene produced. milli. U. min^{-1}, milli units. minutes $^{-1}$

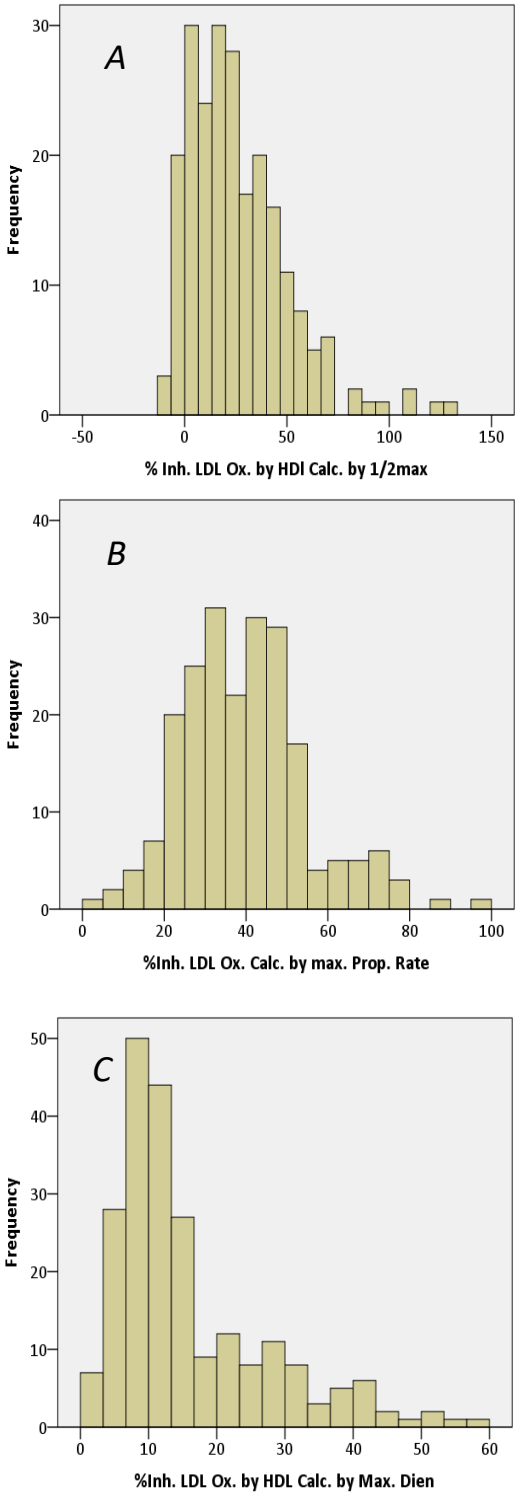


Figure 4.2 Percentage of inhibition of LDL oxidation revealed by pSoBid HDLs on one LDL control measured by: T1/2max (A) ; maximum propagation rate (B); maximum conjugated diene produced (C). T1/2max, time at half maximum; Inh. LDL Ox. By HDL Calc. by T1/2max, Inhibition of LDL oxidation by HDL calculated by T1/2max; Inh. LDL Ox. By HDL calc. by Max. Prop. Rate, Inhibition of LDL oxidation by HDL calculated by maximum propagation rate; Inh. LDL Ox. By HDL Calc. by Max. Diene, Inhibition of LDL oxidation by HDL calculated by maximum

Table 4.2 Oxidation results. Total cohort was little different for some factors; n= 257 for T1/2max HDL oxidation; n= 258 HDL oxidation (Max-Min OD), n=227 for %inhibition by HDL (T1/2max); n=213 %inhibition of oxidation by HDL (Vmax); n=226 %inhibition of oxidation by HDL (max-min).

Variable		Total Cohort (n=259)	Gender		Area		Age band			P Value	
		Mean or Median	Male n=123	Female n=136	L.D n=144	M.D n=115	35-44 n=63	45-54 n=95	55-64 n=101	Sex	Area	Age
HDL Oxidation	(T1/2max) (min)	82.44 (10.71)	84.03 (11.07)	80.98 (10.19)	81.00 (10.33)	84.24 (10.95)	80.91 (9.84)	83.57 (10.28)	82.35 (11.55)	0.021	0.015	0.317
	*Max. Prop. R. (milli.U.min^{-1})	1.43 (0.32)	1.34 (0.32)	1.51 (0.30)	1.49 (0.33)	1.35 (0.28)	1.47 (0.29)	1.42 (0.35)	1.41 (0.30)	<0.001	<0.001	0.442
	Max. Diene Prod. (Units)	0.151 (0.02)	0.144 (0.02)	0.157 (0.02)	0.156 (0.02)	0.144 (0.02)	0.152 (0.02)	0.15 (0.02)	0.15 (0.02)	<0.001	<0.001	0.917
% Inhibition of LDL Oxidation by HDL	T1/2max	25.95 (24.40)	30.41 (27.10)	21.90 (21.02)	21.78 (20.30)	31.34 (28.06)	23.00 (20.89)	28.78 (26.19)	25.08 (24.62)	0.008	0.003	0.370
	Max. Prop. R.	39.50 (15.08)	38.43 (13.91)	40.44 (16.04)	38.95 (15.60)	40.19 (14.43)	39.42 (15.69)	41.01 (14.46)	38.11 (15.32)	0.366	0.506	0.508
	Max. Diene Prod.	11.97 (8.51-21.29)	12.36 (9.00-21.87)	10.90 (8.10-20.72)	11.79 (8.23-21.87)	12.09 (8.51-20.52)	11.50 (7.58-28.50)	11.97 (8.86-21.75)	12.16 (8.13-18.09)	0.454	0.923	0.949

Descriptive statistics are presented as mean (standard deviation) for normal median (inter-quartile range) for not normal distributed variables. L.D, least deprived; M.D, most deprived; T1/2max, Time at half maximum; Max. Prop. R. Maximum propagation rate ; Max. Diene Prod., maximum conjugated dienes produced.

4.9.2 Relationship between HDL Oxidation and Carotid Intima Media Thickness

In the pSoBid population, cIMT varied from 0.4-1.5 mm. **Figure 4.3** relates IMT in the common carotid artery to the parameters of HDL oxidation. No evidence of a linear correlation was observed with HDL measured by T1/2max but a significant inverse relationship was seen between cIMT and maximum propagation rate of HDL oxidation (r= -0.15, P= 0.021). A trend of 0.071 mm reduction in cIMT was revealed for each increase of 1 milli.U.min-1 (95% CI, - 0.013 to - 0.01) in propagation rate of HDL oxidation. Maximum amount of conjugated diene produced for HDL oxidation also showed a correlation of r = 0.12 but this was only borderline of significance (P= 0.06). On the other hand, the mean intermediate thickness in the common carotid artery showed no evidence of a linear correlation to any of the parameters of %inhibition of LDL oxidation by HDL calculated by any of the three factors: (r = 0.08, P= 0.242) by T1/2max, (r = -0.056, P= 0.431) calculated by maximum propagation rate and (r = -0.061, P= 0.379) by maximum conjugated diene produced (**Figure 4.4 –A, B and C)**.

4.9.3 Relationship between HDL Oxidation and % Inhibition of LDL Oxidation by HDL

As seen in **Figure 4.5**, HDL oxidation potential measured by T1/2max was significantly correlated with its inhibition of LDL oxidation (r = 0.43, P< 0.001). There was a trend of 0.981% of increase of % inhibition of LDL oxidation by HDL for each 1% increase of HDL oxidation potential (95% CI, 0.71 to 1.26) calculated by T1/2max. On the other hand, no evidence of a linear correlation was revealed between HDL oxidation potential and % inhibition of LDL oxidation by HDL measured by the other two factors, maximum propagation rate or maximum conjugated dienes produced (r = 0.034, P= 0.62) and (r = -0.21, P= 0.75) respectively.

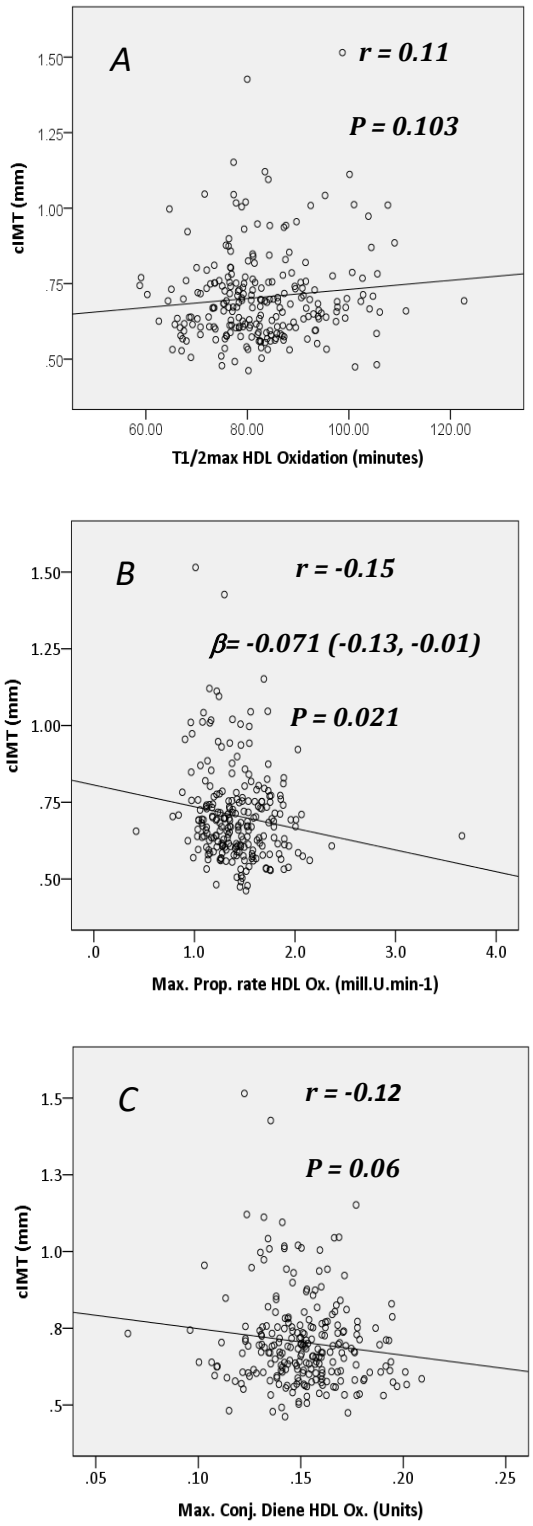


Figure 4.3 Scatter plot (with linear regression line of best-fit) illustrating the relationship between cIMT (mm) of pSoBid participants versus HDL oxidation results calculated by : T1/2max, Time at half maximum (A); Max. Prop. Rate HDL Ox. (milli. $U.min^{-1}$), Maximum conjugated diene produced (milli $Units.mL^{-1}$) (B); Max. Conj. Diene. , Maximum Conjugated Diene produced (C), cIMT, carotid intima media thickness; mm, millimetre.

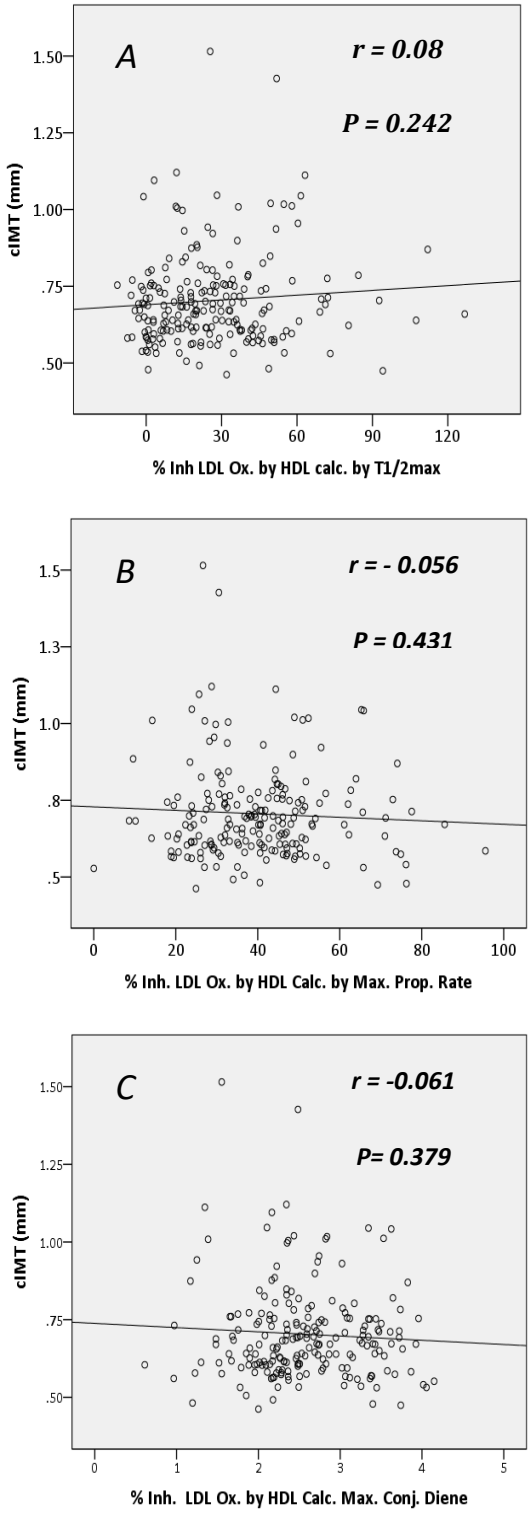


Figure 4.4 Scatter plot (with linear regression line of best-fit) illustrating the relationship between % of inhibition of HDL to LDL oxidation results versus cIMT-ccmean of pSoBid participants calculated by T1/2max (A); by Vmax (B); by Max. Dienes formed (C). Inh. LDL Ox. By HDL Calc. , Inhibition of LDL oxidation by HDL calculated ;T1/2max, time at half maximum; Max. prop. Rate, maximum propagation rate; Max. Conj. Diene, maximum conjugated dienes formed; cIMT, carotid intima media thickness; mm, millimetre.

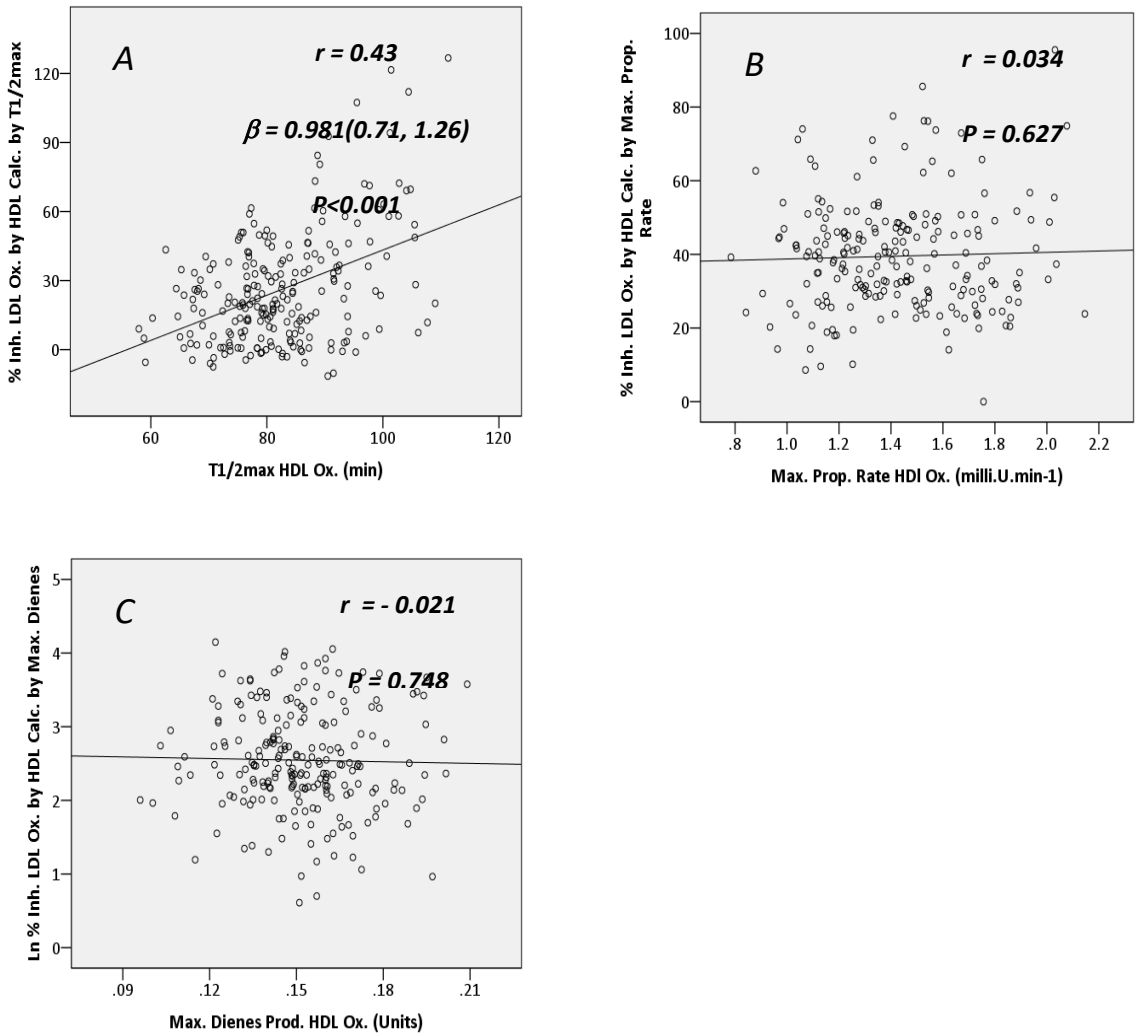


Figure 4.5 Scatter plot (with linear regression line of best-fit) illustrating the relationship between HDL oxidation versus % inhibition of LDL oxidation by HDL of pSoBid individuals calculated by T1/2max (A), Maximum propagation rate (B); Maximum conjugated diene produced (C). Inh. LDL Ox. by HDL Calc. by T1/2max, Inhibition of LDL oxidation by HLD calculated by time at half maximum; Max. Prop. Rate, maximum propagation rate, Max. Dienes Prod., maximum production of conjugated dienes produced.

4.9.4 Paraxonase1 (PON1) Assay

As shown from **Figure 4.6,** pSoBid samples showed a range of PON1 activity from 31-196 units.mL^{-1} with a mean of 94.6 units.mL^{-1}. On the other hand, HDL fractions from the same plasma samples showed a mean of 30.1 units.mL^{-1} with a range of 7.8-73.1 units.mL^{-1}. Standardized HDL (to protein) PON1 activity showed a mean of 29.6 units.mL^{-1} and ranged from 12.9 to 57.9 units.mL^{-1}. Ratio of plasma PON1 activity to HDL PON1 activity ranged from 1.6 to 6.5 with a mean of 3.3. Ratio of plasma PON1 activity to PON1 activity of HDL standardized to protein ranged from 1.6 to 6.2 with a mean of 3.29.

Table 4.3, shows that there was a significant difference between gender groups in HDL PON1 activity as it was higher in females than in males (95% CI, -5.7 to -1.1; P= 0.003). On the hand, plasma PON1/HDL PON1 activity was significantly higher in males than in females (95% CI, 0.12 to 0.43; P<0.001). Those same parameters did not show any significant difference between most deprived and affluent areas or between age bands. Other factors of PON1 activity did not reveal any significant difference between genders, area levels or age groups: plasma PON1 (P= 0.196), (P= 0.08) and (P= 0.913) respectively, HDL standardized to protein PON1 activity (P= 0.980), (P= 0.072) and (P= 0.644) respectively and plasma PON1/ HDL standardized to protein PON1 activity ratio (P= 0.185), (P= 0.654) and (P= 0.357) respectively.

4.9.5 Relationship between PON1 Activity and cIMT

As seen from **Figure 4.7**, PON1 activity was not associated with cIMT; nor plasma PON1 (r = -0.08, P= 0.16), HDL PON1 (r = -0.07, P= 0.22), plasma PON1/HDL PON1 ratio (r = 0.02, P= 0.68) or PON1 for standardized HDL (r = 0.01, P= 0.86). Only the ratio of plasma PON1/ Standardized HDL PON1 was borderline negatively correlated (r= - 0.12, P= 0.06).

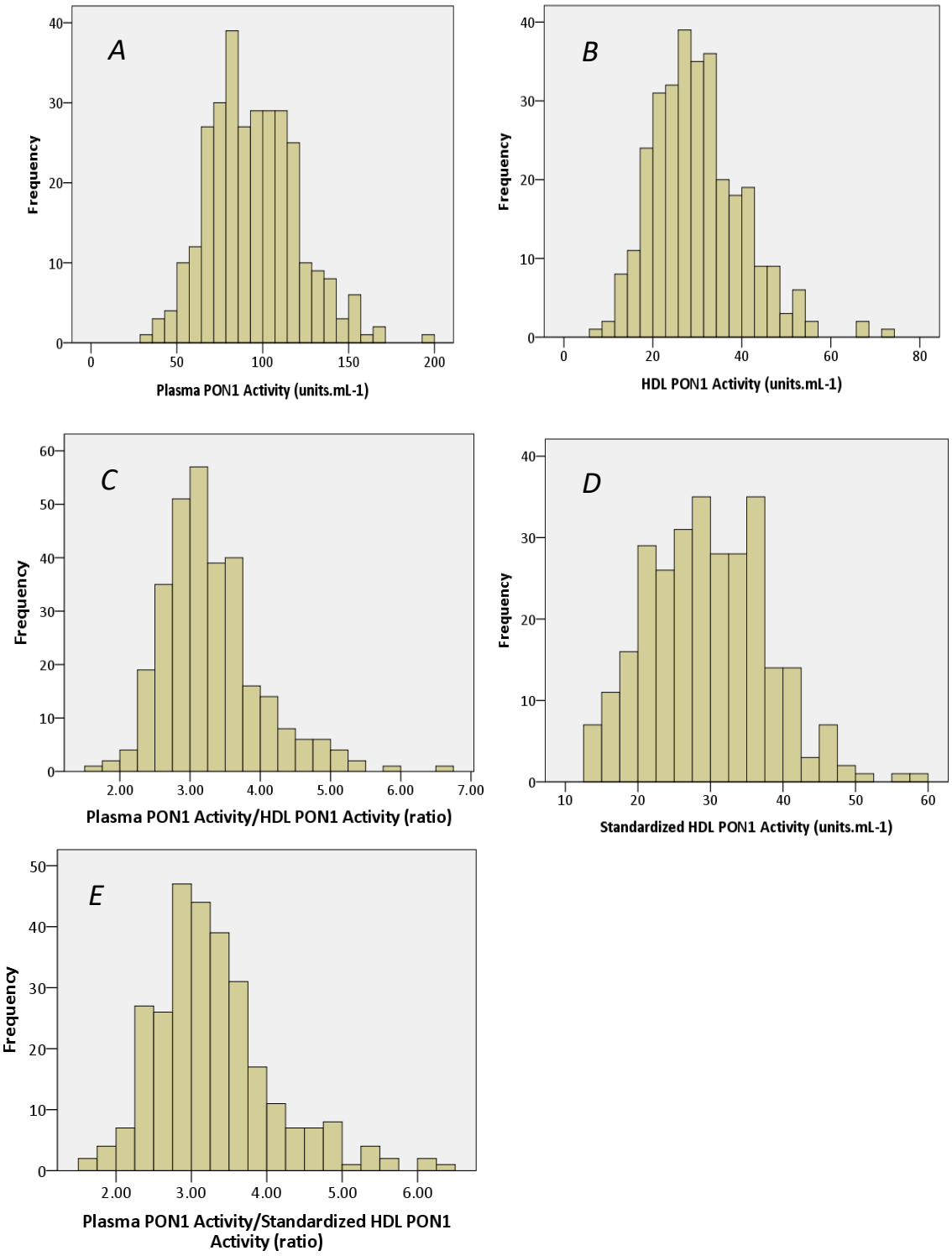


Figure 4.6 PON1 assay results (units.mL^{-1}) for pSoBid samples: plasma PON1 (A); HDLs PON1 (B); Plasma PON1/ HDLs PON1 (C); protein standardized HDLs PON1 (D); plasma PON1/Standardized HDL PON1 (E). PON1, paraxonase1 enzyme.

Table 4.3 Paraxonase results. Total cohort was little different for some factors; n= 305 for plasma Pon1 activity; n= 306 plasma/HDL pon1 activity (units), n=289 for HDL pon1 standardized to protein (units); n=287 for plasma PON1/standardised HDL PON1 activity (units).

Variable	Total Cohort (n=308)	Gender		Area		Age band			P Value		
	Mean or Median	Male n=146	Female n=162	L.D n=168	M.D n=140	35-44 n=80	45-54 n=111	55-64 n=117	Gender	Area	Age band
Plasma PON1 activity (unit.mL^{-1})	94.64 (25.85)	92.63 (24.70)	96.47 (26.80)	97.03 (26.77)	91.83 (24.51)	95.71 (27.75)	94.30 (24.32)	94.24 (26.13)	0.196	0.08	0.913
***HDL PON1 activity (unit.mL^{-1})**	30.08 (10.21)	28.29 (10.02)	31.69 (10.14)	30.90 (10.27)	29.09 (10.09)	29.95 (10.83)	29.42 (9.67)	30.79 (10.32)	0.003	0.120	0.596
Plasma PON1/HDL PON1 Activity	3.29 (0.69)	3.43 (0.71)	3.16 (0.65)	3.28 (0.71)	3.29 (0.68)	3.33 (0.62)	3.34 (0.73)	3.21 (0.7)	<0.001	0.890	0.276
HDL standardized to protein PON1 (units.mL^{-1})	29.55 (8.21)	29.54 (8.43)	29.56 (8.03)	30.34 (8.83)	28.60 (7.31)	29.42 (8.60)	29.07 (7.95)	30.10 (8.23)	0.980	0.072	0.644
Plasma PON1/Stand. HDL PON1 activity (ratio)	3.29 (0.80)	3.23 (0.77)	3.35 (0.82)	3.31 (0.83)	3.27 (0.76)	3.35 (0.80)	3.34 (0.81)	3.21 (0.78)	0.185	0.654	0.357

Descriptive statistics are presented as mean (standard deviation) for normal distributed variables and median (inter-quartile range) for not normal distributed variables.PON1, paraxonase1 enzyme; L.D, least deprived; M.D, most deprived; Stand. , standardized.

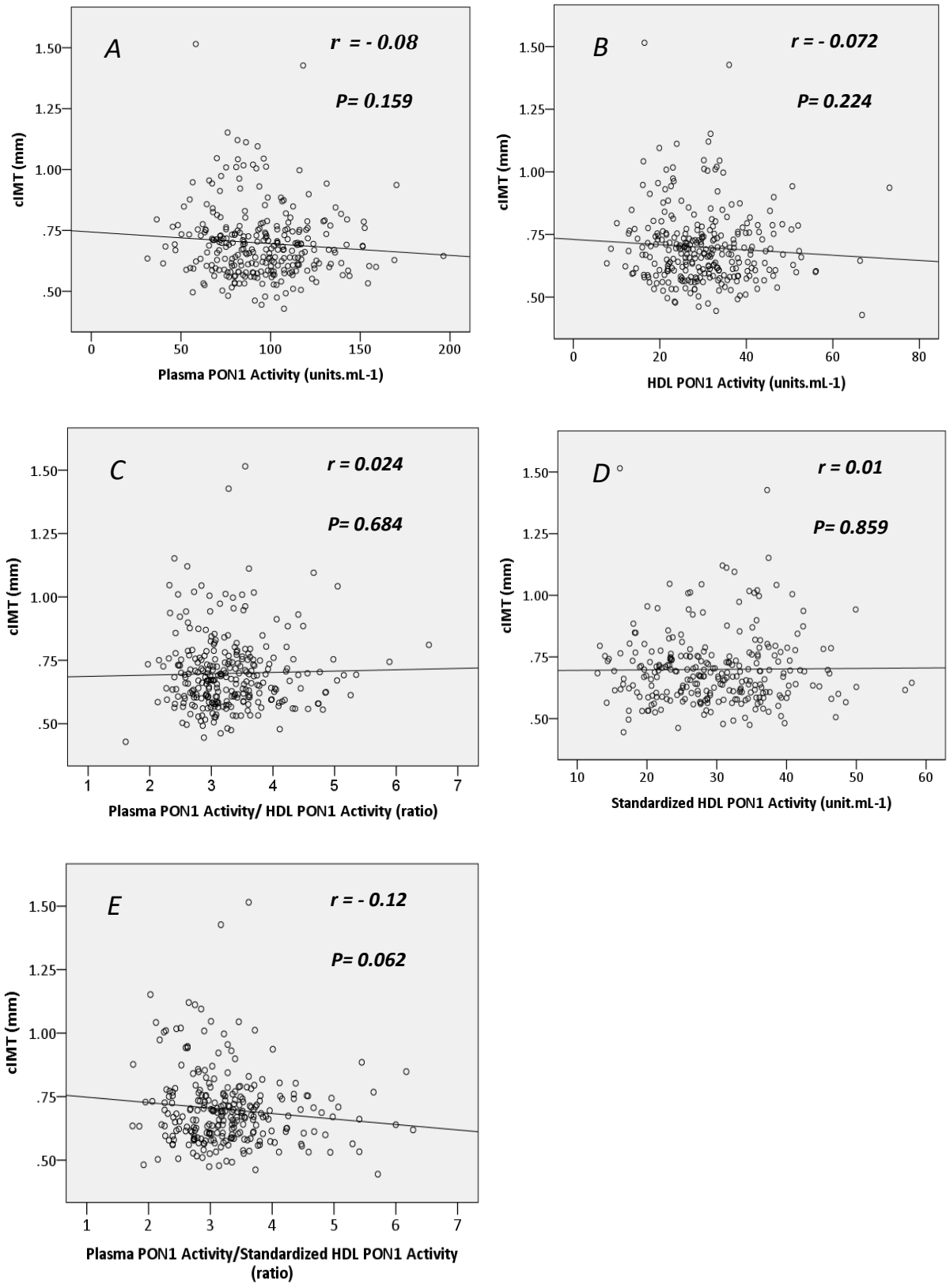


Figure 4.7 Scatter plot (with linear regression line of best-fit) illustrating the relationship between PON1 activities versus cIMT (mm) of pSoBid samples; PON1 plasma activity (A); PON1 HDL activity (B); plasma PON1 activity/ HDL PON1 activity (C); standardized HDL PON1 activity (D); Plasma PON1 activity/standardized HDLPON1 activity (E). cIMT, carotid intima media thickness; (mm), millimetre; PON1, paraxonase enzyme1.

4.9.6 Inter Correlation of HDL Oxidation and HDL Inhibition to LDL Oxidation with PON1 Assay Results

Although HDL PON1 activity did not display a significant correlation with HDL oxidation potential as measured by T1/2max (r = - 0.01, P= 0.830), a significant positive correlation was revealed between HDL PON1 activity and HDL oxidation measured by maximum propagation rate (r = 0.15, P= 0.016) **(Figure 4.8-B).** There was a trend of increase of 0.005 milli.U.min^{-1} in maximum propagation rate of HDL oxidation for each 1 unit.min-1 increase of HDL PON1 activity (95% CI, 0.001 to 0.01). HDL PON1 activity was also significantly correlated with HDL oxidation potential measured by maximum conjugated dienes produced (r = 0.23, P<0.001) **(Figure 4.8-C).** Conversely, HDL PON1 activity did not show any correlations with any other measurement factors for HDL inhibition to LDL oxidation measured by the three factors; r = 0.06, P= 0.370 for T1/2max, r = - 0.01, P= 0.903 for maximum propagation rate and r = 0.02, P= 0.77 for maximum conjugated dienes produced **(Figure 4.9-A, B, C)**.

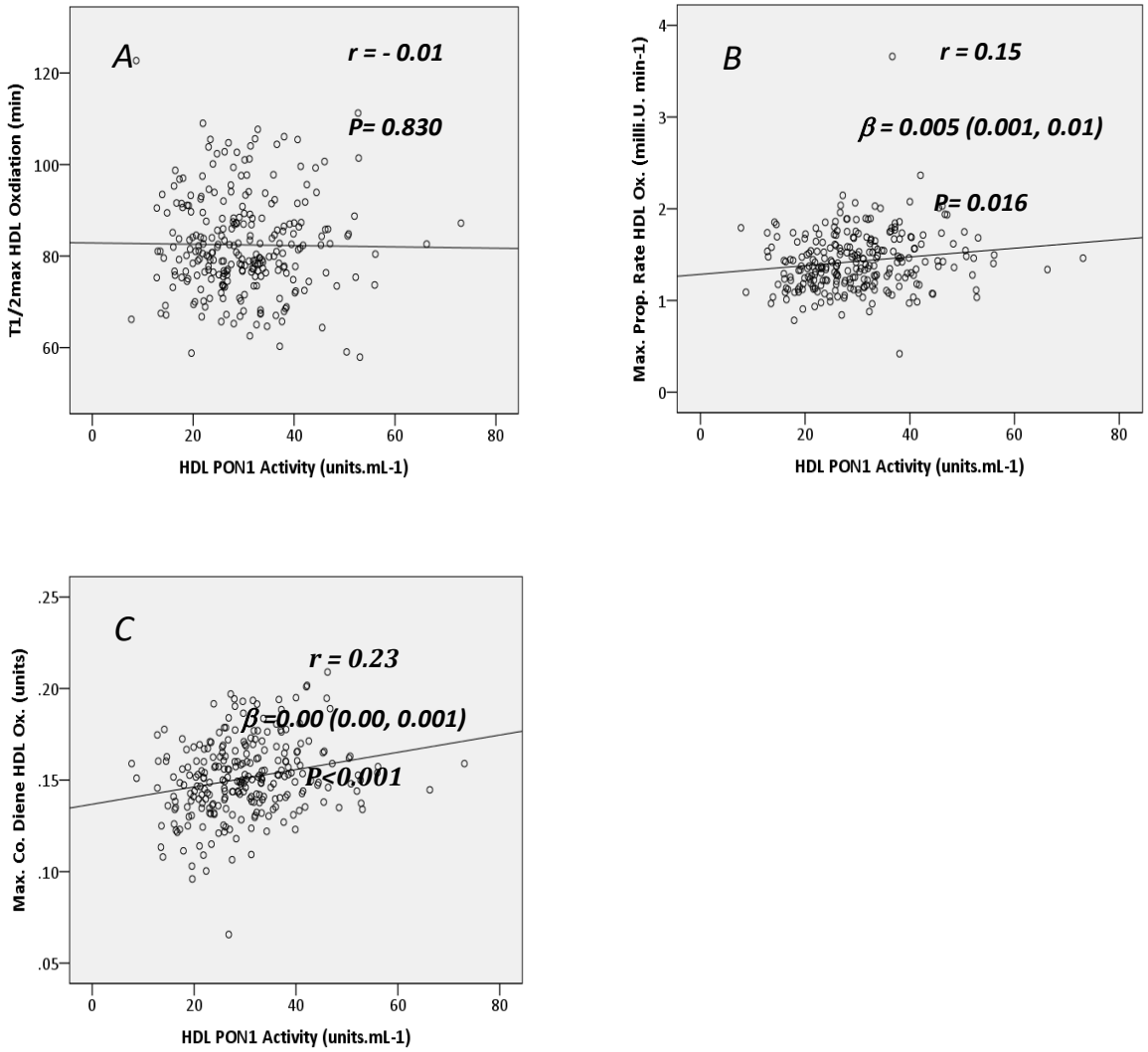


Figure 4.8 Scatter plot (with linear regression line of best-fit) illustrating the relationship between HDL PON1 activities versus HDL oxidation factors; T1/2max HDL oxidation (A); Max. Prop. rate HDL oxidation(B); Max. co. Diene HDL Ox. (C). PON1, paraxonase1 enzyme; T1/2max, time at half maximum; Max. prop. Rate HDL ox., maximum propagation rate of HDL oxidation; Max. Co. Diene HDL Ox., maximum conjugated dienes produced for HDL oxidation ; min, minutes.

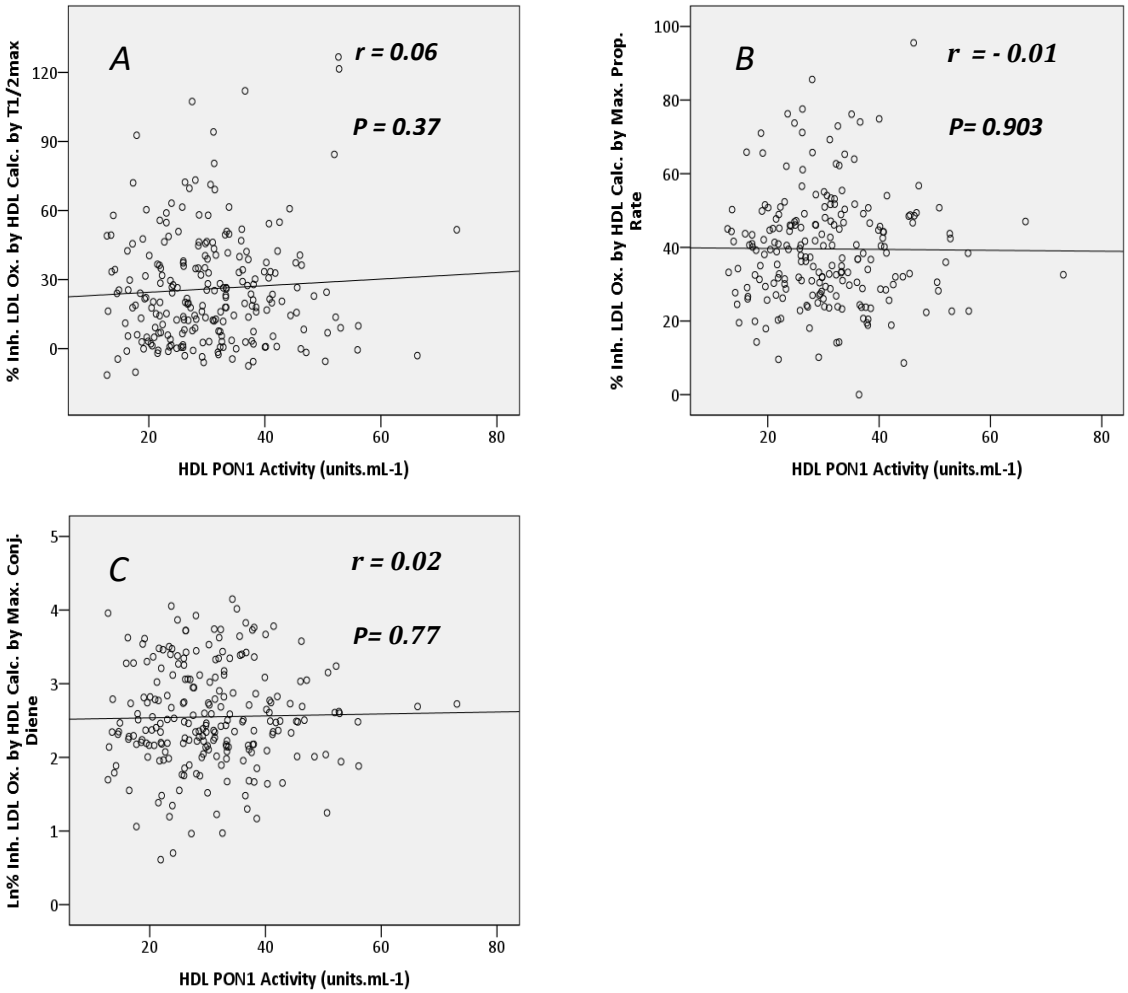


Figure 4.9 Scatter plot (with linear regression line of best-fit) illustrating the relationship between HDL PON1 activities versus % inhibition of HDL to LDL oxidation measured by the 3 factors;T1/2max (A); Vmax (B); Max-Min OD, (C)**.** T1/2max, Time at half maximum; Vmax, Maximum velocity; Max-Min OD (Maximum-Minimum optical density).

4.10 Discussion

In this chapter, it was found that HDL oxidation potential varied over a wide range. It was also found to be higher in females than in males as well as higher in affluent areas compared to more deprived areas. This appears to suggest that in subjects where propensity for atherogenesis is decreased, the susceptibility of HDL to undergo oxidation in the in vitro assay is higher. The three oxidation parameters; T1/2max, maximum propagation rate and maximum conjugated dienes formed indicate that in females and subjects from affluent areas, HDL was not only more extensively oxidized but was more rapidly oxidized as well. This could be because of difference in HDL size and subclasses, or composition especially in the amount of oxidizable lipids that the HDL carries. For example, if females had more polyunsaturated fatty acids in their HDL than in men, this will lead to a greater oxidation. The link between HDL potency for oxidation with higher economic status could be accounted for by a difference in diet. PSoBid study showed that participants from more affluent areas consumed fatty acids and vitamins which are known to increase HDL-C which could also increase their HDL susceptibility for oxidation.

It was of interest to see in **Figure 4.3-B** that the velocity of HDL oxidation was inversely associated with cIMT. This observation again indicates that subjects with less risk atherosclerosis have an HDL that is more susceptible to oxidation. One potential explanation for this association is that in individuals, in whom HDL in the blood is more easily oxidized, there is less oxidation attack on LDL.

In this chapter, the relationship between pSoBid individuals' cIMT and the extent to which their HDL protected LDL against oxidation was also explored. Our hypothesis was that in subjects where HDL had a strong protection to LDL oxidation there will protect against atherosclerosis. That is, we expected to see a negative association of cIMT with the percentage of inhibition of LDL oxidation. However, this was not observed as **Figure 4.4** showed no association between any of the three factors of inhibition of LDL oxidation by HDL and cIMT calculated by T1/2max ($r = 0.08$; $P = 0.24$), by Vmax ($r = -0.056$; $P = 0.43$) or by difference in optical density ($r = -0.06$; $P = 0.379$). This lack of association may have been because in the assay, the amount of HDL added was determined by the HDL-cholesterol content and possibly the number of HDL particle would have been a better way of standardizing the assay. However, measuring HDL particle counts was not undertaken in the

present investigation. Overall, if there are no technical issues, then our findings indicate that the antioxidative potency of HDL to protect LDL form oxidation is not a predictor of extent of atherosclerosis. In the literature, there are mixed reports of the extent to which LDL oxidation itself is related to atherosclerosis (Toshima et al., 2000; Verhoye, Langlois & Investigators, 2009).

Investigating whether HDL oxidation potency was related to its antioxidant potency to protect LDL from oxidation **(Figure 4.5)** revealed that HDL T1/2 max was asscoiated with the antioxidative effect of this lipoprotein on LDL oxidation. There was a trend of 0.98 rise in % inhibition of LDL oxidation for each 1 minute increase in T1/2max of HDL oxidation ($P<0.001$). But this was not confirmed by the other two parameters, Vmax ($r= 0.03$, $P= 0.63$) and maximum optical density ($r= -0.21$, $P= 0.75$) and may be a chance finding.

It was noted that for T1/2max of LDL oxidation in the presence of HDL, a number of the values were negative (23 values out of 227 subjects examined). This implies that the addition of HDL from these subjects slightly accelerated LDL oxidation. This is a theoretical possibility since these HDL may have carried components that cause LDL to become more susceptible to copper mediated oxidation. We did not have time to examine this observation further.

Table 4.3 showed that HDL PON1 activity is significantly higher in females than in males ($P<0.003$) which is consistent with the total amount of HDL-C present. This could be related to one of the antiatherogenic functions of HDL. On the contrary, plasma PON1/ HDL PON1 ratio displayed a significant difference between the two genders with higher mean for males than females. Two reasons could explain this finding. Firstly, plasma PON1 could be higher in males than in females compared to HDL PON1. Secondly, spinning the samples in ultracentrifugation could split PON1 enzyme from the HDL molecules (Cheung & Wolf, 1988; Kunitake, Jarvis, Hamilton & Kane, 1992) as our samples were spun at 27400 g for 2.5 hours followed by another spin of 13800 g for 18 hours. Accepting the second reason will influence the first finding that HDL PON1 is higher in females compared to males. Other expressions of PON1 results did not show any significant difference between the two genders, area deprivation states or age groups which was not expected.

PON1 results (**Figure 4.7**) revealed no significant correlations between measured plasma PON1 or HDL PON1 with cIMT ($r= -0.08$, $P= 0.16$) and ($r= -0.07$, $P= 0.22$) respectively. Other

expressions of PON1 results , ratio of plasma PON1 to HDL PON1, standardized HDL PON1 or ratio of plasma PON1 to standardised HDL PON1, did not show any significant results with cIMT either (r= 0.02, P= 0.68), (r= 0.01, P= 0.86) and (r= -0.12, P =0.06) respectively.

Regarding the relationship between HDL PON1 activity and HDL oxidation (**Figure 4.8**), there was a positive correlation for HDL PON1 activity and the amount of oxidised HDL measured by Vmax (r= 0.15, P= 0.02) as well as with HDL oxidation measured in maximum dienes produced (r= 0.23, P<0.001). This finding confirms that HDL could be protective to LDL oxidation by its content of PON1 enzyme as well as of being more susceptible itself to oxidation. This finding, on the other hand, was not confirmed with HDL T1/2max as it was not found to be significantly correlated to HDL PON1 activity (r= -0.01, P= 0.83)

The outcome displayed in **Figure 4.9** indicated no significant correlation of HDL PON1 with any of the % protection of HDL to LDL oxidation, measured by the 3 factors; T1/2max, Vmax or maximum dienes formed. This was not what was predicted since PON1 was expected to protect LDL from oxidation.

Multivariate analysis was not undertaken in this exploratory analysis since many of the possible confounders were interrelated and interpretation would be difficult.

In summary, this study demonstrated that, HDL oxidation potency could be a better marker for HDL functionality in relation to atherosclerosis than its capacity to protect LDL from oxidation. For PON1 results, it was not found to be related to cIMT. Finally, PON1 does not appear to protect HDL from oxidation. The findings on HDL oxidation potential will be further investigated in relation with HDL structure and subclasses in **Chapter 5** and to explore if it was affected by lifestyle habits in **Chapter 6.**

5 HDL Subclasses and Preβ1-HDL in the pSoBid Cohort

5.1 Introduction

5.1.1 HDL Subclasses

Although the association of HDL with CVD risk is well established, and interpretation of levels have been formally integrated into UK public health guidelines for CVD prevention (NCEP, 2001). ; (NICE guidelines, 2014), there are also reports that the plasma HDL-C level does not always reflect the extent of its anti-atherosclerotic influence, and this issue remains the subject of debate (von Eckardstein & Assmann, 2000). In recent years, Mendelian randomization studies, which are supposed to be valuable observational studies for inferring causal pathways, have cast doubt on the casual association between HDL-C and MI (Voight et al., 2012). Currently, clinical assessment of HDL focuses exclusively on total HDL-cholesterol, i.e., the combined cholesterol content of all HDL particles

(NCEP, 2001). However, HDL particles are not homogenous but rather consist of multiple subclasses that differ by density, migration characteristics on electrophoresis, apolipoprotein content, and perhaps by their relationship to disease. Consequently, it is essential to consider that HDL subpopulations may not all have the same role in preventing cardiovascular disease. It has been assumed that the various physiological functions of HDL are a consequence of heterogeneity of HDL complexes. For this reason, it is important to consider whether different HDL characteristics might mediate propensity for cardiovascular protection in different population groups.

In the present work, a well-established, reproducible non-denaturing method for determination of HDL subspecies was used. This technique, gradient gel electrophoresis (GGE), separates HDL particles on the basis of their differing size and the results are usually described as relative percent distribution.

5.1.2 Preβeta1-HDL

Cholesterol efflux from peripheral tissues is an important function of HDL particles in which cholesterol in peripheral tissues is transferred via the plasma compartment to the liver, where it is either reprocessed back into plasma as a constituent of newly formed lipoproteins or it is excreted from the body via bile. Cholesterol efflux capacity is inversely associated with common atherosclerotic cardiovascular diseases (Khera et al., 2011; Li et al., 2013).

Preβ1-HDL is a lipid poor discoid-shaped HDL of approximately 67 kDa mass which contains apoA-1, PLs, and unesterified cholesterol (Miyazaki et al., 2009). Preβ1-HDL was not documented until 1985 (Kunitake, La Sala & Kane, 1985). Because of its high modal density, it was not included in HDL recovered from serum by ultracentrifugation in the conventional density interval of 1.063-1.21 g/ml. Furthermore, its rapid conversion to a larger alpha HDL species, ex vivo, by LCAT hindered its recognition by other techniques (O'Connor et al., 1998). Preβ1-HDL particles migrate to the Preβ position on agarose gel electrophoresis. They are believed to be one of the primary acceptors of cholesterol efflux from macrophages mediated by several transporters mainly adenosine triphosphate-binding cassette transporter A1 (ABCA1) (de la Llera-Moya, Drazul-Schrader, Asztalos, Cuchel, Rader & Rothblat, 2010; Khera et al., 2011; Tall, Yvan-Charvet, Terasaka, Pagler & Wang, 2008). Thus, preβ1-HDL is proposed to be a key component of reverse cholesterol transport, and high circulating levels might be hypothesised to be protective of CVD. Preβ1-HDL, while originally identified on gel electrophoresis, can be conveniently measured in a commercially available assay that has been used in a range of studies (Kempen et al., 2014; Orsoni et al., 2012; Tashiroa et al., 2009; Vazquez et al., 2012).

5.1.3 pSoBid Study

PSoBid is a cross sectional study carried out in 2007 in Glasgow. 666 Participants aged 35-64, equal number of males & females were recruited from the most and least deprived areas (Deans et al., 2009). Deprivation was associated with increased carotid plaque score and carotid intima-media thickness (cIMT) which is a surrogate measure of atherosclerosis. SBP

was associated with cIMT (p<0.001). HDL cholesterol had a negative association with cIMT (P<0.001) but LDL cholesterol was not correlated with cIMT (P= 0.055).

5.1.4 Aim

In this chapter, we will isolate HDL of pSoBid samples and run gradient gel electrophoresis to detect HDL subclasses percentage and study its relation to CVD markers. We will also estimate preβ1-HDL subclass for each of the pSoBid samples by using a specialist commercial ELISA. It was anticipated that these analyses would be of help in understanding further if specific structure(s) or subclasses of HDL are related to population demographics and extent of atherosclerosis.

5.2 Materials and Methods

5.2.1 Gradient Gel Electrophoresis

A total of 616 Li-Hep samples stored frozen as part of the pSoBid study were used for gradient gel electrophoresis and measurement of Pre-β1-HDL.
The lab work on GGE was undertaken by Mr. Xiaofeng Han, a Master student in our department. Incorporation of these data into the overall analyses was undertaken by myself. All materials and methods are explained in **Chapter 2** (**Section 2.6.4 and 2.11**).

5.2.2 Preβeta1-HDL

Pilot Study

As the pSoBid samples were not stored as recommended in the instructions for the Pre-β1-HDL ELISA kit (Pre-β1-HDL ELISA 289194, American Diagnosta GmbH, Pfungstadt, Germany), a pilot study was undertaken to see how essential it was to use a stabilizing buffer (recommended to be added to the samples before storing). In addition, the type of the sample recommended in the ELISA technical sheet was EDTA plasma with stabilizer while in

the pSoBid study, the available EDTA samples were low in volume and or had been previously thawed and frozen several times. Because we have almost a full set of Li-Hep samples that were taken into ice and frozen immediately with ~ 5 ml aliquots, we ran a pilot study on volunteers to test the differences between fresh EDTA blood samples, EDTA with a stabilizer and Li-Hep samples outcomes.

Five volunteers participated in this pilot study. From each participant, two blood samples were taken - one EDTA sample and one Li-Hep sample. All samples were directly put on ice and were centrifuged at 2060 g for 10 minutes at 4°C. Plasma was then isolated. Li-Hep samples were stored directly in -80°C freezer. For EDTA plasma, the first 20 µl was diluted with 400 µl of stabilizer from the Pre-β1-HDL ELISA kit and then stored in -80°C freezer. The rest of EDTA samples were stored directly in -80°C freezer. After 6 days, all the trial samples were transferred to -20°C freezers and kept for about 16 hours. Before starting the ELISA protocol, all the trial samples were taken out from the freezer and allowed to come to room temperature for about 30 minutes. Li-Hep samples and EDTA samples were diluted (1:20) with the stabilization buffer (20 µl of sample + 400 µl of stabilization buffer). Just prior to the assay, all the 21-fold diluted samples were diluted additionally a further 100-fold with the dilution buffer of the kit by using a dilutor (Hamilton microlab 500 series) i.e. 5 µl of (1:20) plasma + 495 µl of stabilizer (the total dilution was therefore 2,100-fold). The diluted samples were placed into transfer ELISA plate according to the specified layout.

pSoBid Samples

Li-Hep samples left from pSoBid study were stored in -80°C freezers for about 5 years. Since it was impractical to undertake simultaneous extraction and analysis of all, 20 samples each day were taken and had stabilizer (supplied in the assay kit) added immediately after thawing. Diluted samples with stabilizer are stable for 5 days, stored at 4°C, as stated in the kit's technical information sheet. So 79 samples were thawed and run each week for each assay in random order.

A total of 545 Li-Hep samples were used for Preβ1-HDL ELISA in singlicate as explained in **Chapter 2** (**Section 2.10**). The inter-assay CV was 14.2% and intra-assay CV was 9.2%. A plot

of Levey-Jennings for quality control values across 8 plates (1 plate per day) was also generated and did not detect any increase in random error (**Figure 5.1**). Results were accepted for each plate measurements within the range of ± 3X standard deviation.

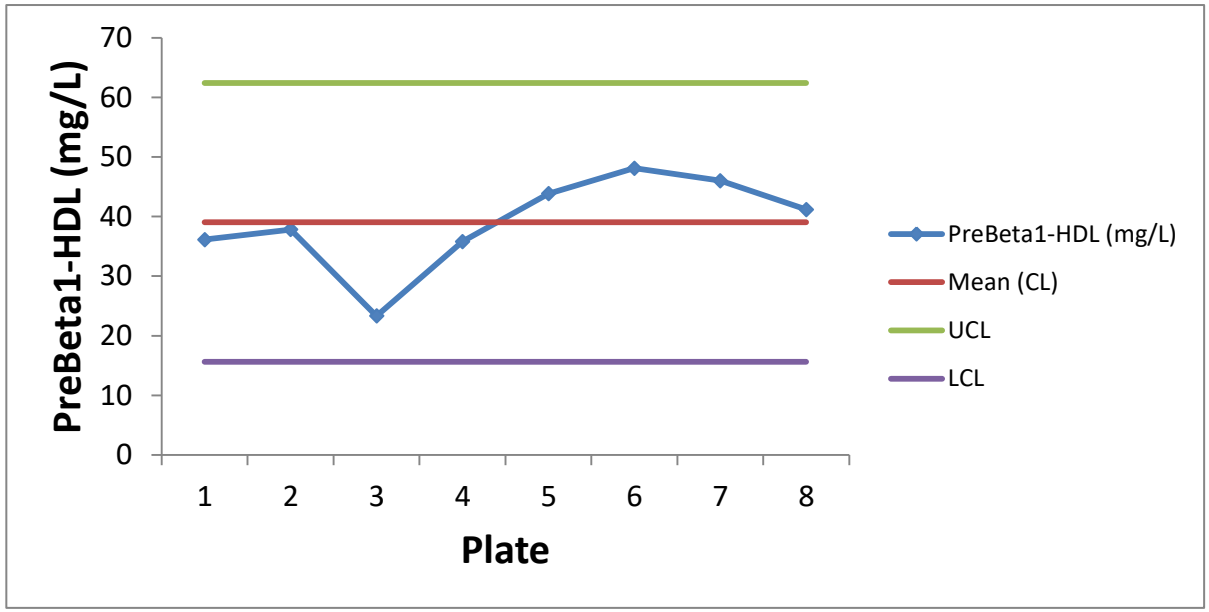


Figure 5.1 Levey-Jennings type plate-to plate plots of quality control values across 8 ELISA plate (one plate per day) showing the mean (CL), upper control limit and lower control limit. CL, control limit; UCL, upper control limit; LCL, lower control limit.

5.3 Calculations and Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics Data Editor (version 22). Normality was checked for all the data using normality plots and the Shapiro-Wilk test. Data were log-transformed when they did not approximate normality. Specifically, Preβeta1-HDL for this chapter was log transformed.

For gender and deprivation, independent-samples T-Test was used to compare means (± standard deviation) for normally distributed or log transformed variables. One-Way ANOVA was used to compare variable means between age groups. Association between variables were determined using Pearson correlation. Data are presented as means (± standard deviation) for normally distributed variables and as median (inter-quartile range) for non-normally distributed ones. Statistical significance was accepted at $P< 0.05$.

5.4 Results

5.4.1 Pilot Study Results

Measurements of Preβeta1-HDL on donors' samples for Li-Hep, EDTA or EDTA+ stabilizer (n= 5 for each) were 18.72, 15.72 and 15.32 mg/L respectively. Since these results were not significantly different ($P= 0.067$), we were content to proceed with Li-Hep samples from the pSoBid study for measurement of Preβeta1-HDL levels.

5.4.2 pSoBid Results

Subject's characteristics along with gradient gel electrophoresis and preβeta1-HDL results are presented in **Table 5.1.** %HDL2a did not reveal any significant difference between the two genders, different areas (deprivation) or between the age groups. cIMT displayed a significant difference between the two genders as it was higher in men ($p< 0.001$) as well as between the age groups ($P< 0.001$). This was parallel with the difference in %HDL3c which has revealed a significant difference between the two genders as it was higher in males (95% CI, 0.50 to 1.2%; $P< 0.001$) and between the age groups ($P= 0.017$). Other HDL subclasses

displayed a significant difference between the genders and area levels along with HDL-C and ApoA-I. For gender the following showed significant differences : HDL-C was higher in females (95% CI, 0.31 to 0.19 mmol/L; P< 0.001); ApoA-I was higher in females (95% CI, -0.20 to 0.12 mmol/L; P<0.001); %HDL2 higher in females (95% CI, 5.39 to -3.00%; P< 0.001); %HDL2b higher in females than in males (95% CI, 4.98 to 2.76%; P< 0.001); %HDL3 was higher in males (95% CI, 3.00 to 5.39%; P< 0.001); %HDL3a higher in males (95% CI, 0.99 to 2.680%; P< 0.001); %HDL3b higher in males (95% CI, 1.06 to 1.93%; P< 0.001).

For the deprivation area levels, HDL2 was higher in least deprived areas (95% CI, 1.08 to 3.56%; P< 0.001); %HDL2b higher in least deprived areas (95% CI, 1.45 to 3.73%; P< 0.001); %HDL3 was higher in most deprived areas (95% CI, 3.56 to 1.08%; P< 0.001); %HDL3a was higher in most deprived areas (95% CI, -2.13 to -0.42%; P= 0.003); %HDL3b was higher in most deprived areas (95% CI, -1.32 to - 0.43%; P< 0.001) and were all significantly different. An additional significant difference was also displayed by %HDL3b across age groups (P= 0.021). Finally, Preβeta1-HDL has revealed a significant difference only between the age bands (P= 0.012).

Table 5.1 Subjects' demographic details used in present chapter including percentage of HDL subclasses measured by gradient gel electrophoresis and Preβ1-HDL (mg/L) measured by ELISA. Total cohort for Preβeta1-HDL was less than gradient gel electrophoresis; n= 545 ; 264 males & 281 females; 284 from least deprived areas & 261 from most deprived areas; 161 from age group (35-44), 186 from age group (45-54) & 195 from age group (55-65).

Variable	Total Cohort (n=616)	Gender		Area		Age band			P Value		
		Male n=301	Female n=315	L.D n=316	M.D n=300	35-44 n=195	45-54 n=201	55-64 n=220	Sex	Area	Age
HDL-C (mmol/L)	1.36 (0.38)	1.23 (0.37)	1.48 (0.36)	1.42 (0.37)	1.30 (0.39)	1.35 (0.38)	1.37 (0.35)	1.36 (0.41)	<0.001	<0.001	0.813
ApoA-I (mmol/L)	1.43 (0.29)	1.35 (0.29)	1.51 (0.27)	1.46 (0.27)	1.40 (0.30)	1.42 (0.30)	1.44 (0.28)	1.43 (0.29)	<0.001	0.007	0.757
cIMT- (mm)	0.69 (0.14)	0.71 (0.15)	0.67 (0.12)	0.68 (0.12)	0.70 (0.15)	0.62 (0.10)	0.69 (0.12)	0.75 (0.15)	<0.001	0.054	<0.001
Preβeta1-HDL (mg/L)	54.19 (40.61-69.88)	51.41 (39.31- 68.14)	56.62 (41.17-72.13)	54.74 (41.07-0.54)	53.81 (40.32-68.89)	49.19 (36.81-63.10)	54.71 (40.24-72.08)	58.62 (43.64-74.26)	0.261	0.898	0.012
%HDL2	56.55 (7.90)	54.41 (7.51)	58.59 (7.73)	57.68 (8.05)	55.36 (7.56)	56.57 (7.96)	56.93 (8.16)	56.19 (7.61)	<0.001	<0.001	0.633
%HDL2a	30.20 (3.84)	30.04 (3.84)	30.35 (3.83)	30.07 (3.91)	30.34 (3.76)	30.03 (3.80)	30.53 (3.86)	30.05 (3.86)	0.312	0.386	0.331
%HDL2b	26.35 (7.29)	24.37 (6.45)	28.24 (7.56)	27.61 (7.74)	25.02 (6.54)	26.54 (7.54)	26.40 (7.08)	26.14 (7.28)	<0.001	<0.001	0.852
%HDL3	43.45 (7.90)	45.59 (7.50)	41.41 (7.73)	42.32 (8.05)	44.64 (7.56)	43.43 (7.96)	43.07 (8.16)	43.81 (7.61)	<0.001	<0.001	0.633
%HDL3a	27.12 (5.41)	28.06 (5.27)	26.23 (5.40)	26.50 (5.47)	27.77 (5.28)	27.16 (5.49)	27.41 (5.68)	26.82 (5.09)	<0.001	0.003	0.533
%HDL3b	10.64 (2.85)	11.41 (2.73)	9.92 (2.77)	10.22 (2.86)	11.09 (2.77)	10.51 (2.83)	10.32 (2.82)	11.06 (2.86)	<0.001	<0.001	0.021
%HDL3c	5.68 (2.22)	6.11 (2.22)	5.26 (2.15)	5.59 (2.28)	5.77 (2.17)	5.75 (2.17)	5.33 (2.08)	5.93 (2.37)	<0.001	0.295	0.017

Descriptive statistics are presented as mean (±standard deviation) for normal distributed variables and median (inter-quartile range) for not normal distributed variables. HDL-C, high density lipoprotein cholesterol;HDL2, high density lipoprotein 2; HDL3, high density lipoprotein 3; HDL2a, high density lipoprotein 2a; HDL2b, high density lipoprotein 2b, HDL3a, high density lipoprotein 3a, HDL3b, high density lipoprotein 3b; HDL3c, high density lipoprotein 3c; Apo-A, Apolipoprotein A-I; cIMT, Carotid intima media thickness; L.D, least deprived; M.D, most deprived.

5.4.3 Relationship cIMT and Preβeta-1HDL, HDL Subclasses and Subpopulations

As seen from **Figure 5.2**, cIMT was negatively correlated with HDL-C (r= -0.158, P<0.001). There was a trend of reduction of 0.57 mm in cIMT for each 1 mmol/L increase in HDL-C (95% CI, 0.086 to 0.027 mm) (**Figure 5.2-A**). On the other hand, cIMT was not correlated with Preβeta1-HDL levels (r= 0.073, P= 0.107) (**Figure 5.2-B**). Likewise, cIMT did not display any correlation with HDL major subclasses: %HDL2 (r= -0.07, P= 0.096); %HDL3 (r= 0.07, P= 0.096) respectively (**Figure 5.2-C, D**).

Exploring if any of the HDL subpopulation was related to cIMT, a significant negative correlation was revealed with %HDL2b (r= -0.095, P= 0.023). A trend of reduction of 0.002 was displayed for each 1% rise of %HDL2b (95% CI, 0.003 to 0.00 nm) (**Figure 5.3-A**). On the other hand, there was a positive correlation for cIMT with %HDL3b (r= 0.107, P= 0.011) with a trend of increase of 0.005 mm in cIMT for each 1% increase of %HDL3b (95% CI, 0.001 to 0.009 nm) (**Figure 5.3-D**). Other subpopulations did not display any significant correlation with cIMT; %HDL2a (r= 0.037, P= 0.375); %HDL3a (r=0.039, P= 0.357); %HDL3c (r= 0.018, P= 0.672) (**Figure 5.3-B, C, E**).

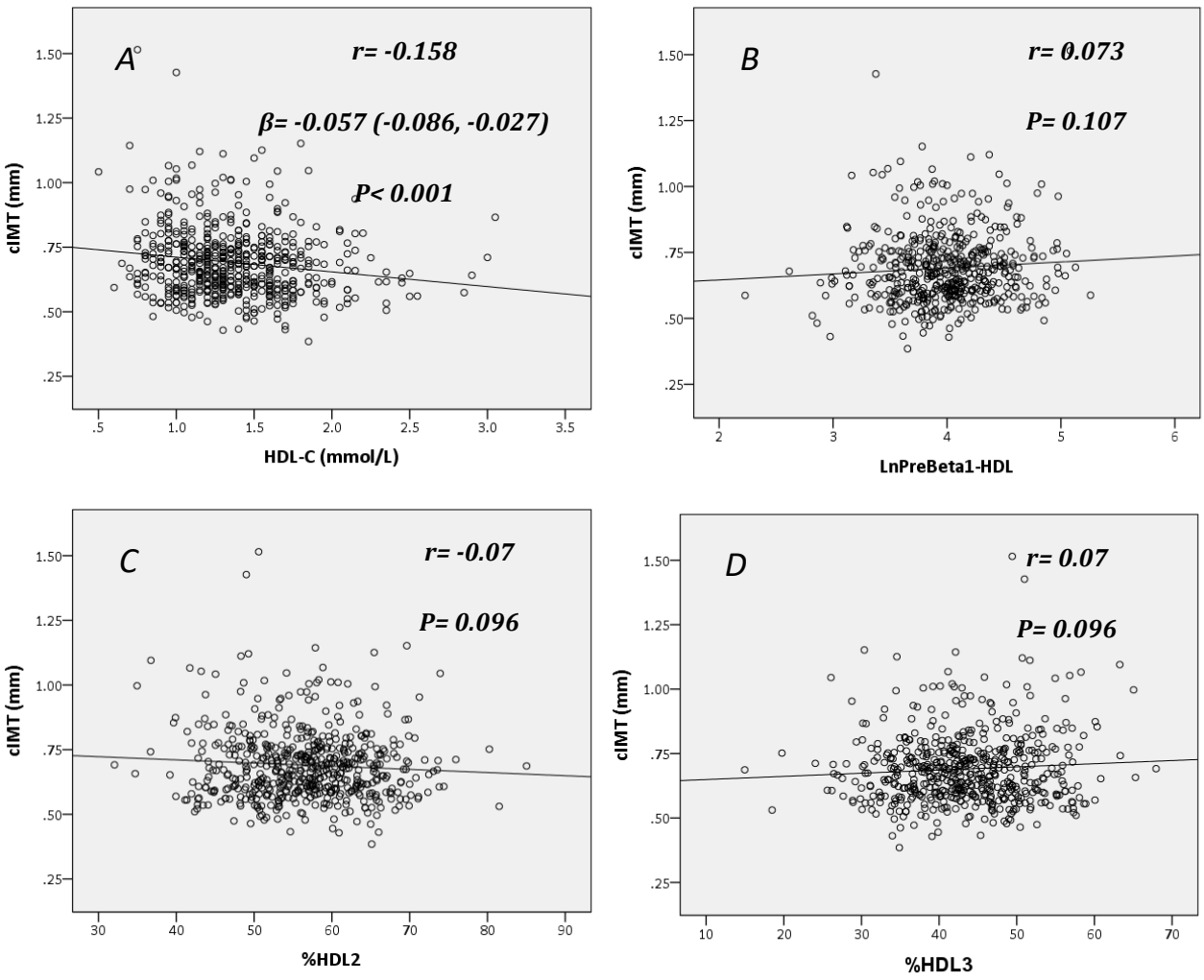


Figure 5.2 Scatter plot (with linear regression line of best-fit) illustrating the relationship between cIMT and; HDL-C (A); Preβeta1-HDL levels (B); %HDL2 (C); %HDL3 (D). cIMT, carotid intima media thickness; mm, millimetre; HDL-C, high density lipoprotein cholesterol; HDL2, high density lipoprotein 2; HDL3, high density lipoprotein 3.

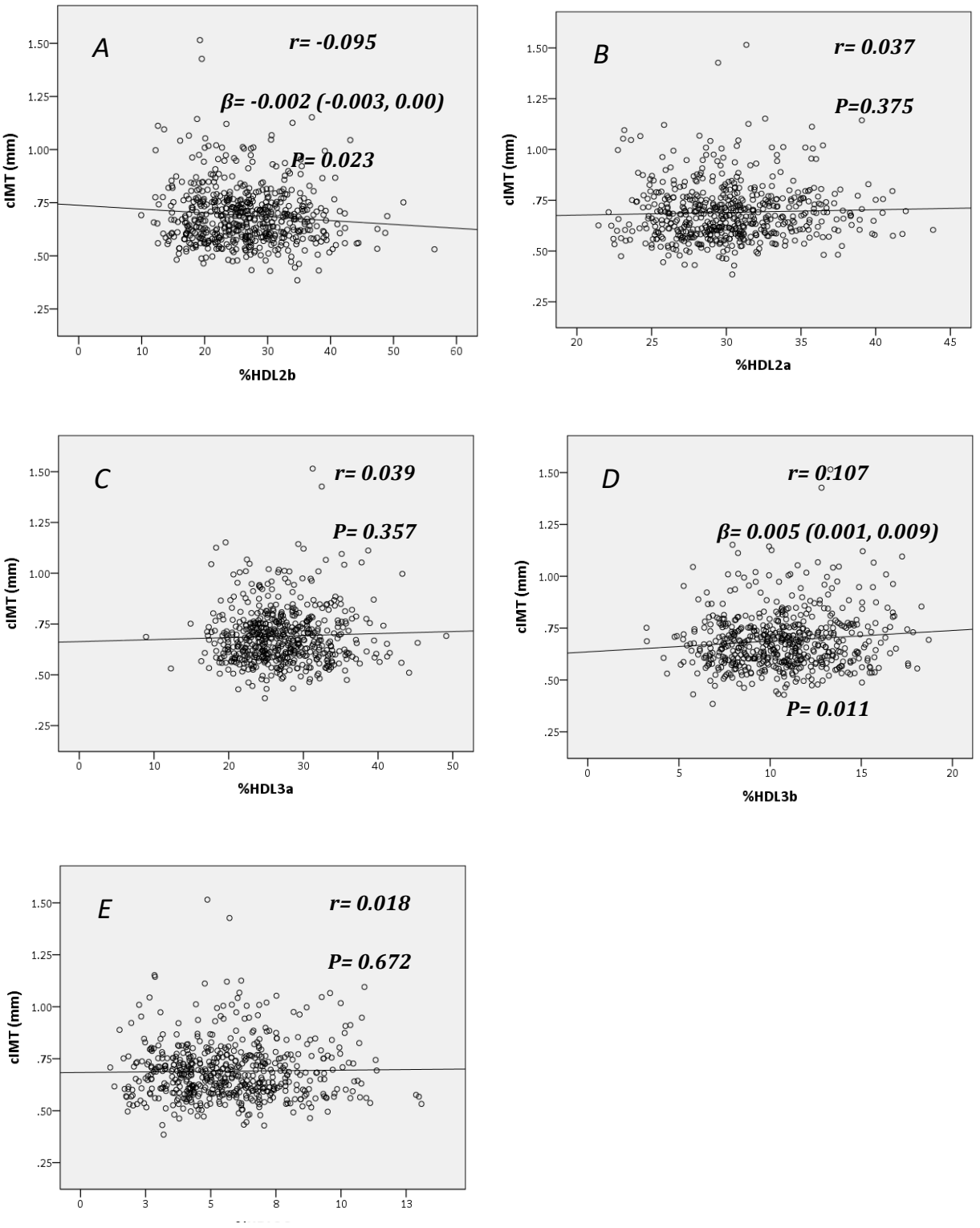


Figure 5.3 Scatter plot (with linear regression line of best-fit) illustrating the relationship between cIMT and; %HDL subpopulations: %HDL2b (A); %HDL2a (B); %HDL3a (C); %HDL3b (D); %HDL3c (E). HDL, high density lipoprotein; cIMT, carotid intima media thickness; mm, millimetre.

5.4.4 Inter Correlation between HDL Subpopulations with HDL Oxidation Factors and PON1 Enzyme Activity

Studying the major HDL subclasses and their relation to HDL oxidation potential measures, %HDL2 displayed an entirely opposite effect to %HDL3. %HDL2 was positively correlated with HDLs' susceptibility to oxidation assessed by the three variables, T1/2max, maximum propagation rate and maximum production of conjugated dienes, (r= -0.16, P= 0.009), (r= 0.42, P<0.001) and (r= 0.45, P<0.001) respectively. There was a trend of a decrease of 0.21 minutes in HDL T1/2max for each 1% increase in HDL2 (95% CI, 0.365 to 0.053) (**Figure 5.4-A**), a trend of increase of 0.016 milli.Units.min^{-1} in HDL oxidation propagation rate for each 1% increase in %HDL2 (95%, 0.012 to 0.02) (**Figure 5.4-C**) and a trend of increase of 0.001 units of conjugated diene produced for each 1% increase in %HDL2 (95% CI, 0.001 to 0.001) (**Figure 5.4-E**). %HDL3 displayed totally opposite correlations with HDL oxidation potential as assessed by the same oxidation measures, (r= 0.16, P= 0.009), (r= -0.42, P<0.001) and (r= -0.45, P<0.001) respectively. The trend of regression was completely the opposite of that of HDL2 (**Figure 5.4-B, D & F**).

Reviewing if any of HDL major classes displayed a correlation with PON1 activity, %HDL2 and %HDL3 displayed another opposed correlations. %HDL2 revealed a positive significant correlation with HDL PON1 activity (r= 0.19, P= 0.001). There was a trend of increase of 0.225 (Units.mL^{-1}) in HDL PON1 activity for each 1% increase in %HDL2 (95% CI, 0.091 to 0.358) (**Figure 5.5-A**). The correlation of %HDL2 with the ratio of plasma PON1 to HDL PON1 activity was significant and negative (r=-0.15, P= 0.009). A trend of decrease of 0.012 in the plasma PON1 to HDL PON1 ratio for each 1% increase in %HDL2 (95% CI, 0.021 to 0.003) (**Figure 5.5-C**). %HDL3 has displayed the opposite correlations with PON1 measurements compared to %HDL2. The percentage of HDL3 particles displayed a negative correlation with HDL PON1 activity (r= -0.19, P= 0.001). There was a trend of 0.225 units.mL^{-1} decrease in HDL PON1 activity for each 1% increase in %HDL3 (95% CI, 0.358 to 0.091 units.mL^{-1}) (**Figure 5.5-B**). Conversely, there was a significant positive correlation between the %HDL3 and the ratio of plasma PON1 to HDL PON1 activity (r= 0.15, P= 0.009). A trend of 0.012 increase in the ratio of plasma to HDL PON1 activity for each 1% increase in %HDL3 (95% CI, 0.003 to 0.21) (**Figure 5.5-D**).

In further exploration, it was observed that %HDL2b or %HDL3b subpopulations were related to HDL oxidation factors As seen in **Figure 5.6-A** %HDL2b was significantly positively correlated with maximum propagation rate of HDL oxidation (R= 0.427, P< 0.001). A trend of increase of 0.017 $m.U.min^{-1}$ in HDL maximum propagation rate for each 1% increase in %HDL2b (95% CI, 0.012 to 0.021 $m.U.min^{-1}$). %HDL2b was also positively significantly correlated with the maximum conjugated diene produced (**Figure 5.6-B**) (r= 0.48, P<0.001). There was a trend of increase of 0.001 units of conjugated diene production for each 1% increase in %HDL2b (95% CI, 0.001 to 0.001 units). Contrariwise, %HDL2b did not display any correlation with the percentage of inhibition of LDL oxidation by HDL calculated by T1/2max (R= -0.02, P= 0.724) (**Figure 5.6-C**). Relating %HDL2b with PON1 activity revealed a significant positive correlation with HDL PON1 activity (R= 0.18, P= 0.002).There was a trend of increase of 0.23 units/mL for 1% rise in %HDL2b (95% CI, 0.09 to 0.37 $units.mL^{-1}$) (**Figure 5.6-D**). Conversely, ratio of plasma PON1 to HDL PON1 displayed a significant negative correlation with %HDL2b (r= -0.187, P= 0.001). A trend of reduction of 0.016 of the ratio of plasma PON1 to HDL PON1 was revealed for each 1% increase of HDL2b (95% CI, 0.026 to 0.007) (**Figure 5.6-E**).

Studying if there was a correlation between %HDL3b with any of the oxidation measures or with PON1 levels, It can be clearly seen from **Figure 5.7-A**, that %HDL3b was significantly negatively correlated with maximum propagation rate of HDL oxidation (R= -0.31, P<0.001). There was a trend of reduction of 0.035 $m.U.min^{-1}$ in HDL maximum propagation rate for each 1% increase in %HDL3b (95% CI, 0.048 to 0.022 $m.U.min^{-1}$). %HDL3b was also negatively significantly correlated with the maximum conjugated diene produced (**Figure 5.7-B**) (r= 0.37, P<0.001). There was a trend of decrease of 0.003 units of conjugated diene production for each 1% increase in %HDL3b (95% CI, 0.004 to 0.002 units). Contrariwise, %HDL3b did not display any correlation with the percentage of inhibition of LDL oxidation by HDL calculated by T1/2max (r= 0.028, P= 0.672) (**Figure 5.7-C**). Studying %HDL3b correlation with PON1 activity results displayed a significant negative correlation with HDL PON1 activity (r= -0.12, P= 0.035). There was a trend of decrease of 0.416 $units.mL^{-1}$ for each 1% rise in %HDL3b (95% CI, 0.803 to 0.029 $units.mL^{-1}$) (**Figure 5.7-D**). On the contrary, ratio of plasma PON1 to HDL PON1 did not display any significant correlation with %HDL3b (r= 0.044, P= 0.439) (**Figure 5.7-E**).

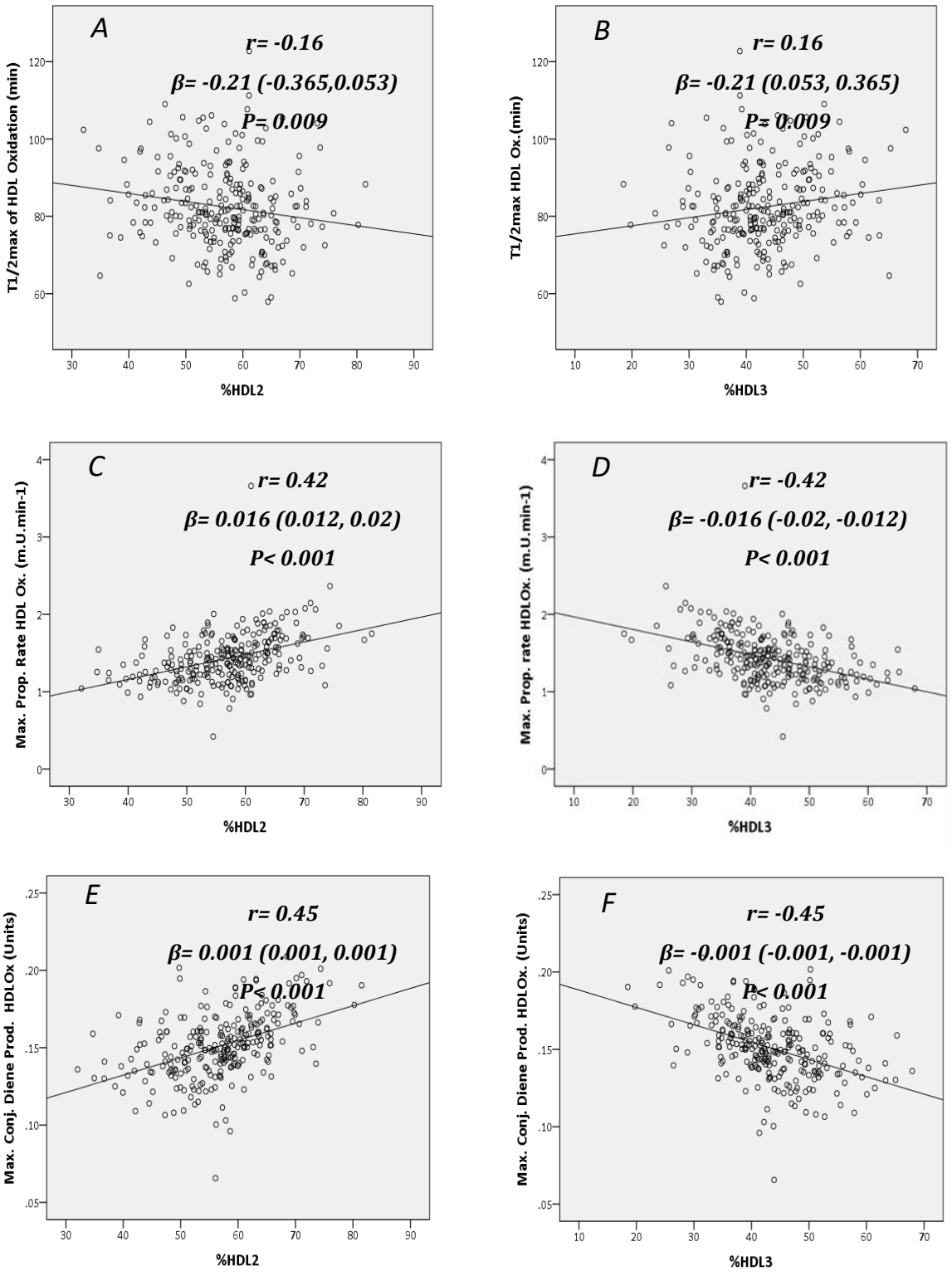


Figure 5.4 Scatter plot (with linear regression line of best-fit) illustrating the relationship between Oxidation factors and HDL major subclasses; HDL2, high density lipoprotein 2; HDL3, high density lipoprotrotein 3; Max. Prop. Rate HDL Ox., maximum propagation rate of HDL oxidation; m.U.min-1, milli units per minutes; Max. Conj. Dien. Prod. HDL Ox., maximum conjugated Diene produced from HDL oxidation; U.Min-1, Units per minutes; PON1, paraxonase1 enzyme.

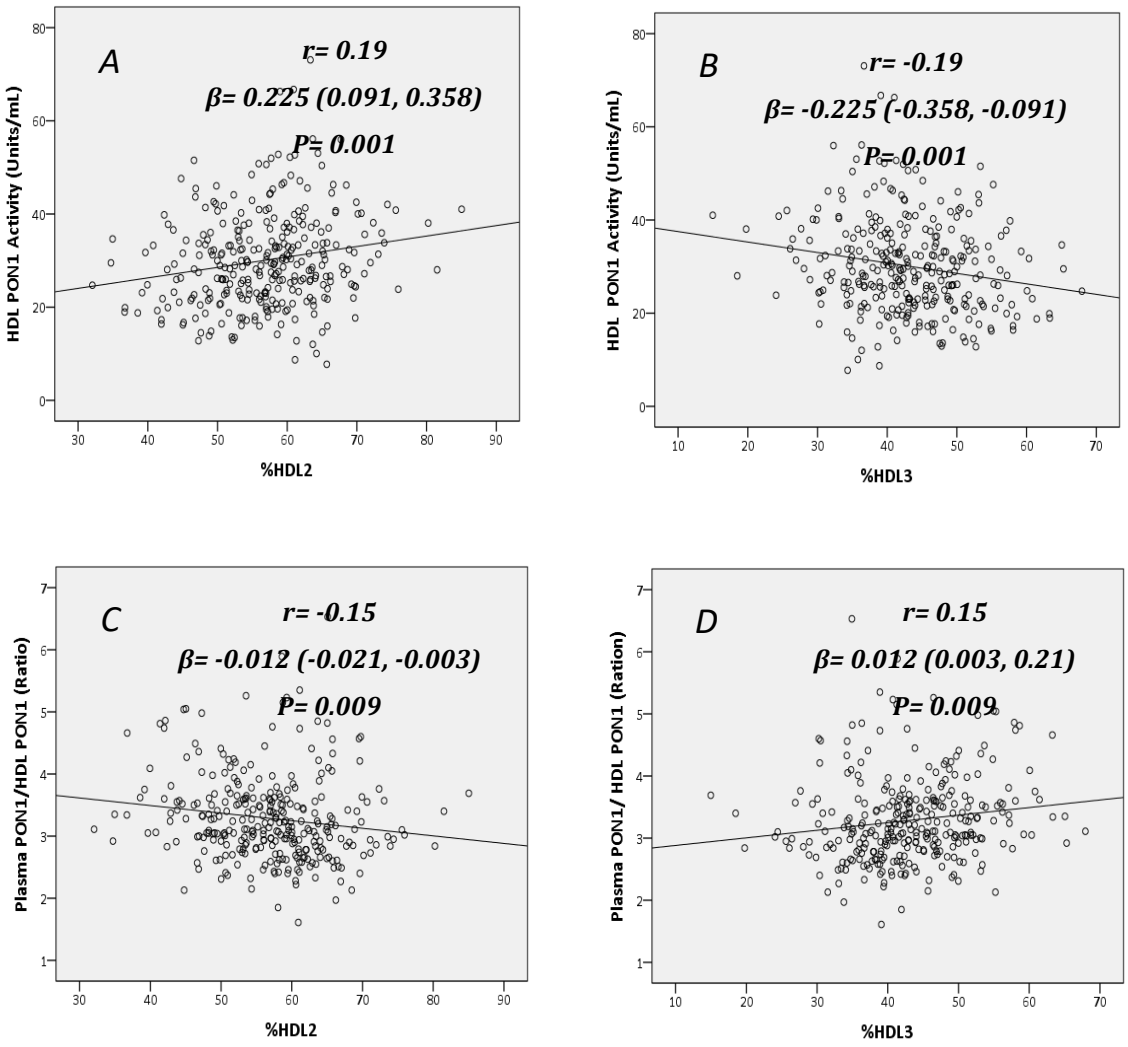


Figure 5.5 Scatter plot (with linear regression line of best-fit) illustrating PON1 activity measurements with %HDL2 (A), with and %HDL3 (B) ;plasma PON1/HDL PON1 with %HDL2 (C), with %HDL3 (D). HDL2, high density lipoprotein 2; HDL3, high density lipoprotein 3; PON1, paraxonase1 enzyme.

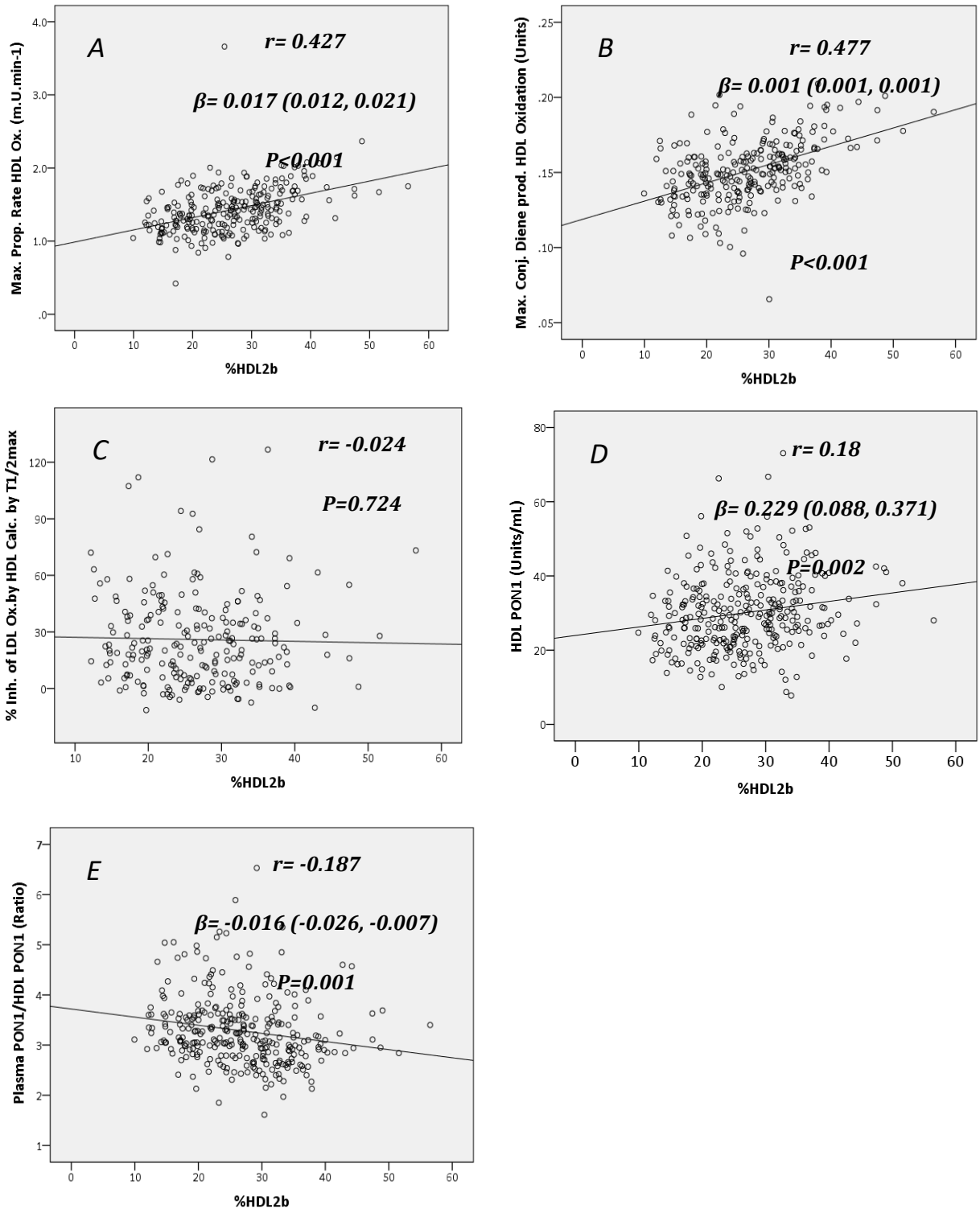


Figure 5.6 Scatter plot (with linear regression line of best-fit) illustrating the relationship between Oxidation factors and PON1 results with %HDL2b subpopulations; HDL2b, high density lipoprotein 2b; Max. Prop. Rate HDL Ox., maximum propagation rate of HDL oxidation; m.U.min-1, milli units per minutes ; Max. Conj. Dien. Prod. HDL Ox., maximum conjugated Diene produced from HDL oxidation; U.Min-1, Units per minutes; % Inh. Of LDL Ox. By HDL Calc. by T1/2max, %Inhibition of LDL oxidation by HDL calculated by time at half maximum; PON1, paraxonase1 enzyme.

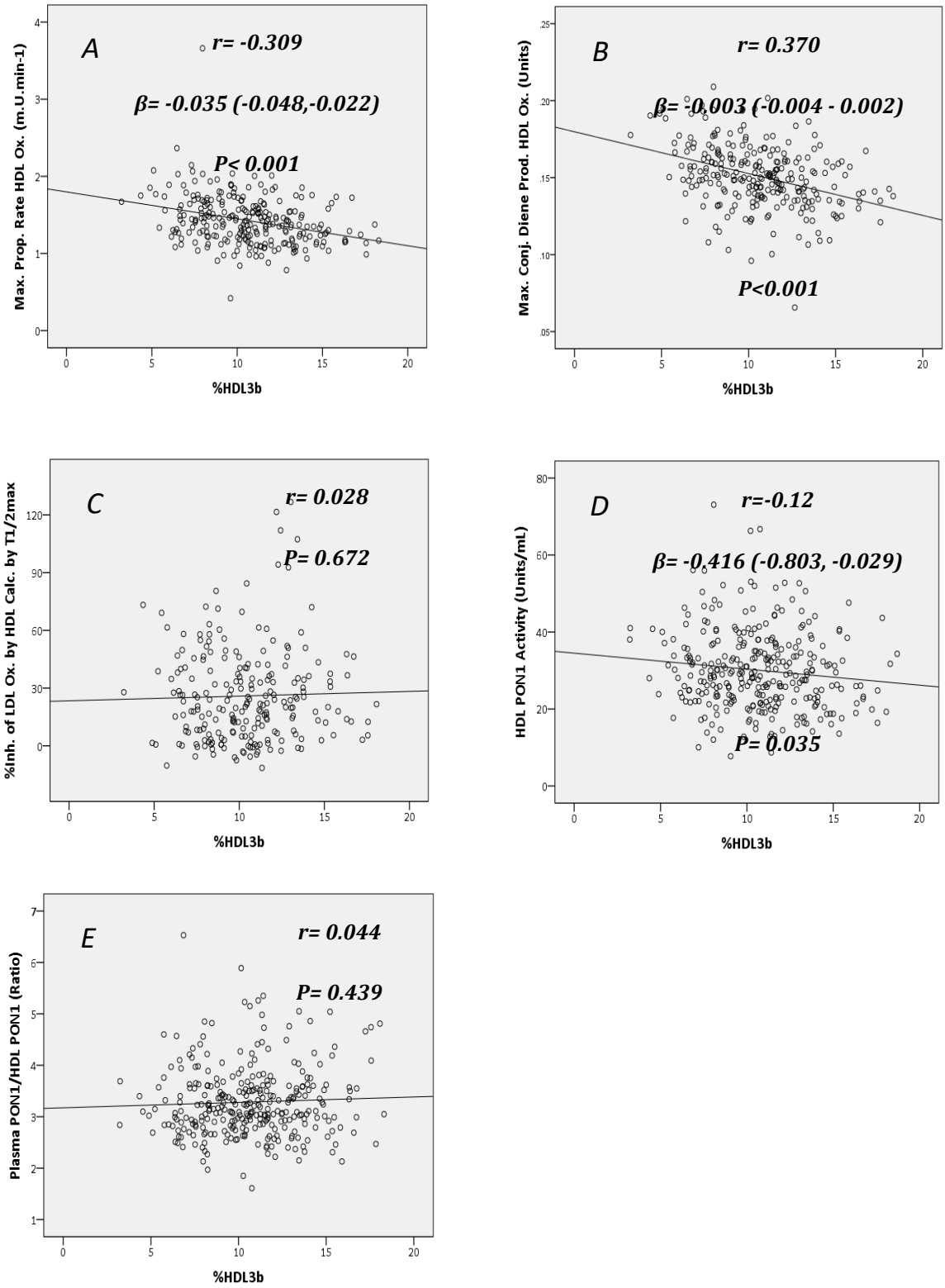


Figure 5.7 Scatter plot (with linear regression line of best-fit) illustrating the relationship between Oxidation factors and PON1 results with %HDL3b subpopulations; HDL3b, high density lipoprotein 2b; Max. Prop. Rate HDL Ox., maximum propagation rate of HDL oxidation; m.U.min-1, milli units per minutes; Max. Conj. Dien. Prod. HDL Ox., maximum conjugated Diene produced from HDL oxidation; U.Min-1, Units per minutes;% Inh. Of LDL Ox. By HDL Calc. by T1/2max, %Inhibition of LDL oxidation by HDL calculated by time at half maximum; PON1, paraxonase1 enzyme.

5.5 Discussion

This chapter explores HDL subfraction distribution and its relationship to carotid atherosclerosis and to HDL oxidation potential and antioxidant potency. We found a number of well-known associations in addition to observations which are novel or less well reported.

HDL size and density varied between the two genders as expected with %HDL2 being higher and %HDL3 lower in females than in males. The major findings were that cIMT is inversely correlated with HDL-C but not with total %HDL2 or total %HDL3. %HDL2b and %HDL3b subpopulations, on the other hand, were like HDL-C associated with cIMT. In terms of risk, %HDL3b had a positive association with atherosclerosis but in terms of protection of atherosclerosis, %HDL2b was negatively correlated with atherosclerosis. Why these specific fractions showed up as the strongest links with atherosclerosis is not very clear but previous publication have also reported similar findings. Watanabe and colleagues reported that B-mode ultrasound measurements of IMT correlated more strongly with %HDL2b in Finnish families (Watanabe et al., 2006). Drexel et al 1992 has also found that %HDL2, in particular the %HDL2b subfraction, was lower in patients with CAD compared to healthy subjects (Drexel et al., 1992). The Johansson et al study of sequential angiographies in premature myocardial infraction survivors showed that a greater percentage of HDL3b predicted more rapid development of coronary atherosclerosis (r= 0.37, P= 0.05), particularly among patients with raised TGs (r= 0.48, P< 0.01). When hypercholesterolemic patients were considered separately, %HDL3b was the only lipoprotein measurement to predict progression rates, accounting for 21% of the variation (Johansson, Carlson, Landou & Hamsten, 1991).

It has been suggested that the cardioprotective properties of HDL2b and atherogenic associations of HDL3b are related to their relationships to the fractional rate of cholesterol esterification (FER-HDL) in VLDL- and LDL-depleted plasma. The FER-HDL is a parameter that defines the capability of the HDL pool to esterify free cholesterol. The FER-HDL is greater in CHD versus healthy controls (Frohlich & Dobiásová, 2003) . There is a strong correlation between FER-HDL and HDL3 (specifically HDL3b, r = 0.89) and an inverse correlation between FER-HDL and HDL2 (specifically HDL2b, r = −0.61, P <0.001) (Dobiasova, Stribrna, Pritchard & Frohlich, 1992). However, there are a number of possible alternative

explanations based on apoA-I/apoA-II content and other compositional and functional features (Pussinen, Jauhiainen & Ehnholm, 1997).

Preβeta1-HDL did not reveal any significant correlation with cIMT. Previous literature has revealed a higher levels preβ1-HDL particles compared to healthy subjects (Guey et al., 2011), due to defective activity of enzyme involved in the HDL maturation cycle or to enhanced enzymatic remodelling of α-HDL induced by high plasma TG levels (Tian, Xu, Fu, Peng, Liu & Long, 2011) . Other literatures, revealed an increased levels of Pre-β-HDL in patients with CAD or ischemic heart disease even when excluding dyslipidemic conditions (Sethi et al., 2010). Although this fraction did not display any significant difference between the two genders or the two deprivation areas, there was a significant increase of about 20% in older ages. The implication of this finding is that the amount of circulating Preβeta1-HDL, the putative initiator of reverse cholesterol transport, does not seem to be a factor in CVD risk as measured by cIMT. It should be noted that the Preβeta1-HDL assay measures a family of particles which are PL complexes with apoA-I where the Preβeta1-HDL epitope is expressed. They may not all be the classical discs of 'nascent HDL' and this may have influenced the findings in this work.

In parallel with their correlation to cIMT and in relation to the findings in **Chapter 4**, HDL2 and specifically %HDL2b displayed a positive correlation with HDL oxidation potential, measured by the maximum propagation rate and by the maximum amount of produced conjugated diene. Likewise, %HDL3 and particularly %HDL3b revealed a negative correlation with HDL oxidizability measured by the same two factors. This finding is in comparable with the study of Stojanovic N. and colleagues who have found that HDL2 was more susceptible to copper-induced oxidation than HDL3 (Stojanović, Krilov & Herak, 2006) and to the study of Shuhei N. et al who has demonstrated that the resistance of HDL3 particles to oxidation is higher than that of total pool of HDL particles and they concluded that small, dense HDL3 particles are less prone to oxidation than large, light HDL2 in vitro (Shuhei, Söderlund, Jauhiainen & Taskinen, 2010).

The resistance of HDL particles to oxidation is affected probably by HDL lipid/apolipoprotein composition, HDL- associated proteins other than apolipoproteins, subclass distribution, and systemic inflammation. The significance of HDL oxidation in terms of atherosclerosis is still

unknown partly because it is not been a property that has been studied in depth. The fact that in this thesis, HDL oxidation was found to be correlated with ur study suggests that this property needs more detailed examination in future experiments. HDL PON1 level was not significantly correlated with atherosclerosis, as seen in **Chapter 4**, but, in this chapter, PON1 activity (**Figure 5.6-D**) was positively and significantly associated with the %HDL2b and negatively with %HDL3b (**Figure 5.7-D**). Additionally, because %HDL2b was negatively associated with the ratio of plasma PON1 to HDL PON1 while %HDL3b was not, this could mean that the HDL2b sub-species has a higher PON1 content compared to HDL3b. This finding , however, is not in agreement with the finding of Bergmeier et al who has revealed that HDL3 fraction carries the highest PON1 activity than HDL2 (Bergmeier, Siekmeier & Gross, 2004). The interrelations of HDL subfractions, HDL oxidation and atherosclerosis are explored further in the following chapters.

6 Alcohol, Smoking, Physical Activity and Diet Association with HDL in the pSoBid Cohort

6.1 Introduction

Low HDL-cholesterol (HDL-C) is well recognized as a major risk factor for coronary heart disease (Gordon et al., 1989). The levels of this lipoprotein are regulated by genetic and lifestyle factors (see **Chapter 1**). Recognition that certain habits such as alcohol consumption, cigarette smoking, exercise and diet can influence HDL has led to advice to alter these to reduce risk.

Alcohol is the fifth leading risk factor for death and overall burden of disease accounting for 4% of life years lost due to disease (Lim et al., 2012). Although the adverse effect of alcohol include liver cirrhosis and malignancy, accidental injuries, malignancies of other organs including colorectum, breast, and upper aerodigestive tract have been firmly established, uncertainity remains concerning the potential protective effects of light to moderate alcohol consumption on risk of coronary heart disease and stroke. Observational studies have consistently reported that compared with non-drinkers, light to moderate drinking exhibits a reduced cardiovascular risk, with the lower risk found at approximately 12-25 British units per week, while heavier and more hazardous drinking is associated with an increased risk, resulting in the well-established U shaped association. This possibly causal link was evident in analysis of over 60 ecological, case control, and cohort studies. Moreover, it was concluded from previous reviews that both men and women who drink one to two drinks a day have lower risk of coronary heart disease (Klatsky, Armstrong & Friedman, 1992; Maclure, 1993; Moore & Pearson, 1986). The most widely proposed mechanism for this purported cardioprotective effect of alcohol is an increase in HDL-C (Brien, Ronksley, Turner, Mukamal & Ghali, 2011)

In 2014, Mendelian randomization analysis based on individual participant data has, however, abolished this association and suggested that reduction of alcohol consumption,

even for light to moderate drinkers, is beneficial for cardiovascular health (Holmes et al., 2014)

Based on the risk associations seen in other chapters and giving the previous studies mentioned in the literature review, one of the hypotheses that we decided to test if alcohol consumption would alter the properties of HDL in the pSoBid study. Muth and colleagues has found that regular alcohol consumers had a higher number and percentage of large HDL particles than non-drinkers (Muth, Laughlin, von Mühlen, Smith & Barrett-Connor, 2010). Moderate alcohol consumption has also been reported to raise circulating levels of small, lipid poor, preβeta HDL (Beulens et al., 2004). Alcohol drinking also affects various anti-atherogenic activities of HDL such as elevating the capacity to efflux cholesterol from cells which was revealed commonly (Beulens et al., 2004; Mäkelä et al., 2008), but not always (Rao, Liu, Marmillot, Seeff, Strader & Lakshman, 2000) after alcohol ingestion. Cholesterol efflux may also be influenced from the compositional modifications in HDL like elevating the content of PL and enhancing particle surface fluidity as a consequence of elevating content of PUFA (Perret et al., 2002). Those changes could also account for improving antioxidative and anti-inflammatory activities of HDL particles, suggesting the existence of a common pathway of enhanced HDL function (Kontush & Chapman, 2010; Schäfer et al., 2007). Lastly, Brinton et al and Costa et al found that a rise in the activity of the HDL-associated anti-atherogenic enzyme PON1 is prompted by alcohol intake (Brinton, 2010; Costa, Giordano & Furlong, 2011) which might affect HDL antioxidative properties. These findings was not consistent with the finding of Schwedhelm et al. which did not find a strong correlation between alcohol consumption and enzymatic activities of PON1 and arylesterase (Schwedhelm, Nimptsch, Bub, Pischon & Linseisen, 2016).

Participation in regular physical activity is highly recommended for the prevention of CVD in national guidelines (Nice, 2010). One of the main mechanisms by which regular physical exercise is thought to attenuate coronary artery disease is by its impact on HDL metabolism. Kesaniemi et al. reviewed 51 papers describing physical activity intervention, and reported a mean rise in HDL cholesterol of 4.6% (Kesaniemi, Danforth, Jensen, Kopelman, Lefebvre & Reeder, 2001). In contrast, the effects on LDL-C and TGs were reported as being inconsistent (Mann, Beedie & Jimenez, 2014). As a consequence, lack of exercise is accompanied by low HDL-C concentrations, as documented in the NHANES cohort (Healy, Matthews, Dunstan,

Winkler & Owen, 2011). Likewise, the AVENA cross- sectional study found that a sedentary lifestyle involving excessive television viewing is associated with low HDL-C in adolescence (Martinez-Gomez et al., 2010). Regarding HDL profile, physical exercise was found to increase large HDL2-C and decrease HDL3-C (Brown et al., 2009; Nye, Carlson, Kirstein & Rössner, 1981). Exploring the effect of exercise on preβeta-HDL level, some studies has found that aerobic exercise raised plasma levels (Jafari et al., 2003; Khabazian, Ghanbari-Niaki, Safarzadeh-Golpordesari, Ebrahimi, Rahbarizadeh & Abednazari, 2009; Olchawa et al., 2004) while Jafari et al. found no difference of Prebeta1-HDL level between trained athletes and sedentary subjects (Kontush & Chapman, 2011). The effect of exercise on HDL antioxidant activity was also studied and it was found to be higher in athletes compared to controls. This was also demonstrated in a study for Ribeiro et al. in patients with Type 2 diabetes (Ribeiro et al., 2008) and in another study for Casella-Fihlo et al. in patients with metabolic syndrome (Casella-Filho et al., 2011). Such functional enhancement for HDL antioxidative effect, which is characterized for both HDL2 and HDL3 subclasses, could be due to elevated HDL content of apoA-I and increased content and activities of LCAT and PON1 (Kontush & Chapman, 2011).

Diet is thought be an important factor in the maintenance of optimal cardiovascular health, although a lack of data from randomised controlled trials is problematic. To date, confirmation that fruit and vegetable intake reduces the risk of cardiovascular disease remains limited (Dauchet, Amouyel & Dallongeville, 2009). Numerous constituents of fruits and vegetables have cholesterol-depressing properties. Observational studies suggest dietary fibre modifies biliary physiology and the enterohepatic cholesterol cycle which promotes exclusion of cholesterol through the feces (Lampe, 1999). Numerous studies have reported effects of fruit and vegetable consumption on lipid profiles (Smith-Warner et al., 2000; Zino, Skeaff, Williams & Mann, 1997). Most have revealed slight or no effect. Nevertheless, not all were precisely intended to examine the effects of fruits and vegetable consumption in patients with hypercholesterolemia (Dauchet, Amouyel & Dallongeville, 2009). In cancer prevention trials, interventions aimed at increasing fruit and vegetable consumption and reducing lipid intake decreased levels of both LDL cholesterol and HDL cholesterol (Howard et al., 2006; Lanza et al., 2001; Pierce et al., 2004; Rock et al., 2004).

Several components of fruits and vegetables could have a role in HDL modification. Polyphenols, which are abundant micronutrients found in plants, possess HDL-raising properties and can increase HDL activities (Kontush & Chapman, 2011). Mechanisms responsible for the HDL-raising action of polyphenols may involve CETP inhibition (Lam, Zhang, Yu, Tsang, Huang & Chen, 2008; Qin et al., 2009), improving the antiatherogenic activities of HDL like increasing cellular cholesterol efflux (Qin et al., 2009) and increasing PON1 action (Gugliucci & Bastos, 2009). On the other hand, Anti-oxidant vitamins, which are found in vegetables and fruits too, did not show any significant influence on HDL-C levels in humans (Abdollahzad, Eghtesadi, Nourmohammadi, Khadem-Ansari, Nejad-Gashti & Esmaillzadeh, 2009; Maki, Rubin, Wong, McManus, Jensen & Lawless, 2011; McRae, 2008; Rajpathak et al., 2010; Sutken, Inal & Ozdemir, 2006).

Smoking has been recognized to account for 14% of deaths from CVD (Health and Social Care Information Centre (HSCIC), 2012). The risk is significantly reduced within two years of smoking cessation (Salonen, 1980). Smokers have a 2 to 4 times increased risk of heart disease and of stroke (U.S. Department of Health and Human Services, 2004) and in fact smoking is associated with increased risk of all types of CVD-CHD, ischemic stroke, peripheral artery disease, and abdominal aortic aneurysm (Perk et al., 2013). The exact mechanisms by which smoking increases the risk of atherosclerosis are not fully understood. It is proven that smoking boosts both the progress of atherosclerosis and the incidence of thrombotic phenomena. Reactive oxygen species-free radicals- present in inhaled smoke may cause oxidation of plasma LDL and oxidized LDL activates the inflammatory process in the intima of the arteries by stimulation of monocyte adhesion to the vessel wall, resulting in increased atherosclerosis (Yamaguchi, Matsuno, Kagota, Haginaka & Kunitomo, 2001) (Weber, Erl, Weber & Weber, 1996; Yamaguchi, Haginaka, Morimoto, Fujioka & Kunitomo, 2005).

In **Chapter 4** and **Chapter 5**, we described HDL properties: HDL oxidation potential, HDL potency to protect LDL from oxidation, HDL PON1 activity and HDL subclass distribution along with preβ1-HDL content. These were explored in detail and related to cIMT. As explained above, there is a large body of literature that suggests that alcohol, exercise and diet affect the total amount of HDL but there is less information on the extent to which these life factors influence the detailed properties of HDL.

Aim

In this chapter, we will examine the determinants of these HDL properties in pSoBid population. So our questions are: to what extent does alcohol intake, smoking, exercise, and diet influence these properties of HDL. The hypothesis is that these lifestyle factors will be associated with recognised changes in HDL properties that have consequences for risk.

6.2 Participants

Data for 666 individuals were available from the pSoBid study. PSoBid is a cross sectional study carried out in 2007 in Glasgow. 666 Participants aged 35-64, equal number of males & females were recruited from the most and least deprived areas (Deans et al., 2009). Participants were invited to come for the first visit at their General practice's clinic. The first visit involved completion of lifestyle and psychology questionnaires as shown in **appendix 1** , assessment of health status and measurement of blood pressure, pulse rate and indexes of obesity. At the second visit, a fasting blood sample was taken to measure total plasma cholesterol, TGs, the cholesterol in VLDL-C, LDL-C and HDL-C. In addition, participants underwent ultrasound assessment of carotid intima media thickness (cIMT). Furthermore, participants completed lifestyle questionnaire which had 13 sections including basic demographic data, past and present health status, current medications, smoking history, alcohol intake, diet, physical activity.

Diet: A score for the consumption of fruit and vegetables was calculated from self-reported food frequency questionnaire participant responses (**Appendix 1**). Participants were asked on average how often they consumed of a range of food categories (21 food categories listed). Responses for each question ranged from daily consumption (number of portions per day) to weekly and monthly consumption. Participants selected one response per food category. For the purposes of the present analysis responses to four questions from the food frequency questionnaire relating to fruit and vegetable intake were aggregated to give an overall indicative diet score (i.e. frequency of intake of fresh fruit, cooked green vegetables (fresh or frozen), cooked root vegetables (fresh or frozen) and raw vegetables or salad (including tomatoes). Monthly diet scores were calculated on the basis of a 28 day month.

The maximum possible total diet score was 672 (6 portions per day X 28 days per month X 4 food category questions).

Exercise: Participants' physical activity was assessed over questions on habitual physical activity at work and in recreation were included in the lifestyle questionnaire (**Appendix 1**), allowing participants to be classified as inactive, moderately inactive, moderately active, or active using a previously validated protocol (Khaw et al., 2006). In our project, the physical activity level was a combination of activity at work and recreational exercise. The ''active group'' included those who had non sedentary work and those who undertook active or very active recreational exercise (> 0.25 h/day). All other subjects were included in the ''inactive group''.

Smoking: Participants' smoking behaviours were also assessed. As part of the participant lifestyle questionnaire, as shown in **appendix 1**, participants were asked whether they ever smoked regularly (at least one cigarette a day for 12 months or more), what they smoked, on what age they started and stopped smoking.

Alcohol: based on the participants' lifestyle questionnaire (**appendix 1**), alcohol consumption was divided into non-alcohol consumer if the individual reported consuming 0 units of alcohol per week, moderate alcohol consumer if the individual reported consuming less than 14 units per week (as recommended by UK Department of Health) (Department of Health, 2016) or excess alcohol consumer if the individual was consuming >14 units per week. Those consuming excess alcohol were excluded from some analyses.

6.3 Statistics

Statistical analyses were performed using IBM SPSS Statistics Data Editor (version 22). Normality was checked for all the data using normality plots and the Shapiro-Wilk test. Data were log-transformed or square rooted when they did not approximate normality. For alcohol and physical activity, independent-samples T-Test was used to compare means (± standard deviation) for normally distributed or medians for log transformed variables. One-Way ANOVA was used to compare variable means between smoking status and diet score groups. Association between variables were determined using Pearson correlation. Statistical significance was accepted at $P< 0.05$. The relationship between variables was explored further in bivariate regression models. These included the independent variable

and a series of adjustment variables to test the extent to which the association was attenuated by inclusion of the second factor. The regression modelling was conducting using the linear regression module within SPSS.

6.4 Results

From **Table 6.1**, it can be seen that alcohol was associated with significantly altered HDL oxidation potential, as it was higher in moderate alcohol drinkers than non-drinkers (P= 0.003) (excess alcohol drinkers were excluded from this comparison). The effect was seen in maximum propagation rate and by conjugated dienes formed (P<0.001) while this was not the case when measured by T1/2max (P= 0.398). On the other hand, no significant difference was revealed looking at the effects of alcohol intake on the %inhibition of LDL oxidation by HDL (P= 0.413, P= 0.984, and P=0.986 for T1/2max, maximum propagation rate and maximum dienes formed respectively).

Smoking altered HDL's susceptibility to oxidation as it was higher in non-smokers than in smokers when measured by the two factors, maximum propagation rate and conjugated dienes formation, (all P <0.001). But this effect was the opposite when measured by T1/2 max as it was significantly higher in smokers than in non- smokers (P= 0.014).

Exercise, as shown in **Table 6.2**, had a significant effect on HDL oxidation as it was significantly higher in active individuals than inactive; P= 0.006 for maximum propagation rate and P= 0.013 for conjugated dienes formed. T1/2 max did not show any effect of exercise (P= 0.475). The percentage of inhibition of HDL to LDL oxidation, once again, was not significantly affected by exercise (P= 0.394) when measured by T1/2max, (P= 0.359) by maximum propagation rate and (P= 0.499) by conjugated dienes formed.

HDL oxidation potential was significantly higher in people eating more vegetables and fruits, when measured by maximum dienes formed (P= 0.011) while it was not significantly affected when measured by maximum propagation rate (P= 0.082) or T1/2max (P= 0.463).

Table 6.1 The effect of alcohol and smoking on HDL oxidation properties. Total cohort was little different for some factors; n= 257 for T1/2max HDL oxidation; n= 258 HDL oxidation (Max-Min OD), n=227 for %inhibition by HDL (T1/2max); n=213 %inhibition of oxidation by HDL (Vmax); n=226 %inhibition of oxidation by HDL (max-min)

Variable		Total cohort (n=259)	Alcohol Consumption			Smoking Status			P Value	
			No Alcohol n=71	Mod. Alcohol n=128	Exc. Alcohol n= 59	Non Smokers n= 117	Ex-Smokers n= 67	Curr. Smokers n= 75	Alcohol*	Smoking
HDL Oxidation	T1/2max (min)	82.39 (10.70)	83.07 (11.11)	81.71 (10.63)	83.18 (10.49)	80.76 (9.57)	81.89 (11.87)	85.34 (10.80)	0.398	0.014
	*Max. Rate (milli. U.min^{-1})	1.43 (0.32)	1.36 (0.30)	1.51 (0.34)	1.35 (0.26)	1.51 (0.35)	1.40 (0.26)	1.33 (0.28)	0.003	<0.001
	Max. Diene (units)	0.152 (0.02)	0.145 (0.02)	0.157 (0.02)	0.147 (0.016)	0.157 (0.02)	0.150 (0.02)	0.145 (0.018)	<0.001	<0.001
% Inhibition of LDL Oxidation by HDL	T1/2max	26.05 (24.41)	27.27 (24.73)	24.16 (23.31)	28.05 (26.40)	23.49 (21.60)	24.74 (21.48)	31.22 (29.90)	0.413	0.118
	Max. Rate	39.50 (15.08)	40.26 (14.89)	40.32 (15.81)	36.98 (13.89)	38.95 (14.66)	37.88 (15.39)	41.71 (15.43)	0.984	0.352
	Max. Diene	11.96 (8.50-21.18)	12.95 (8.34-22.59)	11.77 (8.65-23.37)	10.53 (7.92-15.46)	11.27 (8.00-20.96)	11.96 (8.51-21.33)	13.33 (8.66-20.80)	0.986	0.658
HDL-C (mMol/L)		1.35 (0.38)	1.18 (0.30)	1.42 (0.39)	1.41 (0.360)	1.38 (0.39)	1.34 (0.31)	1.32 (0.40)	<0.001	0.540
ApoA-1 (mg/dL)		1.40 (0.28)	1.25 (0.27)	1.44 (0.27)	1.50 (0.26)	1.41 (0.26)	1.43 (0.21)	1.36 (0.31)	<0.001	0.335
cIMT (mm)		0.70 (0.15)	0.71 (0.13)	0.71 (0.16)	0.69 (0.16)	0.67 (0.13)	0.74 (0.18)	0.72 (0.15)	0.881	0.004

Descriptive statistics are presented as mean (standard deviation) for normal distributed variables and median (inter quartile range) for not normal distributed variables. Mod. Alcohol, moderate alcohol consumers; Exc. Alcohol, Excessive alcohol consumers; Curr. Smokers, current smoker; T1/2max, Time at half maximum; Max. Rate, Maximum propagation rate; Max. Diene, maximum dienes formed; HDL-C, high density lipoproteins- cholesterol, ApoA-1, apolipoprotein A-I; cIMT, carotid intima media thickness. * P value was calculated only between non-alcohol consumers and moderate alcohol consumers.

Table 6.2 The effect of diet and exercise on HDL oxidation properties. Total cohort was little different for some factors; n= 257 for T1/2max HDL oxidation; n= 258 HDL oxidation (Max-Min OD), n=227 for %inhibition by HDL (T1/2max); n=213 %inhibition of oxidation by HDL (Vmax); n=226 %inhibition of oxidation by HDL (max-min)

Variable		Total cohort n=259	Exercise		Diet				P Value	
			Inactive n=138	Active n= 121	Q1 n=61	Q2 n=72	Q3 n=60	Q4 n=66	Exercise	Diet
HDL Oxidation	T1/2max (min)	82.39 (10.71)	82.84 (10.99)	81.88 (10.39)	83.71 (11.15)	81.53 (10.70)	83.36 (82.70)	81.26 (9.94)	0.475	0.463
	*Max. Prop. R. (milli. U.min^{-1})	1.43 (0.32)	1.38 (0.28)	1.48 (0.35)	1.36 (0.28)	1.44 (0.41)	1.41 (0.29)	1.50 (0.25)	0.006	0.082
	Max. Diene (units)	0.152 (0.02)	0.149 (0.02)	0.155 (0.019)	0.15 (0.018)	0.15 (0.02)	0.152 (0.02)	0.16 (0.02)	0.013	0.011
% Inhibition of LDL Oxidation by HDL	T1/2max	26.05 (24.41)	27.40 (24.51)	24.62 (24.34)	24.56 (24.53)	29.68 (27.04)	28.33 (21.64)	21.56 (23.34)	0.394	0.250
	Max. Rate	39.50 (15.08)	38.60 (15.06)	40.50 (15.11)	39.90 (16.46)	38.98 (12.30)	39.02 (12.36)	40.12 (18.52)	0.359	0.969
	Max. Diene	11.96 (8.50-21.18)	11.39 (7.91-20.52)	12.01 (8.78-21.35)	12.05 (8.36-27.44)	11.96 (8.56-16.88)	11.98 (7.25-21.39)	11.73 (8.68-21.96)	0.499	0.709
HDL-C (mMol/L)		1.35 (0.37)	1.33 (0.38)	1.38 (0.37)	1.28 (0.37)	1.34 (0.40)	1.34 (0.38)	1.44 (0.33)	0.264	0.114
ApoA-1 (mg/dL)		1.40 (0.28)	1.38 (0.30)	1.43 (0.26)	1.35 (0.31)	1.40 (0.27)	1.38 (0.26)	1.47 (0.28)	0.145	0.138
cIMT (mm)		0.70 (0.15)	0.72 (0.17)	0.69 (0.13)	0.73 (0.18)	0.70 (0.14)	0.69 (0.15)	0.70 (0.14)	0.200	0.577

Descriptive statistics are presented as mean (standard deviation) for normal distributed variables and median (inter quartile range) for not normal distributed variables. T1/2max, Time at half maximum; Max. Rate, Maximum propagation rate; Max. Diene, maximum dienes formed; HDL-C, high density lipoproteins- cholesterol, ApoA-1, apolipoprotein A-; cIMT, carotid intima media thickness.

As displayed in **Table 6.3**, none of the parameters reflecting PON1 activity was related to alcohol consumption or smoking status. The effect of exercise and diet was also not significant on any of the parameters of PON1 assay (**Table 6.4).** Only exercise revealed an effect on the ratio of plasma PON1 activity to standardized HDL PON1 activity as it was higher in active individuals than in non-active ones (95% CI, - 0.50 to - 0.13 ; P= 0.001).

Except for %HDL2a, all %HDL's major subclasses and their subpopulations were significantly affected by alcohol consumption and smoking status (**Table 6.5**). Total %HDL2 subclass and %HDL2b subpopulation went up with alcohol intake significantly (P<0.001) and (P<0.001) respectively and went down with smoking (P=0.002) and (P= 0.005) respectively. On the other hand, total %HDL3 along with its subpopulations, %HDL3a, %HDL3b and %HDL3c went down with alcohol (P< 0.001, P= 0.006, P= 0.001 and P= 0.007 respectively) and up with smoking (P= 0.002, P= 0.036), P= 0.002 and P= 0.041 respectively). Preβ1-HDL was not effected by alcohol intake or by smoking status (P= 0.069 and P= 0.432 respectively).

The effect of exercise on % HDL subclasses and subpopulations was significant for %HDL2 subclasses. Active people displayed a higher %HDL2 than inactive individuals (P= 0.004) and that was the same for %HDL2b subpopulation (P= 0.003) but that was not seen for %HDL2a (P= 0.744). An opposite effect was revealed by exercise on %HDL3 subclass along with its subpopulations, %HDL3a and %HDL3b as it was lower in active individuals; (P= 0.004, P= 0.029 and P= 0.004 respectively). Nonetheless, exercise did not display a significant effect on %HDL3c or preβ1-HDL (P= 0.165 and P= 0.372 respectively).

Investigating the effect of consuming vegetables and fruits, diet score had a significant association with %HDL3b subpopulation as it was lowest in people with highest fruit and vegetables intake (P= 0.002). Total %HDL2 along with their subpopulations, HDL2a and HDL2b, did not reveal any effect from consuming fruit and vegetables: (P= 0.101), (P= 0.495), (P= 0.118). That was the same for total %HDL3 subclass along with %HDL3a and %HDL3c subpopulations; (P= 0.101, P= 0.229 and P= 0.062 respectively). Diet habit had a significant effect on %HDL3b as it was higher in people who are less likely to consume vegetables and fruits in their diets (P= 0.002). Finally, there was a trend to lower preβ1-HDL level with higher diet score but it was only border line significant (P= 0.042).

Table 6.3 The effect of alcohol and smoking on Paraxonase results. Total cohort was little different for some factors; n= 305 for plasma Pon1 activity; n= 306 plasma/HDL pon1 activity (units), n=289 for HDL pon1 standardized to protein (units); n=287 for plasma PON1/standardised HDL PON1 activity (units)

Variable	Total cohort n=308	Alcohol Consumption			Smoking Status			P Value	
		No Alcohol n=86	Mod. Alcohol n=151	Exc. Alcohol n= 70	Non Smoker n=137	Ex-Smoker n=84	Curr. Smoker n=87	Alcohol*	Smoking
Plasma PON1 activity (unit.ml^{-1})	94.64 (25.85)	88.83 (21.24)	93.72 (25.2)	104.19 (29.73)	96.31 (26.12)	96.55 (25.78)	90.16 (25.23)	0.132	0.165
***HDL PON1 activity (unit.ml^{-1})**	30.08 (10.21)	27.41 (8.84)	29.64 (9.52)	34.46 (11.83)	30.21 (9.34)	30.70 (9.63)	29.26 (11.99)	0.076	0.641
Plasma PON1/HDL PON1 Activity	3.29 (0.69)	3.38 (0.75)	3.31 (0.72)	3.12 (0.51)	3.29 (0.64)	3.26 (0.73)	3.30 (0.74)	0.456	0.931
HDL stand. to protein PON1 (units.ml^{-1})	29.55 (8.21)	28.16 (7.16)	29.17 (7.70)	32.45 (9.69)	29.83 (8.49)	30.40 (8.43)	28.34 (7.48)	0.342	0.253
Plasma PON1/Stand. HDL PON1 activity (ratio)	3.29 (0.80)	3.25 (0.87)	3.31 (0.83)	3.29 (0.63)	3.32 (0.77)	3.35 (0.95)	3.20 (0.67)	0.618	0.472
HDL-C	1.35 (0.37)	1.19 (0.30)	1.42 (0.38)	1.40 (0.34)	1.38 (0.38)	1.35 (0.31)	1.31 (0.40)	<0.001	0.425
ApoA-1	1.41 (0.28)	1.27 (0.27)	1.45 (0.27)	1.50 (0.24)	1.42 (0.26)	1.44 (0.27)	1.37 (0.31)	<0.001	0.265
cIMT	0.70 (0.15)	0.69 (0.13)	0.71 (0.15)	0.68 (0.15)	0.67 (0.12)	0.73 (0.17)	0.71 (0.15)	0.465	0.010

Descriptive statistics are presented as mean (standard deviation) for normal distributed variables and median (inter quartile range) for not normal distributed variables. Mod. Alcohol, moderate alcohol consumers; Exc. Alcohol, Excessive alcohol consumers; Curr. Smokers, current smoker; PON1, paraxonase 1 enzyme; HDL-C, high density lipoproteins- cholesterol, ApoA-1, apolipoprotein A-I; cIMT, carotid intima media thickness. * P value was calculated only between Non alcohol consumers and moderate alcohol consumers.

Table 6.4 The effect of diet and exercise on Paraxonase. Total cohort was little different for some factors; n= 305 for plasma Pon1 activity; n= 306 plasma/HDL pon1 activity (units), n=289 for HDL pon1 standardized to protein (units); n=287 for plasma PON1/standardised HDL PON1 activity (units)

Variable	Total cohort n=308	Physical Activity		Diet				P Value	
		Not Active n=159	Active n=149	Q1 n=77	Q2 n=83	Q3 n=71	Q4 n=77	Exercise	Diet
Plasma PON1 activity (unit.ml^{-1})	94.64 (25.85)	93.00 (26.25)	96.40 (25.38)	92.70 (25.39)	96.42 (27.45)	93.14 (24.75)	96.07 (25.81)	0.252	0.734
***HDL PON1 activity (unit.ml^{-1})**	30.08 (10.21)	30.06 (10.75)	30.09 (9.64)	29.38 (9.97)	30.71 (11.90)	28.65 (9.23)	31.41 (9.26)	0.979	0.339
Plasma PON1/HDL PON1 Activity	3.29 (0.69)	3.24 (0.71)	3.33 (0.61)	3.30 (0.73)	3.32 (0.72)	3.40 (0.67)	3.14 (0.62)	0.258	0.140
HDL stand. to protein PON1 (units.ml^{-1})	29.55 (8.21)	30.34 (8.09)	28.75 (8.28)	28.75 (8.08)	29.50 (8.95)	29.51 (7.55)	30.46 (8.20)	0.098	0.669
Plasma PON1/Stand. HDL PON1 activity (ratio)	3.29 (0.80)	3.14 (0.72)	3.45 (0.84)	3.31 (0.89)	3.34 (0.70)	3.23 (0.71)	3.29 (0.88)	0.001	0.844
HDL-C	1.35 (0.37)	1.32 (0.37)	1.38 (0.36)	1.31 (0.39)	1.32 (0.39)	1.36 (0.36)	1.42 (0.33)	0.147	0.295
ApoA-1	1.41 (0.28)	1.38 (0.30)	1.44 (0.25)	1.39 (0.31)	1.40 (0.26)	1.40 (0.26)	1.45 (0.27)	0.053	0.504
cIMT	0.70 (0.15)	0.71 (0.16)	0.69 (0.13)	0.72 (0.17)	0.70 (0.14)	0.69 (0.14)	0.69 (0.14)	0.128	0.612

Descriptive statistics are presented as mean (standard deviation) for normal distributed variables and median (inter quartile range) for not normal distributed variables. Mod. Alcohol, moderate alcohol consumers; Exc. Alcohol, Excessive alcohol consumers; Curr. Smokers, current smokers; PON1, paraxonase 1 enzyme; HDL-C, high density lipoproteins- cholesterol, ApoA-1, apolipoprotein A-I; cIMT, carotid intima media thickness. * P value was calculated only between Non alcohol consumers and moderate alcohol consumers.

Table 6.5 The effect of alcohol and smoking on HDL subclasses percentages

Variable	Total cohort n=616	Alcohol Consumption			Smoking			P Value	
		No Alcohol n=190	Mod. Alcohol n=278	Exc. Alcohol n= 144	Non Smoker n= 287	Ex-Smoker n= 160	Curr. Smoker n= 169	Alcohol	Smoking
%HDL2	56.55 (7.90)	54.93 (7.31)	57.81 (8.22)	56.25 (7.46)	57.71 (7.65)	55.83 (8.18)	55.57 (7.80)	<0.001	0.002
%HDL2a	30.20 (3.83)	30.36 (3.57)	29.97 (3.97)	30.46 (3.85)	30.46 (3.89)	29.60 (3.51)	30.32 (4.00)	0.269	0.066
%HDL2b	26.35 (7.29)	24.57 (6.41)	27.84 (7.77)	25.79 (6.79)	27.24 (7.48)	26.23 (7.14)	24.96 (6.92)	<0.001	0.005
%HDL3	43.45 (7.90)	45.07 (7.31)	42.19 (8.22)	43.75 (7.46)	42.29 (7.65)	44.17 (8.18)	44.73 (7.80)	<0.001	0.002
%HDL3a	27.12 (5.41)	27.98 (4.91)	26.55 (5.86)	27.07 (4.90)	26.61 (5.17)	27.14 (5.58)	27.96 (5.57)	0.006	0.036
%HDL3b	10.64 (2.85)	11.07 (2.88)	10.17 (2.73)	11.00 (2.88)	10.22 (2.80)	11.06 (2.88)	10.98 (2.80)	0.001	0.002
%HDL3c	5.68 (2.22)	6.03 (2.39)	5.46 (2.14)	5.68 (2.11)	5.45 (2.19)	5.97 (2.25)	5.79 (2.22)	0.007	0.041
Preβeta1-HDL (mg/L)	54.19 (40.62-69.88)	48.97 (36.42-63.06)	56.28 (40.72-68.68)	56.53 (44.92-80.96)	53.62 (40.43-68.26)	56.74 (41.03-76.47)	53.19 (40.24-71.15)	0.069	0.432
HDL-C	1.36 (0.38)	1.20 (0.32)	1.44 (0.39)	1.40 (0.39)	1.38 (0.37)	1.35 (0.36)	1.34 (0.43)	<0.001	0.543
ApoA-1	1.43 (0.29)	1.29 (0.27)	1.48 (0.28)	1.51 (0.27)	1.44 (0.27)	1.45 (0.28)	1.40 (0.33)	<0.1	0.291
cIMT	0.69 (0.14)	0.69 (0.13)	0.69 (0.14)	0.69 (0.14)	0.67 (0.12)	0.70 (0.15)	0.71 (0.14)	0.695	0.013

Descriptive statistics are presented as mean (standard deviation) for normal distributed variables and median (inter quartile range) for not normal distributed variables. Mod. Alcohol, moderate alcohol consumers; Curr. Smoker, current smokers; HDL-C, high density lipoproteins- cholesterol, ApoA-1, apolipoprotein A-I; cIMT, carotid intima media thickness.

Table 6.6 The effect of exercise and diet on HDL subclasses percentages

Variable	Total cohort (n=616)	Physical activity		Diet				P Value	
		Not Active n= 330	Active n= 286	Q1 n=156	Q2 n=162	Q3 n=145	Q4 n=153	Exer.	Diet
%HDL2	56.55 (7.90)	55.70 (7.70)	57.54 (8.02)	55.38 (8.20)	56.32 (8.24)	57.45 (7.72)	57.13 (7.27)	0.004	0.101
%HDL2a	30.20 (3.83)	30.15 (3.71)	30.25 (3.98)	30.04 (3.80)	30.23 (3.94)	30.59 (3.67)	29.95 (3.92)	0.744	0.495
%HDL2b	26.35 (7.29)	25.54 (7.08)	27.28 (7.43)	25.34 (7.10)	26.09 (7.27)	26.86 (7.65)	27.17 (7.09)	0.003	0.118
%HDL3	43.45 (7.90)	44.31 (7.70)	42.46 (8.02)	44.61 (8.20)	43.68 (8.24)	42.55 (7.72)	42.87 (7.27)	0.004	0.101
%HDL3a	27.12 (5.41)	27.56 (5.46)	26.61 (5.31)	27.71 (5.80)	26.89 (5.26)	27.37 (5.82)	26.53 (4.68)	0.029	0.229
%HDL3b	10.64 (2.85)	10.94 (2.81)	10.29 (2.85)	10.98 (2.73)	11.03 (3.14)	9.93 (2.51)	10.58 (2.82)	0.004	0.002
%HDL3c	5.68 (2.22)	5.79 (2.28)	5.54 (2.15)	5.90 (2.32)	5.75 (2.33)	5.25 (1.94)	5.77 (2.22)	0.165	0.062
Preβeta1-HDL (mg/L)	54.19 (40.62-69.88)	55.25 (38.83-(71.15)	53.50 (42.56-69.56)	56.61 (43.52-76.25)	53.53 (40.76-67.76)	49.27 (38.29-62.74)	56.10 (42.12-72.67)	0.372	0.042
HDL-C	1.36 (0.38)	1.33 (0.39)	1.40 (0.37)	1.34 (0.42)	1.33 (0.40)	1.38 (0.36)	1.39 (0.34)	0.019	0.477
ApoA-1	1.43 (0.29)	1.40 (0.31)	1.46 (0.26)	1.42 (0.33)	1.42 (0.28)	1.43 (0.27)	1.44 (0.27)	0.006	0.915
cIMT	0.69 (0.14)	0.70 (0.15)	0.68 (0.13)	0.70 (0.15)	0.70 (0.14)	0.68 (0.13)	0.68 (0.13)	0.147	0.168

Descriptive statistics are presented as mean (standard deviation) for normal distributed variables and median (inter quartile range) for not normal distributed variables. ; HDL-C, high density lipoproteins- cholesterol, ApoA-1, apolipoprotein A-I; cIMT, carotid intima media thickness

The data in **Table 6.1- 6.6** were explored in further detail by looking at the effect of alcohol and exercise according to two major demographic divisions (i.e. men versus women and least deprived versus most deprived (by pSoBid study design approximately equal number of individuals were in these divisions). **Figure 6.1** shows that HDL oxidation was affected by alcohol intake for both sexes (P= 0.072) for males and (P= 0.001) for females but no effect was seen in either of the areas. **Figure 6.2** shows that physical activity had an effect on the maximum propagation rate of HDL oxidation in men and people who lived in affluent areas but not in women or people who lived in most deprived areas.

Reviewing the effect of alcohol consumption on %HDL2b according to the same demographic division, revealed a significant difference (**Figure 6.3**) in %HDL2b in males (P= 0.006) and females (P< 0.001) as well in people who lived in most deprived areas (P= 0.007) and people who lived in affluent areas (P= 0.023). The effect of physical activity, on the other hand, on %HDL2b was revealed to be significantly different in females (P= 0.028) and least deprived areas (P= 0.028) while it was not significant in most deprived areas (P= 0.220) and was border line for females (P= 0.053) (**Figure 6.4**).

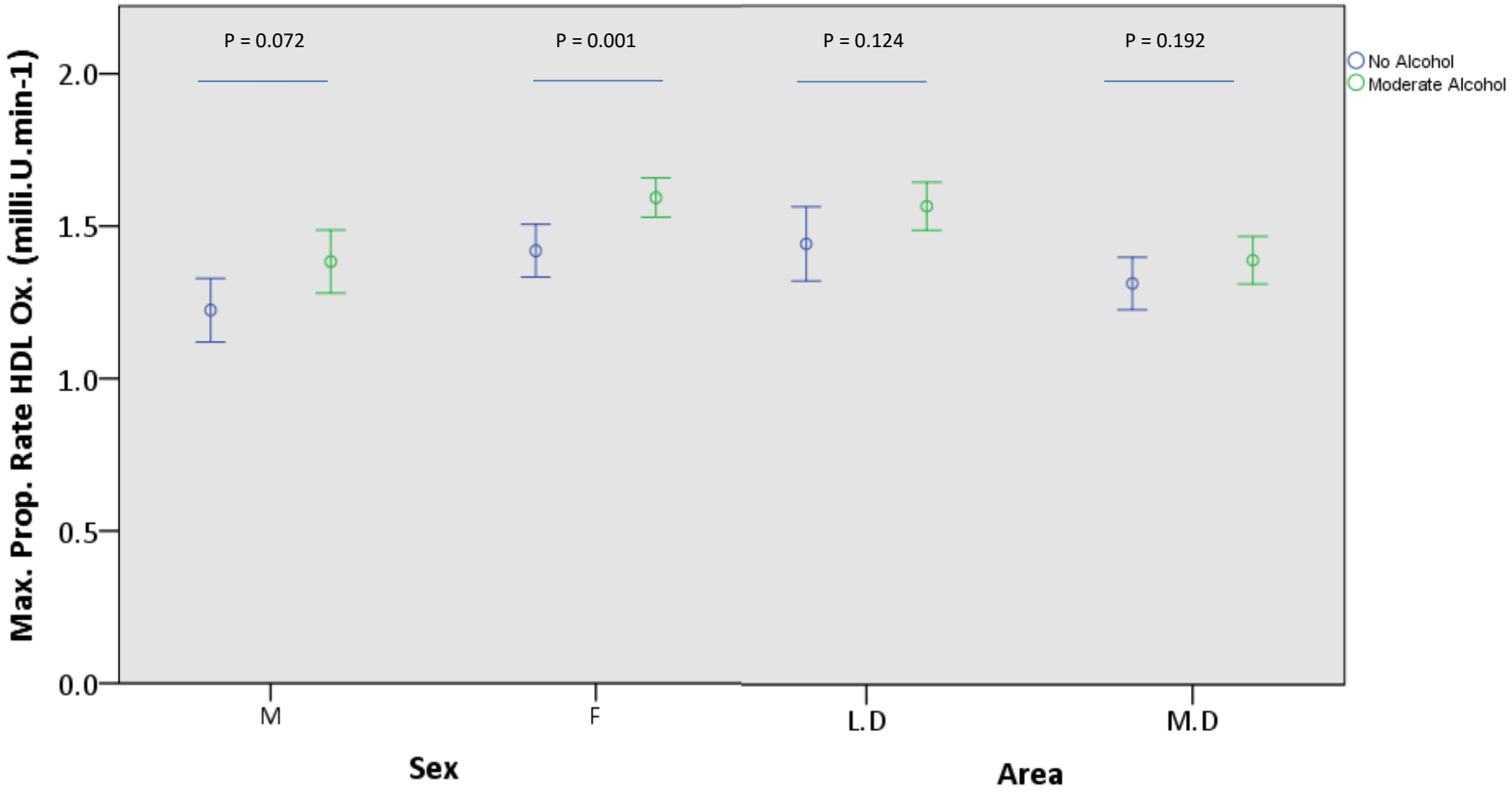


Figure 6.1 Association of alcohol intake with HDL oxidation (measured by maximum propagation rate) by sex and deprivation. Max. Prop. Rate HDl Ox. , maximum propagation rate of HDL oxidation; milli.U.min^{-1}, milli units.minutes^{-1}; M, males; F, Females. L.D, least deprive; M.D., most deprived

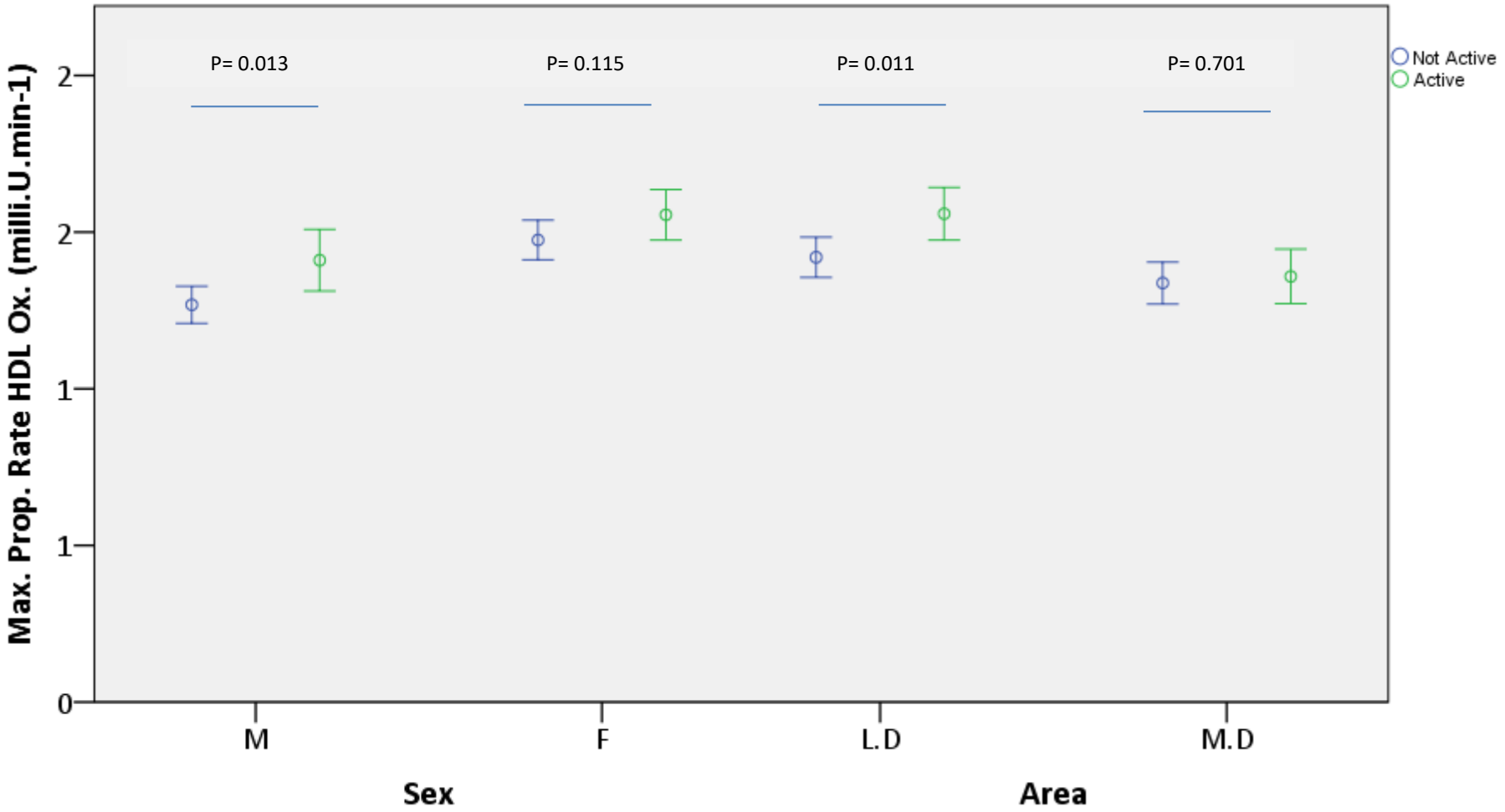


Figure 6.2 Association of physical activity with HDL oxidation (measured by maximum propagation rate) by sex and deprivation. Max. Prop. Rate HDl Ox. , maximum propagation rate of HDL oxidation; milli.U.min^{-1}, milli units.minutes^{-1}; M, males; F, Females. L.D, least deprive; M.D., most deprived

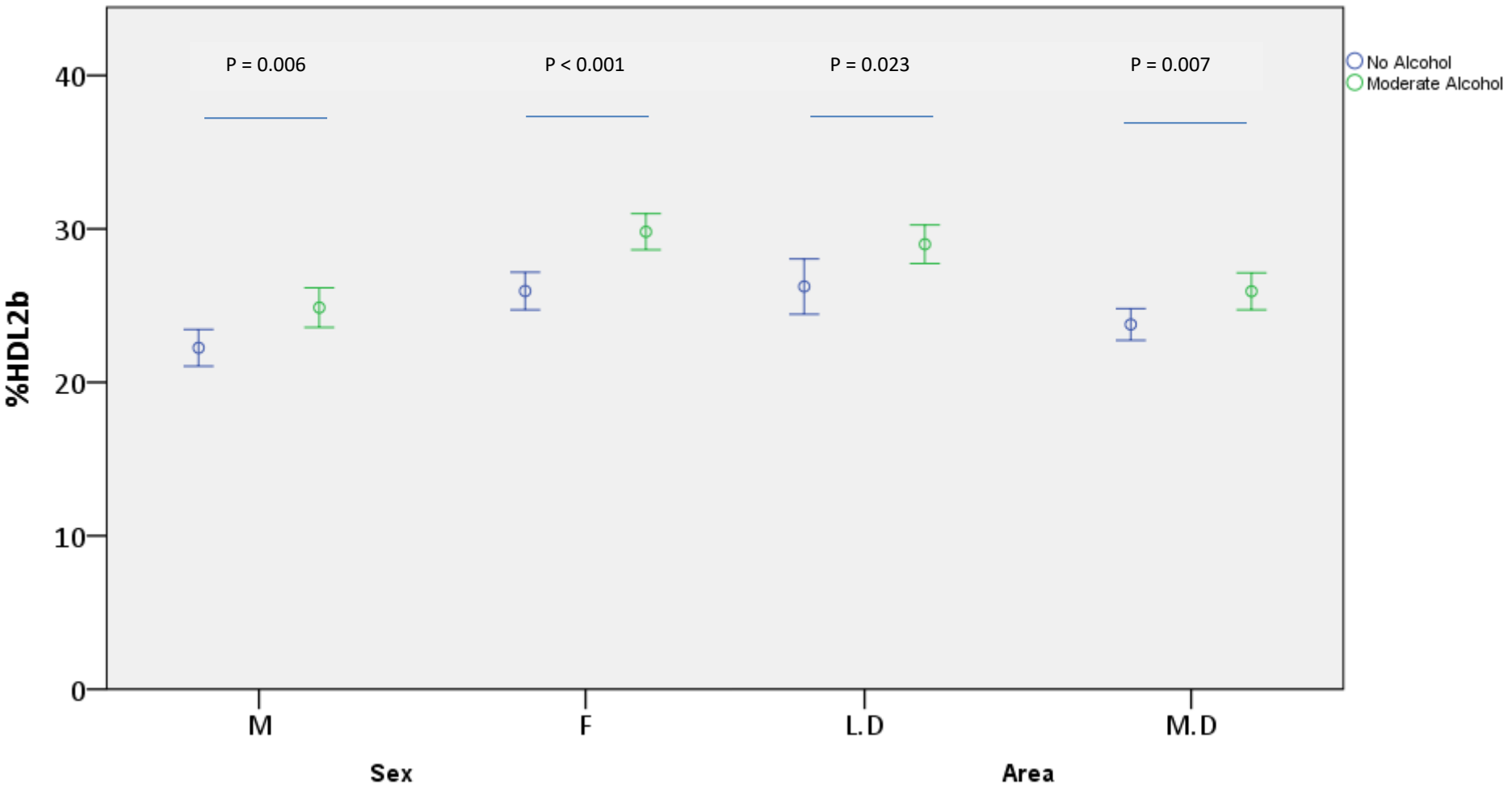


Figure 6.3 Association of alcohol intake with %HDL2b by sex and deprivation. M, males; F, Females. L.D, least deprive; M.D., most deprived

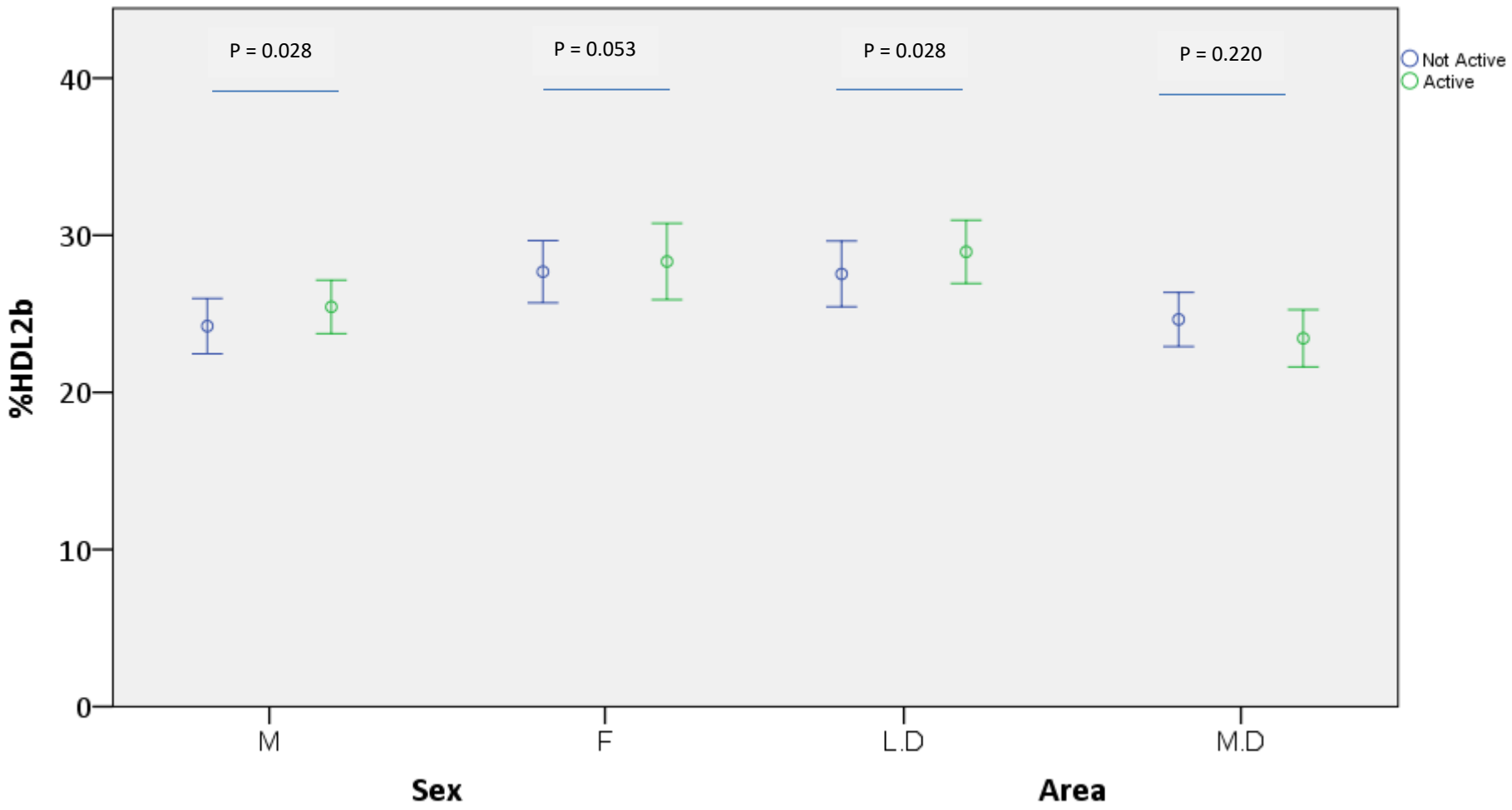


Figure 6.4 Association of physical activity with %HDL2b by sex and deprivation. M, males; F, Females. L.D, least deprive; M.D., most deprived

Regression Models

In **Chapter 5**, it was seen that %HDL2b was negatively correlated to atherosclerosis, as measured by cIMT. There was a trend of decrease of 0.002 mm in cIMT for each 1% increase in HDL2b (95% CI, -0.003 to 0.000; P= 0.023). We therefore investigated whether the lifestyle markers discussed in this chapter might explain the association of %HDL2b and %HDL3b with cIMT in a bivariate regression models.

As displayed in **Table 6.7**, %HDL2b effect was still significant after adjustment in a series of two-variable models for : age (β= -0.002) (95% CI, -0.003 to 0.000; P= 0.01), smoking status (β= -0.002) (95% CI, -0.003 to 0.000; P= 0.047), alcohol consumption (95% CI, -0.003 to -0.001: P= 0.011), diet (95% CI, -0.003 to -0.001: P= 0.036) and physical activity (95% CI, -0.003 to -0.001: P= 0.032). On the other hand, the effect of %HDL2b was attenuated when adjusted for sex (P= 0.187) and by HDL oxidation potential, measured by maximum propagation rate (P= 0.441).

In contrast, %HDL3b was positively associated to atherosclerosis. A trend of 0.005 mm increase in cIMT was displayed in each 1% increase in %HDL3b (95% CI, 0.001 to 0.009; P= 0.011) (**Chapter 5**). As revealed from bivariate regression models (**Table 6.8**) , this trend was almost the same when adjusted for smoking habit (β= 0.005) (95% CI, 0.001 to 0.009; P= 0.022), alcohol consumption (β= 0.006) (95% CI, 0.001 to 0.010; P= 0.014), diet score (β= 0.005) (95% CI, 0.001 to 0.009; P= 0.016) or with physical activity (β= 0.005) (95% CI, 0.001 to 0.009; P= 0.016). In contrast, the association was attenuated when adjusted for sex (P= 0.109), age (P= 0.053) and HDL oxidation potential (P= 0.231).

In similar way, we investigated the effect of HDL susceptibility to oxidation, as measured by maximum propagation rate on atherosclerosis. HDL oxidation potential was negatively correlated to cIMT. There was a trend of decrease of 0.07 mm in cIMT (95% CI, -0.130 to -0.011; P= 0.021) for each 1 milli.unit.minutes^{-1} increase in HDL oxidation rate (**Chapter 4**). This effect was still significant when adjusted for age (β= -0.06) (95% CI, -0.115 to -0.004; P= 0.035), alcohol consumption (β= -0.069) (95% CI, -0.135 to -0.002; P= 0.028), , diet (β= -0.067) (95% CI, -0.127 to -0.007; P= 0.028), and physical activity (β= -0.066) (95% CI -0.126

to -0.006; P= 0.032). In contrast, the effect was attenuated when adjusted for sex (P= 0.092), smoking status (P= 0.068) (**Table 6.9**), %HDL2b (P= 0.070) and %HDL3b (P= 0.061).

Table 6.7 Bivariate regression model of carotid atherosclerosis (cIMT) with %HDL2b and exposure assessment with other related variables

Independent variable	Adjustment variable	n	βeta (95% CI)	P-value
%HDL2b	None	567	-0.002 (-0.003, 0.000)	0.023
	HDL Vmax	239	-0.001 (-0.004,0.002)	0.441
	Sex	567	-0.001 (-0.003, 0.001)	0.187
	Age	567	-0.002 (-0.003, 0.000)	0.01
	Cigarette smoking	567	-0.002 (-0.003, 0.000)	0.047
	*Alcohol	429	-0.002 (-0.004, -0.001)	0.011
	Diet	567	-0.002 (-0.003, 0.000)	0.036
	Physical Activity	567	-0.002 (-0.003, 0.000)	0.032

*Comparison for alcohol intake was considered between non-alcohol consumers and moderate alcohol consumers only. Excess alcohol consumers were neglected

Table 6.8 Bivariate regression model of carotid atherosclerosis (cIMT) with %HDL3b and exposure assessment with other related variables

Independent variable	Adjustment variable	n	βeta (95% CI)	P-value
%HDL3b	None	566	0.005 (0.001, 0.009)	0.011
	HDL Vmax	239	0.004 (-0.003, 0.011)	0.231
	Sex	566	0.003 (-0.001, 0.007)	0.109
	Age	566	0.004 (0.000, 0.007)	0.053
	Cigarette smoking	566	0.005 (0.001, 0.009)	0.022
	*Alcohol	428	0.006 (0.001, 0.010)	0.014
	Diet	566	0.005 (0.001, 0.009)	0.016
	Physical Activity	566	0.005 (0.001, 0.009)	0.016

* Comparison for alcohol intake was considered between non-alcohol consumers and moderate alcohol consumers only. Excess alcohol consumers were neglected

Table 6.9 Bivariate regression model of carotid atherosclerosis (cIMT) with HDL oxidation potential (maximum propagation rate) and exposure assessment with other related variables

Independent variable	Adjustment variable	n	βeta (95% CI)	P-value
HDL Vmax	None	240	-0.07 (-0.130, -0.011)	0.021
	%HDL2b	239	-0.06 (-0.126, 0.005)	0.070
	%HDL3b	239	-0.059 (-0.122, 0.003)	0.061
	Sex	240	-0.055 (-0.117, 0.008)	0.085
	Age	240	-0.060 (-0.115, -0.004)	0.035
	Cigarette smoking	240	-0.056 (-0.117, 0.004)	0.068
	*Alcohol	183	-0.069 (-0.135, -0.002)	0.044
	Diet	240	-0.067 (--0.127, -0.007)	0.028
	Physical Activity	240	-0.066 (-0.126, -0.006)	0.032

*Comparison for alcohol intake was considered between non-alcohol consumers and moderate alcohol consumers only. Excess alcohol consumers were neglected

In **Chapter 5** it was seen that %HDL2b was related significantly to HDL oxidation potential. A trend of increase of 0.017 milli.Units.minutes^{-1} was displayed for each 1% increase in %HDL2b (95% CI, 0.012 to 0.021; P< 0.001). This effect was not affected when adjusted for sex (β= 0.015) (95% CI, 0.011 to 0.019; P< 0.001), deprivation state (β= 0.015) (95% CI, 0.011 to 0.020; P< 0.001), age band (β= 0.017) (95% CI, 0.012 to 0.021; P< 0.001), smoking status (β= 0.015) (95% CI, 0.011 to 0.020; P< 0.001), alcohol consumption (β= 0.015) (95% CI, 0.010 to 0.021; P< 0.001), diet habit (β= 0.016) (95% CI, 0.012 to 0.021; P< 0.001) or physical activity (β= 0.016) (95% CI, 0.012 to 0.021; P< 0.001) (**Table 6.10**).

Table 6.10 Bivariate regression model of the association of %HDL2b with HDL oxidation potential (maximum propagation rate) and exposure assessment with other related variables

Independent variable	Adjustment variable	n	βeta (95% CI)	P-value
%HDL2b	None	258	0.017 (0.012, 0.021)	<0.001
	Sex	258	0.015 (0.011, 0.019)	<0.001
	Deprivation	258	0.015 (0.011, 0.020)	<0.001
	Age	258	0.017 (0.012, 0.021)	<0.001
	Cigarette smoking	258	0.015 (0.011, 0.020)	<0.001
	*Alcohol	198	0.015 (0.010, 0.021)	<0.001
	Diet	258	0.016 (0.012, 0.021)	<0.001
	Physical Activity	258	0.016 (0.012,0.021)	<0.001

*Comparison for alcohol intake was considered between non-alcohol consumers and moderate alcohol consumers only. Excess alcohol consumers were neglected

6.5 Discussion

The focus of this chapter as set out in the aim was to explore further the findings in **Chapter 4 and Chapter 5** that the HDL properties of oxidation potential and subfraction distribution were significantly associated with carotid atherosclerosis.

The pSoBid population by design was broad ranging in the lifestyle factors including alcohol intake, smoking, amount of exercise and type of diet. The study also included equal numbers of men and women and people from areas of different derivation levels. So this chapter explored the interaction between these variables and the extent to which they predicted cIMT and HDL oxidation potential. The impact of lifestyle on HDL subfractions themselves has been well reported in the literature (Diehl, Fuller, Mattock, Salter, el-Gohari & Keen, 1988; Gardner, Tribble, Young, Ahn & Fortmann, 2000).

It is important to note that adjustment for other risk factors like inflammatory markers, medical status, obesity or BMI was not included in this analysis. Incorporation of these potential confounders in complex multivariate models would made interpretation difficult. Rather it was decided to address key factors known to affect HDL that had the potential to interact with the oxidation potential.

Alcohol and Cigarette Smoking:

In this chapter, a decision was made to examine the effect of alcohol intake on HDL properties. Individuals who consume > 14 units per week of alcohol were excluded in studying the difference in all factors as well and they were not incorporated in regression models. Consuming alcohol higher that of recommended guidelines will include people who have medical consequence of alcohol excess.

In the pSoBid population, alcohol had a well reported effect of raising total HDL-C and apoA-I. These results were compatible with the findings of Gaziano and Monson (Gaziano & Manson, 1996), Stamfer et al. (Stampfer, Colditz, Willett, Speizer & Hennekens, 1988) and Rimm et al. (Rimm et al., 1991) but smoking did not appear to affect either of these measures. Alcohol and cigarette smoking as lifestyle choices had opposite effects on HDL susceptibility to oxidation and on HDL subfraction distribution. HDL oxidation potential, as measured by maximum propagation rate or maximum dienes formed, went up (comparing

no alcohol to moderate alcohol consumers) whereas current smokers had lower HDL oxidation potentials than non- smokers and ex-smokers were intermediate.

These associations have not been reported previously for HDL oxidation potential and possibly need to be taken into account when trying to understand the relationship between lifestyle and atherosclerosis.

We noted that alcohol and smoking had no significant effect on the ability of HDL to inhibit LDL oxidation. This could be seen in the regression models in **Table 6.4** and **6.8** where %HDL2b and %HDL3b were related to cIMT. Including cigarette smoking and that alcohol intake in the model did not affect the association of these subfractions with carotid atherosclerosis.

On the other hand, when the association of HDL oxidation potential with atherosclerosis was examined, alcohol did not appear to affect this relationship but cigarette smoking appear to weaken it, so that it became borderline non-significant (P= 0.068).

Previous publication that examined the relationship between alcohol (Rao et al., 2003), smoking (Kumar & Biswas, 2011) and PON1 activity have reported. In the present study (**Table 6.3**), no association between these lifestyle factors and PON1 activity in HDL or plasma was detected.

Exercise and Diet

In the pSoBid population, undertaking active exercise compared to no exercise was associated with increase in HDL-C and apoA-I (as shown in **Table 6.6**). The diet score, measuring fruits and vegetable intake, had no influence. Both diet and exercise appeared to affect HDL oxidation (**Table 6.2**). The better choices, that is to be active and eat more fruit and vegetables, were associated with higher HDL susceptibility to oxidation measured by maximum propagation rate of oxidation and maximum conjugated dienes formed.

Neither lifestyle choice had an effect on the potency of HDL to inhibit LDL oxidation measured by any of the 3 parameters. Diet and exercise had variable effects on HDL subfractions (**Table 6.6**). %HDL2b was higher in active people and %HDL3b was lower but

diet had no effect on %HDL2b but did influence %HDL3b. Exercise had a number of significant effects on preβeta1-HDL levels whereas diet had a borderline influence.

Previous publications showed an effect of physical activity (Sviridov, Kingwell, Hoang, Dart & Nestel, 2003) but no significant difference between vegan and omnivore groups (Kuchta et al., 2016) on levels of Prebeta1-HDL as well as HDL subfractions.

Regarding PON1, there was no significant association with diet and exercise apart from finding that active individuals had a higher ratio of plasma PON1: standardized HDL PON1 activity. It is hard to give a reason for this. Nevertheless, the relationship between PON1 activity and cardiovascular risk has been described to be controversial at present (Kontush & Chapman, 2011). Furthermore, Soran et al. have explained that if PON1 were dissociated from HDL and other components like apoA-I and apoM, which is likely to take place once the sample is spun, will not retain their properties (Soran, Hama, Yadav & Durrington, 2012).

HDL properties and atherosclerosis:

Bivariate regression models were used to look at the interaction between lifestyle factors and the association of %HDL subfractions and HDL oxidation potential to cIMT (**Table 6.7-6.9**). Or in another word, we explored to find out to what extent are %HDL2b, %HDL3b and HDL oxidation potentialstatistically independent determinants of atherosclerosis. It appeared that the association between %HDL2b, %HDL3b and HDL oxidation with cIMT was attenuated by gender, which is not a modifiable risk factor, and those associations were not affected by lifestyle factors . Age also explained some of the increase in %HDL3b in the model displayed in **Table 6.8** but it was only borderline significant. Cigarette smoking was a predictor affected the association between HDL oxidation with cIMT in the model displayed in **Table 6.9.** The fact that men and women have different levels of atherosclerosis is well-known and HDL may be a factor in this in addition to other factors such as oestrogen hormones.

The new finding in this study was that HDL oxidation potential was related to cIMT (**Table 6.9**). Inclusion of lifestyle factors in the model did not lead to marked attenuation to the association of HDL maximum propagation rate with cIMT although the P-value became attenuated for gender and smoking. This distinguished HDL oxidation potential from HDL subfraction distribution in relationship to the association to cIMT. However, in the same

model, **Table 6.9,** when %HDL2b or %HDL3b was included, the association of HDL oxidation potential with cIMT was attenuated. In the same way, including HDL oxidation in the model in **Tables 6.7** and **6.8** led to attenuation of the association of HDL subfractions with cIMT. This suggested that at least some of the association of HDL oxidation potential with atherosclerosis is explained by its relationship to the HDL subfraction distribution.

Finally, in **Chapter 5**, it was shown that there was a significant association between %HDL2b and HDL oxidation. In **Table 6.10**, it can be seen that this relationship is not affected by lifestyle factors we examined. In **Table 6.5 and 6.6**, a number of lifestyle factors were determinants of both %HDL2b (including alcohol intake, smoking status and physical activity) and also of HDL oxidizability (including alcohol intake and smoking) (**Table 6.1**). The regression models showed that the association of %HDL2b with HDL oxidation was not affected by adjustment with alcohol, smoking, diet or physical activity. However, the association was explained by sex.

7 General Discussion

HDL in epidemiological studies shows a strong inverse relationship with risk of CVD that seems to be independent and consistent across populations. Some of the early trials where HDL raising drugs were used against placebo like the VA-HIT study (Rubins et al., 1999) revealed that, those on the fibrate, which raised HDL, had a 22% reduction in risk of CVD compared to those on placebo. However, later trials where fibrate was added to statin therapy did not show a risk reduction (e.g. The FIELD study) (Tonkin et al., 2012). Trials of other molecules like niacin in the THRIVE trial and CETP inhibition as in the DAL-outcomes failed to show any further risk reduction (Hassan, 2014). So the question is, are we failing to understand HDL sufficiently, so that we are not using the right intervention? Or alternatively, is HDL raising is a waste of time in people taking statin therapy?

As explained in the introduction, the idea that we needed to know more about structure, function and properties of HDL was the reason why this work was undertaken. The focus of the study was to look at four aspects of HDL: first, its role in oxidation processes, particularly its ability to inhibit LDL oxidation, the second aspect was looking at variations in preβeta1-HDL levels, the third was to examine PON1 activity which is a potential antioxidative agent, and the fourth was to investigate HDL subclass distribution.

7.1 Study population

The advantage of using the pSoBid population was that it was a cross sectional study which included individuals across a wide range of age, both men and women, and people who were likely to have very wide range of lifestyle because they came from extremes of the socioeconomic gradient. The clinical aspects of the study were already performed and so we were able to use stored samples which appeared to work well in the validation assays that we undertook.

7.2 Major findings

In this study, it was hypothesised that HDL antioxidative effects on LDL oxidation would be an important determinant of atherosclerosis. While the assay appeared to work well in that HDL from pSoBid samples inhibited the oxidation of a standardized LDL preparation in a consistent manner, there was no evidence that this inhibitory effect was related to the extent of atherosclerosis of coronary arteries of pSoBid population. However, it was surprising that oxidation of HDL itself was related to carotid atherosclerosis and as far as we can tell, this was a novel observation.

The findings **in Chapter 4**, **5** and **6** show strangely, HDL is more readily oxidized in those subgroups associated with less atherosclerosis. Also increased HDL oxidation susceptibility appeared to be related to lifestyle factors associated with less atherosclerosis disease such as moderate alcohol intake, not smoking, active exercise habit and higher intake of fruits and vegetables. HDL oxidation potential was importantly related to HDL subfractions, particularly the %HDL2b. A key observation in understanding the relationship of HDL oxidation potential to atherosclerosis is that in a model looking at the association of %HDL2b or %HDL3b with cIMT, inclusion of HDL Vmax attenuated strongly the relationship (**Table 6.7** and **Table 6.8**). On the other hand, in model relating HDL Vmax to cIMT, the attenuation upon addition of %HDL2b or %HDL3b was less marked (**Table 6.9**). These data suggest, at least in statistical terms, that the oxidative potential of HDL was more important than the percentage of HDL subfraction distribution in relationship to cIMT. This is a major finding of this thesis and it was a surprise since examining HDL oxidation was initially a secondary objective relative to the main focus which was on the HDL inhibition to LDL oxidation. The data on ability of HDL to inhibit LDL oxidation did not show any association with atherosclerosis.

7.3 HDL Oxidation Potential

There are a very limited number of papers on this topic in the literature. Some researchers have described that HDL oxidation may contribute to the formation of dysfunctional HDL (Navab, Anantharamaiah, Reddy, Van Lenten, Ansell & Fogelman, 2006; Navab et al., 2004). In our findings, however, HDL with more susceptibility to oxidation was a better protector

from atherosclerosis. Why should HDL oxidation be higher in groups protected from CVD risk is not clear? It is important to note that the property we tested was not the amount of HDL oxidized in the circulation, but rather the oxidation susceptibility in standardized oxidation assays particularly the maximum dienes formation or maximum propagation rate.

Lipoprotein oxidation studies, showed that maximum diene formation is related to the amount of polyunsaturated fat that are present (Parthasarathy, Khoo, Miller, Barnett, Witztum & Steinberg, 1990) or the presence of lipid- soluble antioxidant vitamins (Gilligan et al., 1994) so one explanation for our finding is that HDL that is more lipid rich, like HDL2b, and from people eating more fruits and vegetables have a higher oxidation potential. Regarding atherosclerosis, it may be that HDL particles, which are very abundant in the circulation, play a sacrificial role in that they are oxidized first and therefore have the capacity to prevent LDL oxidation in vivo.

7.4 Limitation of the Study

There are a number of limitation concerning the design and conduct of the experimental work:

The validity of the preβ1-HDL assay - ideally that would require further validation e. by testing the specificity of the ELISA with other subclasses of HDL.

1. The studies of the PON1 enzyme may be compromised by the centrifugation step used in HDL population as described by Soran et al as when PON1 are dissociated from the lipid phase and other components of HDL, such as apoA-I and apoM, all of which may be required for HDL (through its PON1 component) to hydrolyse more lipophilic substrates (Soran, Hama, Yadav & Durrington, 2012).

2. In the pSoBid study, we did not study LDL oxidation itself as a predictor of atherosclerosis.

3. Due to resource constraints, we could only study about half of the pSoBid population in the HDL oxidation study. This limited the statistical power of the study.

4. pSoBid is a cross-sectional sectional study, and as such exposure and outcome variables are measured at the same time. We cannot rule out the possibility that atherosclerosis itself and its associated consequences, such as inflammation, may directly influence HDL oxidation susceptibility (reverse causality).

7.5 Future experiments and conclusion

From the work presented in this thesis, it is possible that it is worthwhile exploring in more detail what are the determinants of HDL susceptibility to oxidation; is it regulated by the size of particles, or the lipid composition or the protein or enzyme content. One way to get better insight would be to perform experiments where HDL subclasses are isolated and tested individually for oxidation potential. Other in vivo experiments would be to alter the diet or to get people to stop smoking as only change and then test HDL- susceptibility to oxidation. Repeating these experiments in a prospective large cohort study, although a large undertaking, would improve statistical power, and allow us to investigate whether changes in HDL oxidation susceptibility precede future development in atherosclerosis.

In conclusion, this work has added a further potentially important property to the whole range of HDL functions that have been already described in the literature. It needs to be tested in other subject groups but given the association with atherosclerosis, it is a potentially important finding.

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9 Appendices

Appendix 1: pSoBid's questionnaire-**Chapter 2, 3, 4, 5 & 6**

Appendix 2: Table for pSoBid participants socioeconomic status and biomarkers- **Chapter 3, 4, 5 & 6**

Appendix 3: Consent form-**Chapter 3 & 4**

Appendix 4: Subject information sheet- **Chapter 3 & 4**

Appendix 5: Abstract. Preβ1-HDL as Part of the Lipoprotein Spectrum and Vascular Disease. Atherosclerosis, 2014; 236 (2): E307

Appendix 6: Poster- Preβ-HDL and pSoBid study. Heart UK 28th Annual Conference 2014.

Appendix 7: Lab work plan to run samples for Oxidation and PON1 assay- **Chapter 4**

Appendix 1: pSoBid's questionnaire-Chapter 2, 3, 4, 5 & 6

Version 3.1

Lifestyle Questionnaire

A. ENROLMENT

1. Time started ⌊_⌋⌊_⌋:⌊_⌋⌊_⌋
2. pSoBid1 Id: ⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋
3. Post Code ⌊_⌋⌊_⌋⌊_⌋ ⌊_⌋
4. Participant Initials ⌊_⌋⌊_⌋⌊_⌋
5. Date of Birth ⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋ (D D M M Y Y Y Y)
6. Residence ☐ Owner occupier ☐ Tenant (local authority) ☐ Tenant (private) ☐ Living with parents ☐ Other _____
7. Dependents ☐ None ☐ Dependent children under 16 ☐ Dependent relatives ☐ Both dependent children and relatives

   Total Dependents ⌊_⌋⌊_⌋
8. Sex ☐ Male ☐ Female
9. Marital Status ☐ Married ☐ Single ☐ Divorced ☐ Separated ☐ Widowed ☐ Co-habiting
10. Date of interview ⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋ (D D M M Y Y Y Y)
11. Interviewer _____

Version 3.1

A3. DRUG HISTORY

A3a. Please mention all the "prescribed" drugs you are taking now?

Medication

1. _____
2. _____
3. _____
4. _____
5. _____
6. _____
7. _____
8. _____
9. _____
10. _____

A3b. Please mention all the "over-the-counter" drugs you are taking now?

Medication

1. _____
2. _____
3. _____
4. _____
5. _____
6. _____
7. _____
8. _____
9. _____
10. _____

Version 3.1

A4. CHEST PAIN

A4a. Have you ever had a pain or discomfort in your chest? ☐ No ☐ Yes

If No, go to Question B1
If Yes, continue

A4b. Do you get this pain or discomfort when you walk uphill or hurry? ☐ No ☐ Yes

A4c. Do you get this pain or discomfort when you walk at an ordinary pace on the level? ☐ No ☐ Yes

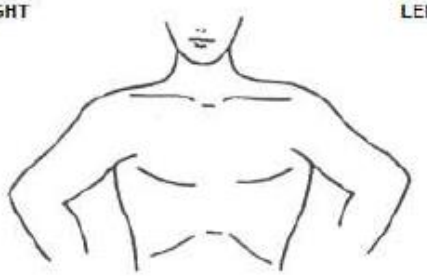
A4d. When you get pain or discomfort in your chest what do you do?
(please tick one box only) ☐ Stop ☐ Slow down ☐ Continue at the same pace

A4e. Does the pain or discomfort go away when you stand still? ☐ No ☐ Yes

A4f. How soon before the pain or discomfort goes away? ☐ 10 minutes or less ☐ More than 10 minutes

A4g. Where do you get this pain or discomfort? *(Mark the place(s) with an 'X' on the diagram below)*

RIGHT LEFT



A4h. Have you ever had a severe pain across the front of your chest last for half an hour or more? ☐ No ☐ Yes

If No, go to Question B1
If Yes, continue

A4i. Did you talk to a doctor about it? ☐ No ☐ Yes

If No, go to Question A4k
If Yes, continue

A4j. What did the doctor say it was? _____

_____

A4k. How many of these attacks have you had? ⌊__⌋

A4l. Have you ever had heart trouble suspected or confirmed? ☐ No ☐ Yes

If No, go to Question B1
If Yes, continue

A4m. When was the first time? *(Give year)* ⌊Y Y Y Y⌋

A4f. Have you ever had either of the following operations to improve the circulation to your heart? (tick all that apply)
☐ Coronary artery bypass surgery ☐ Balloon angioplasty

pSoBid1D

Version 3.1

B. DENTAL QUESTIONS (Periodontal disease)

B1a. Have you seen a dentist? ☐ No ☐ Yes

B1b. What year did you last visit your dentist? ☐☐☐☐ Y Y Y Y

B2. Has your dentist ever told you that you have gum disease or "periodontal disease"? ☐ No ☐ Yes

B3. Do your gums bleed when you brush them? ☐ No ☐ Yes

B4. Do you still have some of your own teeth in your mouth? ☐ No ☐ Yes

C. QUESTIONS ABOUT SMOKING

C1. Have you ever smoked regularly? ☐ No **If No, go to C8** ☐ Yes If Yes, continue

C2. What did/do you smoke? ☐ Cigarettes ☐ Other

If Other, specify_____

C3. Have you ever smoked cigarettes regularly? (by regularly we mean at least one cigarette a day for 12 months or more.) ☐ No ☐ Yes, current smoker ☐ Yes, ex-smoker

C4. If Yes, current smoker, about how many cigarettes a day do you usually smoke? ☐☐☐

C5. If you are an ex-smoker, about how many cigarettes a day did you usually smoke? ☐☐☐

C6. How old were you when you stopped smoking cigarettes regularly? ☐☐ years

C7. How old were you when you started smoking cigarettes regularly? ☐☐ years

C8. Did either of your parents or guardians smoke regularly when you lived with them?

☐ No, neither parent smoked ☐ Yes, mother smoked
☐ Yes, father smoked ☐ Yes, both parents smoked ☐ Don't know

D. QUESTIONS ABOUT DRINKING

D1. Thinking of the last 7 days, how much of each of the following did you drink? (If it helps, think back over each day to this time last week)

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday	NA
Beer, lager, cider:	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐ pints	☐
Wine:	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐ glasses	☐
Martini, sherry, port:	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐ glasses	☐
Spirits:	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐ measures	☐
Other alcoholic drinks:	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐ glasses	☐

D2. In the last year how often have you had a hangover from drinking alcohol? (Select one only)

☐ At least once a week
☐ 2-3 times a month
☐ Once a month
☐ Less than once a month
☐ Not at all in the last year

pSoBid1

Version 3.1

E. QUESTIONS ON EATING HABITS

E1. What kind of bread do you usually eat? (Select one only)

- ☐ White
- ☐ Brown, granary, wheatmeal
- ☐ Wholemeal
- ☐ Do not have usual type
- ☐ Do Not Know
- ☐ Do not eat any type of bread
- ☐ Other kind

E2. What do you usually spread on your bread? (Select one)

- ☐ Butter
- ☐ Margarine
- ☐ Low fat spread
- ☐ Do not have usual type
- ☐ Don't know
- ☐ Do not use fat spread on bread

E3. a. What kind of milk do you usually use for drinks, in tea or coffee and on cereals etc? (Select one)

- ☐ Whole milk
- ☐ Semi-skimmed
- ☐ Skimmed
- ☐ Do not have usual type
- ☐ Dont Know
- ☐ Do not drink milk
- ☐ Other Kind

If Other, specify _____

E4. a. Do you drink tea or coffee? ☐ No ☐ Yes

If yes, do you usually take sugar in: (Do not include sweeteners)

b. Tea ☐ No ☐ Yes

c. Coffee ☐ No ☐ Yes

E5. At the table do you usually add salt to your food.. (Select one)

- ☐ Without tasting it first
- ☐ Generally After tasting
- ☐ Occasionally after tasting
- ☐ Rarely or never

Version 3.1

E6. Which type of breakfast cereal do you normally eat? (Select one)

- ☐ High fibre (eg All bran, Branflakes, Shredded Wheat, Muesli, Porridge, Weetabix)
- ☐ Others (eg, Cornflakes, Rice Krispies, Special K, Sugar Puffs, honey Smacks)
- ☐ Do not have a usual type
- ☐ Do not eat breakfast cereal

E7. At around 11 years of age in school, what did you usually do for lunch? Did you:-

- ☐ Eat a packed lunch
- ☐ Eat a school meal
- ☐ Other (this might include eating at home, at a cafe etc)

E7b. Please give details:_____

E8. On average, how often do you eat each of these foods. (Please only select one for each category of food)

Food	Per Day				Per Week			Per Month		
	6	4-5	2-3	Once	5-6	2-4	Once	1-3	< Once	NA
Breakfast cereal	☐0	☐1	☐2	☐3	☐4	☐5	☐6	☐7	☐8	☐
Fresh fruit	☐0	☐1	☐2	☐3	☐4	☐5	☐6	☐7	☐8	☐
Cooked green vegetables (fresh or frozen)	☐0	☐1	☐2	☐3	☐4	☐5	☐6	☐7	☐8	☐
Cooked root vegetables (fresh or frozen)	☐0	☐1	☐2	☐3	☐4	☐5	☐6	☐7	☐8	☐
Raw vegetables or salad (including tomatoes)	☐0	☐1	☐2	☐3	☐4	☐5	☐6	☐7	☐8	☐
Chips	☐0	☐1	☐2	☐3	☐4	☐5	☐6	☐7	☐8	☐
Potatoes, pasta, rice	☐0	☐1	☐2	☐3	☐4	☐5	☐6	☐7	☐8	☐
Meat	☐0	☐1	☐2	☐3	☐4	☐5	☐6	☐7	☐8	☐
Meat products (e.g. haggis, pâté)	☐0	☐1	☐2	☐3	☐4	☐5	☐6	☐7	☐8	☐
Poultry	☐0	☐1	☐2	☐3	☐4	☐5	☐6	☐7	☐8	☐
White fish	☐0	☐1	☐2	☐3	☐4	☐5	☐6	☐7	☐8	☐
Other types of fish	☐0	☐1	☐2	☐3	☐4	☐5	☐6	☐7	☐8	☐
Cheese	☐0	☐1	☐2	☐3	☐4	☐5	☐6	☐7	☐8	☐
Beans or pulses	☐0	☐1	☐2	☐3	☐4	☐5	☐0	☐7	☐8	☐
Sweets, chocolate	☐0	☐1	☐2	☐3	☐4	☐5	☐6	☐7	☐8	☐
Ice cream	☐0	☐1	☐2	☐3	☐4	☐5	☐6	☐7	☐8	☐
Crisps, savoury snacks	☐0	☐1	☐2	☐3	☐4	☐5	☐0	☐7	☐8	☐
Soft-fizzy drinks	☐0	☐1	☐2	☐3	☐4	☐5	☐6	☐7	☐8	☐
Cakes, scones, sweet pies or pastries	☐0	☐1	☐2	☐3	☐4	☐5	☐6	☐7	☐8	☐
Biscuits	☐0	☐1	☐2	☐3	☐4	☐5	☐6	☐7	☐8	☐

Version 3.1

F. ABOUT YOUR PHYSICAL ACTIVITY

F1. Are you at present in any type of work (including self-employed)? ☐ No, not working ☐ Yes, currently working

F2. We would like to know about your level of physical activity and the type and amount of physical activity involved in your work. Please tick one box that best corresponds to your present activities from the following four possibilities:

- ☐ Sedentary occupation: You spend most of your time sitting (such as in an office) or standing
- ☐ You spend most of your time standing or walking. However, your work does not require intense physical effort (eg shop assistant, hairdresser, guard, etc) or physical work
- ☐ This involves some physical effort, including handling of very heavy objects (eg plumber, cleaner, nurse, sports instructor, electrician, carpenter, etc) or Heavy manual work
- ☐ This involves very vigorous physical activity including handling of very heavy objects (eg docker, miner, bricklayer, construction worker, etc)

F3. Leisure activity: In a typical week during the past 12 months, how many hours did you spend on each of the following activities? (Put 0 if none)

F3. a. Walking, including walking to work, shopping and leisure:

1. in summer ⌊_ _⌋ hours per week ☐ NA
2. in winter ⌊_ _⌋ hours per week ☐ NA

F3. b. Cycling, including cycling to work and during leisure time:

1. in summer ⌊_ _⌋ hours per week ☐ NA
2. in winter ⌊_ _⌋ hours per week ☐ NA

F3. c. Gardening:

1. in summer ⌊_ _⌋ hours per week ☐ NA
2. in winter ⌊_ _⌋ hours per week ☐ NA

F3. d. Housework such as cleaning, washing, cooking, childcare: ⌊_ _⌋ hours per week ☐ NA

F3. e. Do-it-yourself: ⌊_ _⌋ hours per week ☐ NA

F3. f. Other physical exercise such as keep fit, aerobics, swimming, jogging:

1. in summer ⌊_ _⌋ hours per week ☐ NA
2. in winter ⌊_ _⌋ hours per week ☐ NA

F4. a. Vigorous exercise: In a typical week, during the past year did you practise any of these activities vigorously enough to cause sweating or a faster heartbeat? ☐ No ☐ Yes ☐ Don't Know

b. If yes, for how many hours per week in total did you practise such vigorous physical activity? (Put 0 if none) ⌊_ _⌋ hours per week

F5. Other Activity: In a typical day during the past 12 months, how many floors of stairs did you climb up?(Put 0 if none) ⌊_ _⌋ flights per day

Version 3.1

G. ABOUT YOUR CHILDHOOD

A person's experiences in childhood may affect their health in later life so we would like to ask you questions about your own childhood.

G1. Up to the age of 11 years who brought you up? (Select all that apply)

- ☐ Both my natural (biological) parents
- ☐ At least one of my natural (biological) parents
- ☐ Brought up by other relatives
- ☐ Brought up by adoptive parents
- ☐ Lived in children's home or was fostered

G2. At the time you were 11 years old, was your family home:

- ☐ Owned by your family (with or without a mortgage)
- ☐ Rented from the local council
- ☐ Rented from private land lord
- ☐ NV
- ☐ Other

If Other, specify _____

G3. At the time you were 11 years old, did your family own a car? ☐ No ☐ Yes

G4. At the time you were 11 years old, how many living rooms and bedrooms did the family home have?

Number of living rooms (include the kitchen if it was used as a living room): ⌊__⌋

Number of bedrooms: ⌊__⌋

G5. When you were aged 11 years old, how many children and adults lived in your family home?

Number of adults (aged 18 or over): ⌊__⌋

Number of children (aged under 18 including yourself): ⌊__⌋

G6. Have you moved out of the family home? ☐ No ☐ Yes

G6a. What age were you when you finally moved out of your family home? ⌊__⌋ years

G7. How many friends did you have in primary school compared to other children?

- ☐ More friends than other children
- ☐ About the same number of friends as other children
- ☐ Fewer friends than other children

G8. Did you ever experience being bullied by your class mates in primary school?

- ☐ Yes, very often
- ☐ Yes, sometimes
- ☐ No

G9. Since you were 11 years old, have you moved away to anywhere other than Glasgow for more than a year? ☐ No ☐ Yes

G9a. If Yes, what age were you when you first moved away from Glasgow for more than a year? ⌊__⌋ years

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H. ABOUT YOUR BIRTH WEIGHT AND PLACE OF BIRTH

H1. In which city/town or village were you born? ☐ NV

City_____ Town_____ Village_____

H2. Where did the birth take place?
☐ Home ☐ Hospital ☐ Don't Know ☐ Other specify_____ ☐ NV

H3. If Hospital, what was the name of the hospital where you were born? _____

H4. Do you know your birth weight? ☐ No ☐ Yes ☐ NV

H5. If yes, what was your birth weight?
(Birth weight in pounds and ounces) |_|_| pounds |_|_| ounces ☐ NV

H6. Please say where you obtained the information about your birth weight.
☐ Mother ☐ Other family member ☐ Don't Know ☐ Other specify_____ ☐ NV

J. QUESTIONS ABOUT YOUR PARENTS

Your health may be related to the health of your parents. We would therefore like to ask you about them. If you were adopted, please answer the questions in this section with respect to your adoptive parents.

J1. Is your father still alive? ☐ No ☐ Yes ☐ Don't know, Go to J4

J2. If Yes, how old is your father? (please write age in years) |_|_| years ☐ Don't know, Go to J4 ☐ NV

J3. If No, how old was your father when he died?
(please write age in years) |_|_| years ☐ Don't know, Go to J4 ☐ NV

J4. Is your mother still alive? ☐ No ☐ Yes ☐ Don't know, Go to J7

J5. If Yes, how old is your mother? (please write age in years) |_|_| years ☐ Don't know, Go to J7 ☐ NV

J6. If No, how old was your mother when she died?
(please write age in years) |_|_| years ☐ Don't know, Go to J7 ☐ NV

J7. Please give the title of your father's job at the time you were 11 years old (or his last job if he died or retired before this time), and describe what he actually did.

J7. a. Job Title: _____ ☐ NV

b. Job Description: _____

_____ ☐ NV

c. ☐ Don't know

J8. In that job, was your father...
☐ Manager
☐ A foreman or supervisor
☐ An employee (other than manager or foreman)
☐ Self-employed with employees
☐ Self-employed/freelance without employees
☐ Don't know
☐ NV

Version 3.1

K. QUESTIONS ABOUT YOUR EDUCATION

K1. What secondary school did you go to when you left primary school?

School _____ Town/City _____

K2. At what age did you leave secondary school? ☐☐ years

K3. Have you been in further or higher education since you left school? ☐ No ☐ Yes

K4. For how many years in total were you in full or part-time further or highter education? If less than 1 year write 0.

Full-time ☐☐ years

Part-time ☐☐ years

K5. Which of the following qualifications do you have? Select all that apply.

K5. a. No formal qualifications ☐

K5. b. Degree or degree level qualification (including a higher degree) ☐

K5. c. Teaching qualification, HNC/HND, BECT/TECT higher, BTEC (higher) City and Guilds Full Technological Certificate, Nursing Qualification ☐

K5. d. Certificate in Sixth Year Studies, Highers, A-levels, ONC? OND? VEC? TEC (not higher), City and Guilds Advanced/Final Level ☐

K5. e. 'O' Grade Passes, 'O'Level passes, CSE grade 1, School certificate or matric, City and Guild Craft/Ordinary level ☐

K5. f. CSE grades 2-5, Clerical/commercial qualifications ☐

K5. g. CSE ungraded ☐

K5. h. Other ☐

If Other, specify _____

L. EMPLOYMENT QUESTIONS

L1. Which of these best describes your current situation? (Select one)

Go to question L3
- ☐ In paid work(including self-employed)
- ☐ Unemployed

Go to question L2
- ☐ Permanently sick or disabled
- ☐ Retired from paid work
- ☐ Looking after the home or family
- ☐ A full-time student
- ☐ Other

If Other, specify _____

L2. Have you ever been in paid employment or been self-employed?
- ☐ No, Go to M1
- ☐ Yes, Go to L3

L3. Please give the title of your present or most recent paid job (or period of self-employment), and describe what you actually do/did?

Title _____

Description _____

_____

_____

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L. EMPLOYMENT QUESTIONS continued...

L4. In that job, are you or were you... (Select one)

- ☐ a manager
- ☐ a foreman or supervisor
- ☐ an employee (other than manager or foreman)
- ☐ self-employed with employees
- ☐ self-employed/freelance without employees

L5. How many people work(ed) for your employer at the place where you work(ed)? (Select one)

- ☐ 1-9 persons
- ☐ 10-24 persons
- ☐ 25-499 persons
- ☐ 500 or more persons

M. INCOME QUESTIONS

M1. There has been a lot of talk about health and income. I would like to get some idea of your household's income. Can you please tell me which kind of income you (and your husband/wife/partner) receive?

- ☐ Earnings from employment or self-employment
- ☐ State retirement pension
- ☐ Pension from former employer
- ☐ Personal pension
- ☐ Child Benefit
- ☐ Job-Seekers Allowance
- ☐ Income Support
- ☐ Working families tax credit, Child tax credit or working tax credit
- ☐ Housing benefit
- ☐ Other state benefits
- ☐ Interest from savings and investments (eg stocks and shares)
- ☐ other kinds of regular allowance from outside your household (eg maintenance, student's grants, rent)
- ☐ No source of income
- ☐ Does not want to answer

M2. What is your total income in a year?

1. ☐ Less than £15,000
2. ☐ £16,000 to £25,000
3. ☐ £26,000 to £35,000
4. ☐ £36,000 to £45,000
5. ☐ £45,000 or more

☐ RTA

N. END OF VISIT

N1. Time completed ⌊_⌋⌊_⌋ ⌊_⌋⌊_⌋

N2. Date of 2nd interview ⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋ D D M M Y Y Y Y

N3. Time of 2nd visit ⌊_⌋⌊_⌋ ⌊_⌋⌊_⌋

Appendix 2: pSoBid's characteristics of study participants

	Least deprived (n=342)	Most deprived (n=324)	Least minus most deprived (adjusted for age and sex (95% CI))	P value
Classic risk factors (behavioural)				
Smoking				
Ever smoked regularly	121 (35.4%)	241 (74.4%)	12.07 (7.33 to 19.88)†	<0.0001
Current cigarette smoker	21 (6.1%)	131 (40.4%)		
Data missing	0 (0)	0 (0)		
Physical activity				<0.0001
Inactive	82 (24%)	160 (49%)		
Moderately inactive	84 (25%)	37 (11%)		
Moderately active	87 (25%)	71 (22%)		
Active	89 (26%)	56 (17%)		
Data missing	0 (0)	0 (0)		
Classic risk factors (physiological)				
Cholesterol (mmol/l)	5.29 (1.03)	4.95 (1.05)	0.35 (0.19 to 0.51)	<0.0001
Data missing	7 (0)	14 (0)		
Triglycerides (mmol/l)	1.19	1.44	−0.20 (−0.27 to −0.12)‡	<0.0001
Data missing	7 (0)	14 (0)		
Low density lipoprotein cholesterol (mmol/l)	3.16 (0.87)	2.86 (0.88)	0.31 (0.17 to 0.44)	<0.0001
Data missing	7 (0)	18 (0)		
High density lipoprotein cholesterol (mmol/l)	1.43 (0.38)	1.30 (0.39)	0.13 (0.08 to 0.19)	<0.0001
Data missing	7 (0)	14 (0)		

	Least deprived (n=342)	Most deprived (n=324)	Least minus most deprived (adjusted for age and sex (95% CI))	P value
Glucose (mmol/l)	5.15 (0.69)	5.42 (1.90)	-0.41 (-0.69 to -0.14)	0.0088
Data missing	19 (7)	35 (16)		
Weight (kg)	78.7 (15.3)	78.2 (18.4)	0.34 (−2.06 to 2.74)	0.78
Data missing	1 (0)	1 (0)		
Waist/hip ratio	0.88(0.08)	0.92(0.09)	-0.04 (-0.05 to -0.03)	<0.0001
Data missing	3 (1)	4 (2)		
BMI	26.9 (4.49)	28.7 (6.34)	−1.81 (−2.64 to −0.98)	<0.0001
Data missing	2 (0)	2 (0)		
Blood pressure (mm Hg)	135 (17.8)/81.4 (10.3)	136 (20.0)/81.1 (11.6)	−0.75 (−3.44 to 1.93)/0.27 (−1.32 to 1.87)	0.58/0.74
Data missing	2 (0)	2 (0)		
Emerging risk factors: insulin resistance/fat mass				
Insulin (mU/l)	6.62 (4.91)	7.72 (5.97)	−1.81 (−3.29 to −0.34)	0.011
Data missing	18 (8)	41 (26)		
Homeostasis model of assessment-insulin	1.52 (1.22)	1.81 (1.60)	−0.80 (−1.15 to −0.44)	0.015
Data missing	24 (10)	49 (31)		
Leptin (ng/ml)	18.7 (16.8)	23.7 (24.0)	−4.46 (−7.24 to −1.67)	0.0017
Data missing	14 (0)	20 (0)		
Emerging risk factors: inflammation/endothelial dysfunction				
C reactive protein (mg/l)	1.16	2.07	−0.57 (−0.74 to −0.41)‡	<0.0001
Data missing	11 (4)	19 (5)		

	Least deprived (n=342)	Most deprived (n=324)	Least minus most deprived (adjusted for age and sex (95% CI))	P value
Interleukin 6 (pg/ml)	1.36	2.08	-0.43(-0.53 to -0.33)‡	<0.0001
Data missing	13 (3)	24 (8)		
Intercellular adhesion molecule 1 (ng/ml)	235.8	302.4	−0.25 (−0.29 to −0.21)‡	<0.0001
Data missing	10 (0)	20 (4)		
Emerging risk factors: haemostasis				
von Willebrand factor (IU/dl)	129 (39)	155 (47)	−26 (−33 to −20)	<0.0001
Data missing	8 (0)	23 (0)		
Fibrinogen (g/l)	3.23 (0.60)	3.50 (0.80)	−0.26 (−0.36 to −0.15)	<0.0001
Data missing	10 (0)	23 (0)		
D-dimer (ng/ml)	130 (97)	155 (102)	−24 (−39 to −8.9)	0.0018
Data missing	8 (0)	23 (0)		
Markers of individual socioeconomic status				
Height (cm)	171.0 (9.4)	165.0 (8.7)	5.72 (4.77 to 6.66)	<0.0001
Data missing	2 (0)	1 (0)		
Leg length (cm)	81.9 (6.0)	78.7 (5.4)	3.07 (2.31 to 3.83)	<0.0001
Data missing	41 (0)	21 (0)		
People per room at age 11 years	1.2 (0.5)	1.8 (0.9)	−0.67 (−0.78 to −0.56)	<0.0001
Data missing	0 (0)	2 (0)		

	Least deprived (n=342)	Most deprived (n=324)	Least minus most deprived (adjusted for age and sex (95% CI))	P value
Father's Register General Social class				<0.0001
0 Data not classifiable	15 (4%)	17 (5%)		
I Professional	30 (9%)	1 (0.3%)		
II Managerial & technical	130 (38%)	27 (8%)		
IIIN Skilled non-manual	30 (9%)	13 (4%)		
IIIM Skilled manual	98 (29%)	155 (48%)		
IV Partly skilled	22 (7%)	43 (13%)		
V Unskilled	10 (3%)	42 (13%)		
Unknown to participant	4(1%)	16 (5%)		
Unemployed	1 (0.3%)	10 (3%)		
Data missing	2 (0)	0 (0)		
Participant's Registrar General social class				<0.0001
0 Data not classifiable	1 (0.3%)	16 (5%)		
I Professional	58 (17%)	5 (2%)		
II Managerial & technical	193 (57%)	57 (18%)		
IIIN Skilled non-manual	59 (17%)	52 (16%)		
IIIM Skilled manual	16 (5%)	87 (27%)		
IV Partly skilled	10 (3%)	70 (22%)		
V Unskilled	2 (0.6%)	35 (11%)		
Unemployed	1 (0.3%)	2 (0.6%)		
Data missing	2 (0)	0 (0)		

	Least deprived (n=342)	Most deprived (n=324)	Least minus most deprived (adjusted for age and sex (95% CI))	P value
Annual household income				<0.0001
<£15 000	12 (4%)	186 (57%)		
£16-25 000	29 (9%)	78 (24%)		
£26-35 000	40 (12%)	21 (7%)		
£36-45 000	44 (13%)	13 (4%)		
>£45 000	187 (55%)	10 (3%)		
Data missing	30 (0)	16 (0)		
Total education (years)	16.1 (3.6)	11.8 (2.5)	4.32 (3.85 to 4.79)	<0.0001
Data missing	0 (0)	0 (0)		

Descriptive statistics are presented as mean (standard deviation) for continuous variables and count (%) for categorical outcomes. Geometric means are shown for trigycerids, C reactive protein, interleukin 6 , and intercellular adhesion molecule 1.
In ''Data missing'' fields, figures in brackets indicate the number of values that were removed on the basis of known pre-analytical factors or biological implausibility.

Appendix 3: Informed Consent and Health Questionnaire Sheet oxidation trials and plasma/ LDL/ & HDL Pools- (Chapter 2, 3 & 4)

INFORMED CONSENT FOR QUALITY CONTROL PUROSES

You are being invited to donate a small amount of blood which will be used in the Vascular Biochemistry Laboratory, 2nd floor, McGregor Building for method development or quality control purposes.

No significant risks are associated with taking blood samples. However, it can cause minor bruising and only in rare cases may cause inflammation and possible infection. The sample will be taken by a member of staff who has qualifications in phlebotomy.

Please sign below if you are willing to participate.

I agree to donate blood for the above purpose.

Sign …… Date ……..

Print Name …….

Researcher:

Sign ……. Date …….

Print Name …….

Appendix 4: Health Questionnaire – Chapter 2,3 & 4

Title of Project: Structure and Function of HDL

Participant number [ ]	age:_____	**M** [ ]	**F** [ ]

Please complete this brief questionnaire to confirm fitness to participate:

Have you been diagnosed with or currently taking medication for:

	Diagnosis	**Medication**
(a) High cholesterol	[ ]	[ ]
(b) High triglycerides	[ ]	[ ]
(c) Hypertension	[ ]	[ ]
(d) Diabetes	[ ]	[ ]
(e) Heart Disease	[ ]	[ ]
(f) Other diseases	[ ]	[ ]

Please mention the other diseases if any:

_____

Do you currently smoke? **Yes [ ]** **No [ ]**

Have you ever smoked? **Yes [ ]** **No [ ]**

Are you fasting for 12 hours **Yes [ ]** **No [ ]**

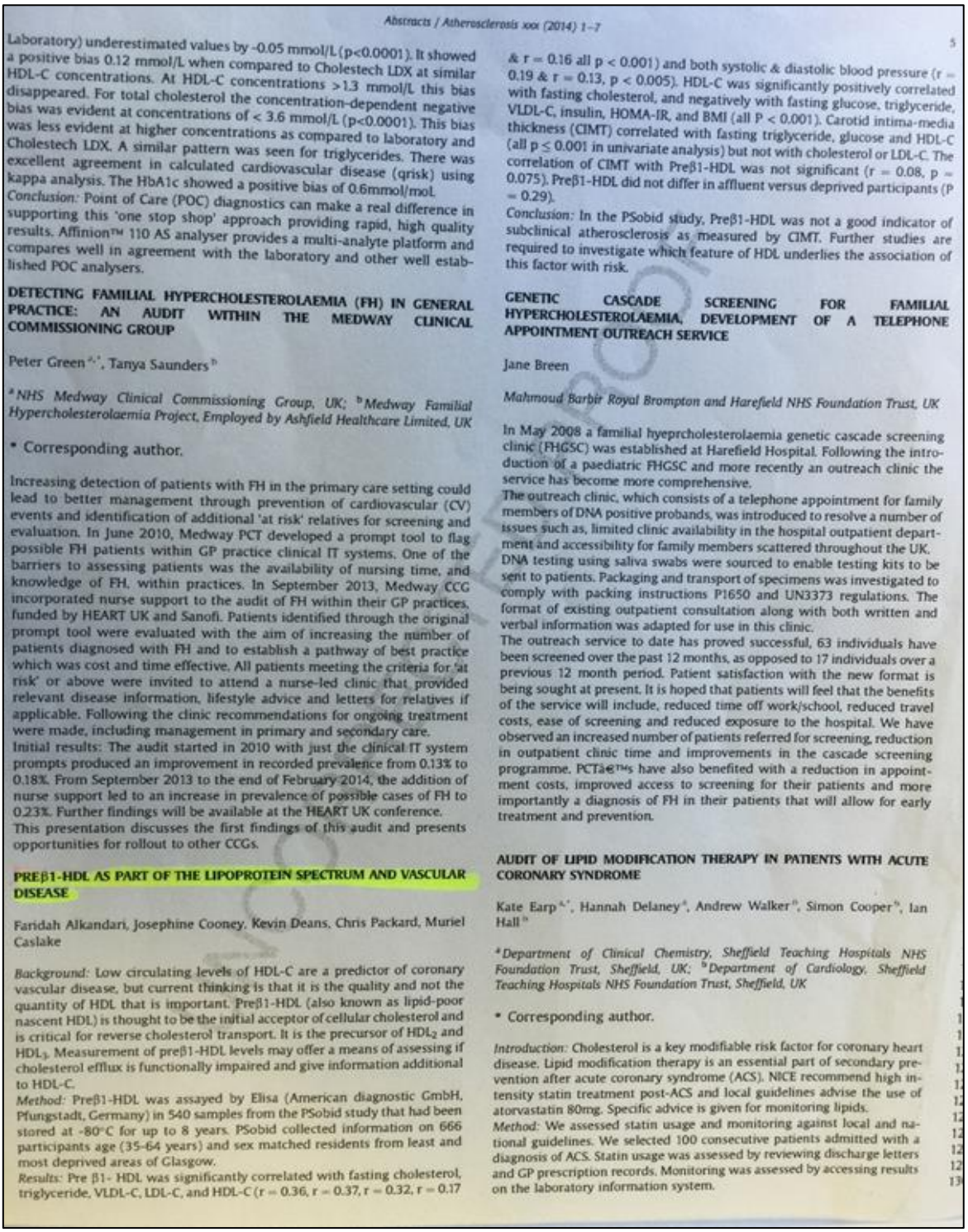
Do you take any vitamins or minerals **Yes [ ]** **No [ ]**

Do you take any type of supplement? **Yes [ ]** **No [ ]**

If yes, please mention their names? _____

_____	_____	_____
Name of the Participant	Date	Signature
_____	_____	_____
Name of the researcher	Date	Signature

Appendix 5: Abstract. Preβ1-HDL as Part of the Lipoprotein Spectrum and CVD. - Chapter 5.



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Laboratory) underestimated values by -0.05 mmol/L ($p<0.0001$). It showed a positive bias 0.12 mmol/L when compared to Cholestech LDX at similar HDL-C concentrations. At HDL-C concentrations >1.3 mmol/L this bias disappeared. For total cholesterol the concentration-dependent negative bias was evident at concentrations of < 3.6 mmol/L ($p<0.0001$). This bias was less evident at higher concentrations as compared to laboratory and Cholestech LDX. A similar pattern was seen for triglycerides. There was excellent agreement in calculated cardiovascular disease (qrisk) using kappa analysis. The HbA1c showed a positive bias of 0.6mmol/mol.
Conclusion: Point of Care (POC) diagnostics can make a real difference in supporting this 'one stop shop' approach providing rapid, high quality results. Affinion™ 110 AS analyser provides a multi-analyte platform and compares well in agreement with the laboratory and other well established POC analysers.

DETECTING FAMILIAL HYPERCHOLESTEROLAEMIA (FH) IN GENERAL PRACTICE: AN AUDIT WITHIN THE MEDWAY CLINICAL COMMISSIONING GROUP

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* Corresponding author.

Increasing detection of patients with FH in the primary care setting could lead to better management through prevention of cardiovascular (CV) events and identification of additional 'at risk' relatives for screening and evaluation. In June 2010, Medway PCT developed a prompt tool to flag possible FH patients within GP practice clinical IT systems. One of the barriers to assessing patients was the availability of nursing time, and knowledge of FH, within practices. In September 2013, Medway CCG incorporated nurse support to the audit of FH within their GP practices, funded by HEART UK and Sanofi. Patients identified through the original prompt tool were evaluated with the aim of increasing the number of patients diagnosed with FH and to establish a pathway of best practice which was cost and time effective. All patients meeting the criteria for 'at risk' or above were invited to attend a nurse-led clinic that provided relevant disease information, lifestyle advice and letters for relatives if applicable. Following the clinic recommendations for ongoing treatment were made, including management in primary and secondary care.
Initial results: The audit started in 2010 with just the clinical IT system prompts produced an improvement in recorded prevalence from 0.13% to 0.18%. From September 2013 to the end of February 2014, the addition of nurse support led to an increase in prevalence of possible cases of FH to 0.23%. Further findings will be available at the HEART UK conference.
This presentation discusses the first findings of this audit and presents opportunities for rollout to other CCGs.

PREβ1-HDL AS PART OF THE LIPOPROTEIN SPECTRUM AND VASCULAR DISEASE

Faridah Alkandari, Josephine Cooney, Kevin Deans, Chris Packard, Muriel Caslake

Background: Low circulating levels of HDL-C are a predictor of coronary vascular disease, but current thinking is that it is the quality and not the quantity of HDL that is important. Preβ1-HDL (also known as lipid-poor nascent HDL) is thought to be the initial acceptor of cellular cholesterol and is critical for reverse cholesterol transport. It is the precursor of HDL_2 and HDL_3. Measurement of preβ1-HDL levels may offer a means of assessing if cholesterol efflux is functionally impaired and give information additional to HDL-C.
Method: Preβ1-HDL was assayed by Elisa (American diagnostic GmbH, Pfungstadt, Germany) in 540 samples from the PSobid study that had been stored at -80°C for up to 8 years. PSobid collected information on 666 participants age (35-64 years) and sex matched residents from least and most deprived areas of Glasgow.
Results: Pre β1- HDL was significantly correlated with fasting cholesterol, triglyceride, VLDL-C, LDL-C, and HDL-C ($r = 0.36$, $r = 0.37$, $r = 0.32$, $r = 0.17$ & $r = 0.16$ all $p < 0.001$) and both systolic & diastolic blood pressure ($r = 0.19$ & $r = 0.13$, $p < 0.005$). HDL-C was significantly positively correlated with fasting cholesterol, and negatively with fasting glucose, triglyceride, VLDL-C, insulin, HOMA-IR, and BMI (all $P < 0.001$). Carotid intima-media thickness (CIMT) correlated with fasting triglyceride, glucose and HDL-C (all $p \leq 0.001$ in univariate analysis) but not with cholesterol or LDL-C. The correlation of CIMT with Preβ1-HDL was not significant ($r = 0.08$, $p = 0.075$). Preβ1-HDL did not differ in affluent versus deprived participants ($P = 0.29$).
Conclusion: In the PSobid study, Preβ1-HDL was not a good indicator of subclinical atherosclerosis as measured by CIMT. Further studies are required to investigate which feature of HDL underlies the association of this factor with risk.

GENETIC CASCADE SCREENING FOR FAMILIAL HYPERCHOLESTEROLAEMIA, DEVELOPMENT OF A TELEPHONE APPOINTMENT OUTREACH SERVICE

Jane Breen

Mahmoud Barbir Royal Brompton and Harefield NHS Foundation Trust, UK

In May 2008 a familial hyeprcholesterolaemia genetic cascade screening clinic (FHGSC) was established at Harefield Hospital. Following the introduction of a paediatric FHGSC and more recently an outreach clinic the service has become more comprehensive.
The outreach clinic, which consists of a telephone appointment for family members of DNA positive probands, was introduced to resolve a number of issues such as, limited clinic availability in the hospital outpatient department and accessibility for family members scattered throughout the UK. DNA testing using saliva swabs were sourced to enable testing kits to be sent to patients. Packaging and transport of specimens was investigated to comply with packing instructions P1650 and UN3373 regulations. The format of existing outpatient consultation along with both written and verbal information was adapted for use in this clinic.
The outreach service to date has proved successful, 63 individuals have been screened over the past 12 months, as opposed to 17 individuals over a previous 12 month period. Patient satisfaction with the new format is being sought at present. It is hoped that patients will feel that the benefits of the service will include, reduced time off work/school, reduced travel costs, ease of screening and reduced exposure to the hospital. We have observed an increased number of patients referred for screening, reduction in outpatient clinic time and improvements in the cascade screening programme. PCTâ€™s have also benefited with a reduction in appointment costs, improved access to screening for their patients and more importantly a diagnosis of FH in their patients that will allow for early treatment and prevention.

AUDIT OF LIPID MODIFICATION THERAPY IN PATIENTS WITH ACUTE CORONARY SYNDROME

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Introduction: Cholesterol is a key modifiable risk factor for coronary heart disease. Lipid modification therapy is an essential part of secondary prevention after acute coronary syndrome (ACS). NICE recommend high intensity statin treatment post-ACS and local guidelines advise the use of atorvastatin 80mg. Specific advice is given for monitoring lipids.
Method: We assessed statin usage and monitoring against local and national guidelines. We selected 100 consecutive patients admitted with a diagnosis of ACS. Statin usage was assessed by reviewing discharge letters and GP prescription records. Monitoring was assessed by accessing results on the laboratory information system.

Appendix 6: Poster- Preβ-HDL and pSoBid study. Heart UK 28th Annual Conference 2014- Chapter 5

Pre β1-HDL and PSoBid study

Faridah Alkandari, Josephine Cooney, Kevin Deans, Chris Packard and Muriel Caslake

UNIVERSITY of GLASGOW

Vascular Biochemistry, University of Glasgow

Introduction:

PSoBid is a cross sectional study carried out in 2007 in Glasgow. 666 Participants aged 35-66, equal number of males & females from the most and least deprived areas [1]. Deprivation was associated with increased carotid plaque score and intima-media thickness (cIMT) which is a surrogate measure of atherosclerosis. Systolic blood pressure was associated with cIMT ($p<0.001$) . HDL cholesterol had a negative association with cIMT ($p<0.001$) but LDL cholesterol was not correlated with cIMT ($p=0.055$).

Pre-β1-HDL is the initial plasma acceptor of cell-derived cholesterol. It is important in reverse cholesterol transport. Pre-β1-HDL consist of one or two molecules of apolipoprotein A-I (apoA-1) and small amounts of phospholipids and unesterified cholesterol [2]

Methods

540 Li-Hep PsoBid samples, not previously thawed, were measured for Pre-β1-HDL using an ELISA Kit (American diagnostica GmbH, Pfungstadt, Germany).
Inter-assay C.V. of 14.2% & intra-assay C.V. of 9.2%
Statistics was done by using SPSS -21

Results

Variable	Tot. Cohort	Male	Female	Least Deprived	Most Deprived	P values
	n=540	n=263	n=277	n=279	n=261	
Cholesterol	5.12	5.04	5.05	5.25	4.95	*N.S
(mmol.l^{-1})	(1.02)	(1.05)	(0.059)	(0.99)	(1.02)	**0.001
TG	1.20	1.3	1.15	1.15	1.33	*<0.001
(mmol.l^{-1})	(0.04)	(0.073)	(0.048)	(0.04)	(0.08)	**<0.001
VLDL	0.65	0.70	0.65	0.63	0.70	*0.001
(mmol.l^{-1})	(0.02)	(0.03)	(0.02)	(0.22)	(0.31)	**0.002
LDL	2.99	2.98	3.01	3.14	2.84	*N.S
(mmol.l^{-1})	(0.87)	(0.89)	(0.85)	(0.83)	(0.88)	**<0.001
HDL	1.30	1.20	1.50	1.40	1.25	*<0.001
(mmol.l^{-1})	(0.02)	(0.02)	(0.02)	(0.02)	(0.02)	**<0.001
Apo A-1	1.40	1.30	1.50	1.40	1.40	*<0.001
g.l^{-1}	(0.01)	(0.02)	(0.02)	(0.02)	(0.02)	**0.001
Apo B	0.90	0.90	0.90	0.90	0.90	*0.04
g.l-1	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)	**N.S
IMT-ccMean	0.67	0.69	0.66	0.66	0.69	*<0.001
(mm)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)	**N.S
Plaque Score	1.00	1.00	0.00	0.00	1.00	*0.007
	(0.035)	(0.05)	(0.047)	(0.043)	(0.053)	**<0.001
Pre-β1-HDL	54.62	51.6	56.62	54.85	53.81 (2.19)	* N.S
(mg.ml^{-1})	(1.29)	(2.04)	(1.60)	(1.43)		**N.S

Descriptive statistics are presented as mean (standard deviation) for normal distributed variables and median (standard error of mean) for not normal distributed variables. *P value for Male X Females groups **P values for Least Deprived X Most Deprived groups

Table1: Subjects' Characteristic including Preβ1-HDL

Model	Ln IMT-ccmean		
	Coefficient	t	P-Value
(Constant)	-0.921	-10.720	0.000
Systolic BP	0.003	4.706	0.000
Statin	0.125	4.314	0.000
LDL	0.032	3.239	0.001
Plaque count	0.017	3.345	0.001
Other Heart Trouble	0.106	2.935	0.004
Diastolic BP	-0.003	-2.543	0.011
Area	-0.043	-2.529	0.012

R^2=0.305 , P-Value <0.001

Table2: Effect of independent variables on IMT-ccmean using forward selection

Model	Ln Preβ1-HDL		
	Coefficient	t	P-value
(Constant)	2.295	12.850	0.000
VLDL	0.319	6.974	0.000
APOA1	0.571	8.145	0.000
Systolic BP	0.003	3.134	0.002
APOB	0.236	2.688	0.007

R^2=0.307, P-Value < 0.001

Table3: Effect of independent variables on pre-β1-HDL using forward selection

- Fitted model in table 2 was very highly significant (P-Value <0.001).
- The independent variables were able to explain 30.5 % (R^2=0.305) of variation in the IMT-ccmean.
- Fitted model in table 3 was a very highly significant (p-value <0.001)
- The independent variables were able to explain 30.7 % (R^2=0.307) of variation in the pre-β1-HDL

Discussion & Interpretation

In this subsection of PSoBid participants, Pre-β1-HDL was not associated with IMT-ccMean. Therefore, other subfractions of HDL might be implicated in these findings

References

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Castro G. et al 1988. Bioch. 27

Appendix 7: Lab work plan to run samples for Oxidation and PON1 assay- Chapter 4

DAY	TIME	TASK
Sat	11:00	Save oxidation data on Soft max pro files in J & M drive
Sun	11:00	Take 10 new samples of PSOBIDs from freezers to defrost
	11:00	Take 1 LDL control from Freezer to defrost
	11:30-12:00	Prepare 2 L PBS & Tube for Dialysis
	12:00-12:30	Prepare 10 PSOBIDS for HDL fist spin/ Store the rest for PON assay & Analyse
	11:30	put LDL control for dialysis
	12:30	Change dialysis buffer
	3:00-3:30	Isolate VLDL/LDL form PSOBIDS and start second spin
Mon/Tues/Wed/ Thurs./Fri	8:00-8:15	Save Oxidation data/ Prep HDL & LDL dil sheets
(Grey bits not on Fridays)	8:15-8:30	Get d.d.H_2O & ice
	08:30	Change LDL dialysis Buffer
	8:30-9:00	prepare 2 liters of PBS/ Prepare boats/ bottles& reagents (Cu/ PON1& Brad)/Prep PON & Ox. sheets & programs (PBS +Ca)
	9:00-9:30	prepare desalting columns with PBS+Ca/ Taking Plasma and HDL **controls** from Freezers to defrost
	9:30-10:30	Isolate HDLs of 10 PSOBIDS & Desalt along 1 HDL control/ put HDLs on ice
	10:30-11:00	Running PON1 assay for 10 HDLs + 10 Li-Hep Plasma of PSOBIDS+ 1 plasma & 1 HDL controls
	11:00-11:30	Measuring HDL-Protein by Bradford for 10 HDLs of PSOBIDS +1 Plasma & HDL controls
	11:30	Taking LDL from Dialysis/ Give aliquot for measuring LDL-C/Put new LDL control for Dialysis
	11:30	Take out 10 new samples of PSOBIDS from freezers to defrost
	11:30-12:00	Dilute PSOBIDS HDLs& HDL control to (250Ug/ml) along with LDL control (100Ug/ml)
	12:00-12:30	Prepare soft Max pro for Oxidation/ 5 UM $Cucl_2$ sol. / Start oxidation /re store left HDLs
	12:30	Change dialysis buffer
	12:30-1:00	Prepare HDLs first spin/ defrost a new LDL control/ Prepare Dialysis tube
	3:30-4:00	Isolate VLDL/LDL form PSOBIDS and start second spin