# Understanding the relationship between high-density lipoprotein (HDL) subclass distribution and functionality in patients at risk of cardiovascular disease

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“The difference between screwing around and science is writing it down.”

**Adam Savage**

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## DEDICATION

This thesis is dedicated to my saviour Jesus Christ, without whom I would be nothing, to my beloved family, my father, Chris and my mother, Charlotte, my sister, Laura and my grandmother, Tilly. All of their constant love and support have helped me reach the end of this journey. Finally, it is dedicated to my supervisors, Sandrine Lecour, Julia Goedecke and Miguel Frias, whose wise mentorship has been invaluable.

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**TABLE OF CONTENTS**

ABBREVIATIONS:

| | | | |
|---|---|---|---|
| AAPH | 2,2’ – azobis (2-amidinopropane) dihydrochloride | ORAC | Oxygen radical absorbance capacity |
| ABCA1 | ATP-binding cassette transporter A1 | OxLDL | Oxidized low-density lipoprotein |
| ABCG1 | ATP-binding cassette transporter G1 | PAF | Platelet activating factor |
| ACAT | Acyl-CoA cholesterol acyltransferase | PAF-AH | Platelet activating factor acetylhydrolase |
| Apo | Apolipoprotein | PBS | Phosphate buffered saline |
| AU | Arbitrary units | PI-3 | Phosphatidyl-inositol-3 |
| BMI | Body mass index | PLTP | Phospholipid transfer protein |
| BNP | Brain natriuretic peptide | PON | Paraoxonase |
| cAMP | Cyclic adenosine monophosphate | PWD | Posterior Wall Diameter in Diastole |
| CETP | Cholesteryl ester transfer protein | RAA | Right Atrial Area |
| CPM | Counts per minute | RCT | Reverse cholesterol transport |
| CVD | Cardiovascular disease | Rf | Retention factor |
| DALY's | Disability adjusted life years | rHDL | Reconstituted high-density lipoprotein |
| DCF | Dichlorofluorescein | RVD | Right ventricular diameter |
| DGAT2 | Diacylglycerol acyltransferase-2 | SAA | Serum Amyloid A |
| DMEM | Dulbecco’s modified eagle medium | SAT | Subcutaneous adipose tissue |
| DNTB | 5, 5’-dithio-bis-(2-nitrobenzoic acid) | SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| DT | Deceleration time | SEM | Standard error of mean |
| EDD | End Diastolic Diameter | SRB1 | Scavenger receptor type 1 |
| ESD | End Systolic Diameter | TAPSE | Tricuspid Annular Pulmonary Excursion |
| FBS | Foetal bovine serum | TE | Trolox equivalents |
| HbA1c | Glycated haemoglobin | TNF-α | Tumor necrosis factor alpha |
| HDL | High-density lipoprotein | TTBS | Tween in tris-buffered saline |
| HDL-C | High-density lipoprotein cholesterol | VAT | Visceral adipose tissue |
| HF | Heart failure | VCAM | Vascular cell adhesion molecule |
| HUVEC | Human umbilical vein endothelial cells | VEGF | Vascular endothelial growth factor |
| ICAM | Intercellular adhesion molecule | VLDL | Very low density lipoprotein |
| IVSD | Inter-ventricular septal diameter in diastole | $VO_{2max}$ | Maximal oxygen consumption |
| IQR | Interquartile range | WHR | Waist/hip ratio |
| LAA | Left Atrial Area | | |
| LCAT | Lecithin cholesterol acyltransferase | | |
| LC-MS | Liquid chromatography mass spectroscopy | | |
| LDL | Low-density lipoprotein | | |
| LDL-C | Low-density lipoprotein cholesterol | | |
| LVH | Left ventricular hypertrophy | | |
| MA | Atrial Filling | | |
| MACE | Major adverse cardiovascular events | | |
| MCP-1 | Monocyte chemotactic protein | | |
| ME | Early Mitral Filling | | |
| MEM | Minimum essential eagle | | |
| MI | Myocardial infarction | | |
| NF-κβ | nuclear factor kappa-light-chain-enhancer of activated B cells | | |

**LIST OF FIGURES AND TABLES**

ABSTRACT

Background:

Risk factors for cardiovascular disease (CVD) include obesity, ethnicity and hypertension. High-density lipoprotein (HDL) has traditionally served as a marker for CVD risk. Latest studies, however, propose that the composition and subclass distribution and the anti-atherogenic function of HDL are more accurate predictors of CVD risk. We therefore explored whether obesity, ethnicity, exercise and hypertension may modulate HDL composition, subclass and function in three different sample populations of patients affected with these CVD risk factors.

Methods:

The first study sample population consisted of black and white obese and normal-weight South African women (n=40). In the second sample population, obese black South African women were randomly assigned to exercise (combined aerobic and resistance exercise 4 times/week) or control (sedentary) conditions for 12-weeks (n=32). The third sample population included Nigerian out-patients, divided into healthy controls, hypertensive patients and hypertensive patients with heart failure (HF) (n=80). HDL composition measurements included apolipoproteins A1 and M (ApoA1 and ApoM), paraoxonase (PON1) and platelet activating factor acetylhydrolase (PAF-AH) expression (using Western blotting) and sphingosine-1-phosphate (S1P) content (using mass spectometry). Levels of large, intermediate and small HDL subclasses were measured using the Lipoprint® system. HDL functionality was assessed by measuring PON1 activity, PAF-AH activity, reverse cholesterol efflux capacity, HDL-mediated activation of endothelial nitric oxide synthase (eNOS) and quantification of the expression of vascular cell adhesion molecule in endothelial cells.

Results:

In all sample populations, HDL-cholesterol concentration was not different between groups. PON1 activity was lower in white compared to black women (0.49±0.09 U/L vs 0.78±0.10 U/L, $p<0.05$). Obese black women had lower PAF-AH activity compared to obese white women (9.34±1.15 U/L vs 13.89±1.21 U/L, $p<0.05$). Compared to normal-weight women, obese women had lower large HDL, greater intermediate and small HDL. Compared to the sedentary control condition, exercise training was associated with a decrease in PON1 activity (-8.7±2.4% vs +1.1±3.0%, $p<0.05$), PAF-AH serum expression (-22.1±8.0% vs +16.9±9.8, $p<0.005$) and small HDL subclasses (-10.1±5.4% vs +15.7±6.6%, $p<0.005$). S1P content in HDL was lower in hypertensive and HF patients compared to controls (165 ± 55 vs 201 ± 73 pmol/mg, $p < 0.05$). HDL subclass distribution was different in hypertensive and HF patients with lower large HDL (48 ± 15 vs 63 ± 7%, $p<0.005$), higher intermediate (45 ± 7 vs 34 ± 5%, $p<0.005$) and small HDL (7 ± 9 vs 2 ± 4%, $p<0.05$). In contrast to HDL from control patients, HDL from all hypertensive patients failed to activate eNOS.

Conclusions:

In all three sample populations, there were associations between CVD risk factors and measures of HDL quality. HDL subclass distribution differences were associated with obesity and hypertensive heart failure, both in cross-sectional studies and in an exercise intervention study. In African sample populations, consideration of HDL quality rather than total HDL quantity may be a more sensitive marker to assess CVD risk.

## CHAPTER ONE: LITERATURE REVIEW

### 1. Introduction

Cardiovascular disease (CVD) is the leading cause of death worldwide (Mathers and Loncar, 2006; World Health Organization, 2011). From a reported 16.7 million deaths in 2002, global cardiovascular related deaths are estimated to rise to 23.3 million by 2030 (Mathers and Loncar, 2006). No longer mainly prevalent in developed or high-income countries, ischemic heart disease is also predicted to be the leading cause of death in low-income countries by 2030 (Mathers and Loncar, 2006). In an African setting, progressive changes in socio-economic status has raised the burden of preventable cardiovascular disease (Akinboboye *et al.*, 2003; Mayosi *et al.*, 2009; Sliwa *et al.*, 2012). Individuals in newly-industrialised countries have improved access to Westernized diets with greater wealth and status often linked to increased inactivity and potential obesity.

The Global Burden of Diseases, Injuries and Risk Factors study of 2010 indicated that much of the burden of CVD in sub-Saharan Africa can be attributed to haemorrhagic stroke, hypertension and heart failure (HF), whilst there was a relatively low incidence of atherosclerosis and ischemic heart disease (Moran *et al.*, 2013). Recent studies, however, indicate tremendous growth in the rates of ischemic heart disease in North Africa and sub-Saharan Africa (Kakou-Guikahue *et al.*, 2016; Traina *et al.*, 2017). Indeed, myocardial infarction, cardiomyopathy and coronary artery disease continue to increase and contribute to the burden of CVD in sub-Saharan Africa (Keates *et al.*, 2017). In a clinical setting, incidence of periprocedural myocardial infraction in a tertiary South African hospital are even consistent with international rates (Tsabedze *et al.*, 2016). Clearly, the epidemiological climate of CVD in Africa is unique, and is demonstrative of an epidemiological transition. It therefore remains important to use applicable measures to assess the CVD risk in these populations.

Blood lipids have traditionally served as accurate risk factors for cardiovascular events. Higher low-density lipoprotein cholesterol (LDL-C) and lower high-density lipoprotein cholesterol (HDL-C) favour cardiovascular risk (Gordon *et al.*, 1977; Barter and Rye, 1996; Kontush *et al.*, 2003). The protective capacity of HDL can be attributed to several atheroprotective functions, including reverse cholesterol transport (RCT), antioxidative, anti-inflammatory, anti-apoptotic and anti-thrombotic properties (reviewed by Nofer et al. 2002). It would then seem intuitive that decreased levels of HDL-C would be associated with increased CVD risk. This has been confirmed in the literature by epidemiological studies that show a negative correlation between HDL-C and CVD risk (Acharjee et al. 2013; Gordon et al. 1989; Gordon et al. 1977; Sharrett et al. 2001). More recently, however, the trend has been to move away

from total HDL as a strict marker of CVD risk in favour of measures of functionality and composition of HDL (Egom *et al.*, 2013; Santos-Gallego, 2015).

HDL composition refers to the relative concentration of different subclasses, defined by density, electrophoretic mobility, particle size and lipoprotein composition (Asztalos & Schaefer 2003). Differences in HDL subclasses relate not only to differences in HDL functionality but also have the potential as independent predictors of CVD risk (Asztalos & Schaefer 2003; Berrougui et al. 2007; Camont et al. 2013; Julia et al. 2010; Kontush et al. 2003; Lee et al. 2010; Martin et al. 2014; Rader 2003; Stampfer et al. 1991).

This review explored the apparent disparities between the "quantity "and the so-called "quality" of the HDL particle in relation to CVD risk. Accordingly, HDL function, composition and subclass were examined as a possible marker of CVD risk. In this regard, the structural significance of HDL, its multiple anti-atherosclerotic functions and the distribution of individual subclasses will be proposed as improved measures of CVD risk in place of traditional HDL-C measurement.

## 2. HDL, the smallest of the plasma lipoproteins

Plasma lipoproteins are water-soluble macromolecules made up of a complex of lipids, namely cholesterol, triglycerides, and phospholipids, as well as lipoprotein-specific proteins known as apolipoproteins (Morrisett *et al.*, 1975). Lipoproteins are classified according to density, particle size, electrophoretic mobility or affinity chromatography (Assmann 1982; Asztalos et al. 2011; Mahley et al. 1984). Chylomicrons are the largest lipoproteins with a diameter of 70-1000nm (Figure 1). They are synthesized in the intestine and transport dietary cholesterol and triglycerides from the site of absorption (Mahley *et al.*, 1984). Triglycerides from the chylomicrons are enzymatically digested by plasma lipoprotein lipase, liberating fatty acids for energy or storage by adipocytes. Lipoproteins generated by the hydrolysis of triglycerides associated with chylomicrons are known as chylomicron remnants (Mahley *et al.*, 1984). Very low-density lipoproteins (VLDL) are 30-90 nm particles with a density less than 1.006 g/ml, which transport triglycerides and cholesterol from the liver (Mahley *et al.*, 1984). Hydrolysis of VLDL-associated triglycerides by hepatic or plasma lipoprotein lipase generates cholesterol-enriched lipoproteins including intermediate-density lipoproteins (IDL) (density = 1.006-1.019 g/ml) and LDL (density = 1.019-1.063 g/ml), which are the principal cholesterol transporting lipoproteins (Mahley *et al.*, 1984). HDL, the smallest (8-12 nm in diameter) and most dense (> 1.21 g/ml) of all lipoproteins perform protective roles through the removal of cellular cholesterol. The formation of HDL is a complex and intricate process, which will now be focussed on in greater detail.

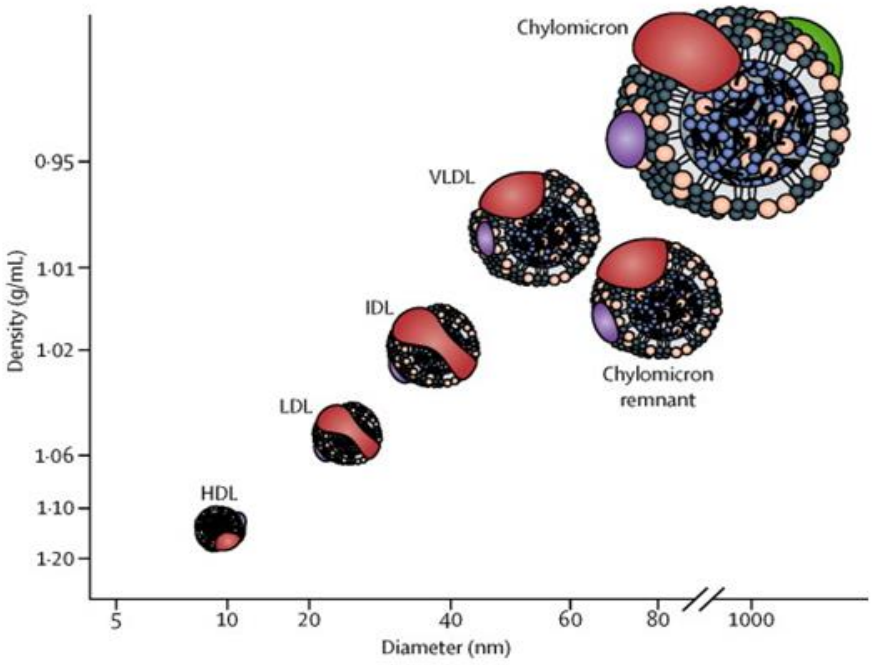



**Figure 1. Lipoprotein heterogeneity. Adapted from** (Ridker, 2014)

### 2.1 The formation of HDL

HDL, like other lipoproteins, exist in their mature form as spherical complex structures comprising a lipid bilayer surrounding a hydrophobic core of triglycerides and cholesteryl esters. HDL does, however, originate as discoidal particles, formed within the liver or assembled from lipid and apolipoprotein constituents in circulation (Hamilton *et al.*, 1976). In combination with a phospholipid bilayer, discoidal HDL are surrounded by at least two apolipoproteins (Rye and Barter, 2014).

Apolipoprotein A1 (ApoA1) is the most abundant of the lipoproteins present in HDL, followed by ApoAII, ApoAIV and ApoE. ApoA1, mainly synthesised in the liver, undergoes an initial enzymatic cleavage, rendering a mature form which undergoes lipidation in the endoplasmic reticulum (Stoffel *et al.*, 1983; Gillard *et al.*, 2009). Lipid-poor ApoA1 generates discoidal HDL complexes following the transfer of phospholipids and acceptance of cholesterol from cell membranes by ATP-binding Cassette Transporter A1 (ABCA1) (Wang *et al.*, 2001). The generation of discoidal HDL molecules in the aforementioned processes is summarized in Figure 2.

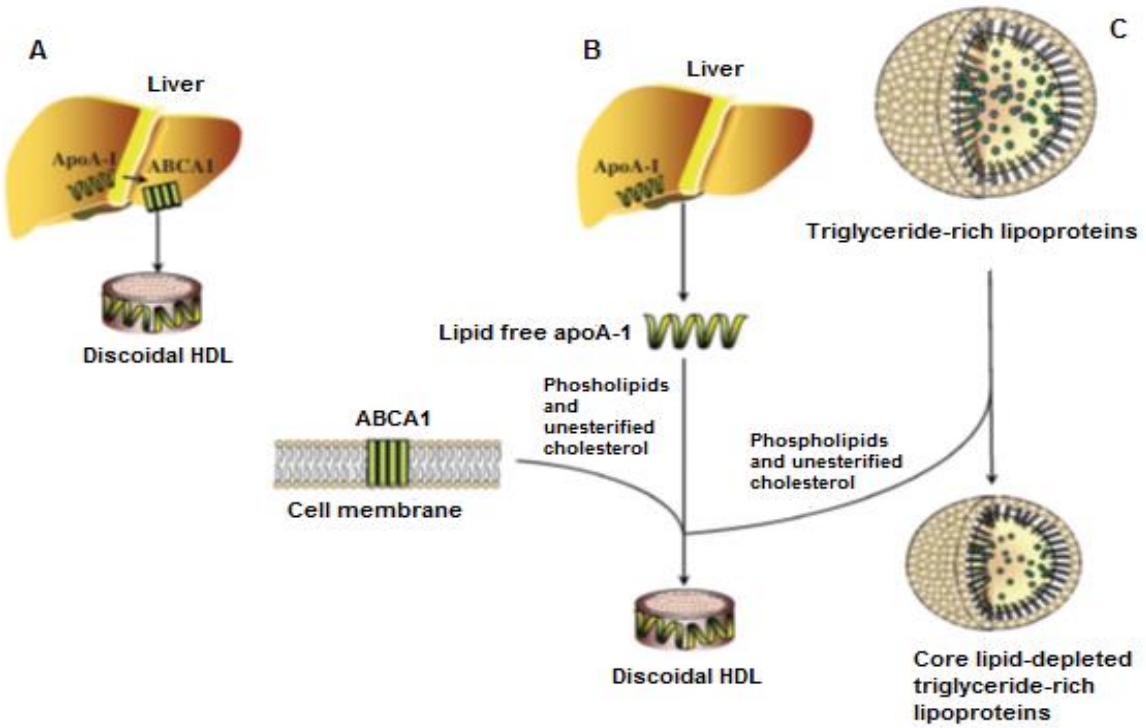



**Figure 2. Biogenesis of discoidal High-Density lipoproteins. ApoA1 can be synthesized in the liver where interaction with ATP binding Cassette Transporter A1 (ABCA1) forms discoidal HDL complexes (A). Lipid-free or lipid-poor ApoA1 can acquire phospholipids and cholesterol from cell membranes (B) or triglyceride rich lipoproteins (C) to generate discoidal HDL by alternative means** (Rye and Barter, 2014)**.**

Lecithin cholesterol acyltransferase (LCAT) is responsible for the conversion of discoidal HDL into mature spherical molecules, forming a gradient of unesterified cholesterol between other lipoproteins (VLDL and LDL), peripheral cells and HDL (Asztalos *et al.*, 2007). Conversion of discoidal HDL to spherical HDL involves hydrolysis of phospholipids in the discoidal HDL to generate lysophosphatidylcholine and free fatty acid groups (Rye and Barter, 2014). LCAT transfers these fatty acyl groups to cholesterol to form the hydrophobic cholesteryl esters which moves to the core of the particle leading to a change in HDL particle conformation (Asztalos *et al.*, 2007). This reaction also depletes discoidal HDL of unesterified cholesterol and forms the gradient between HDL and VLDL or LDL.

All of the processes required for the formation of mature HDL are subject to regulation by additional factors including ApoA1 availability, phospholipid composition and discoidal HDL size, all of which alter LCAT activity (Jonas *et al.*, 1989; Bolin and Jonas, 1996; Scott *et al.*, 2001). HDL function and physiology are associated with several key protein and lipid components including the apolipoproteins, paraoxonase (PON) and sphingosine-1-phosphate (S1P). These components control HDL formation and are linked with overall HDL functionality.

## 2.2 Key components in HDL

### 2.2.1 Apolipoproteins

Apolipoproteins are proteins, associated with lipoproteins and perform following functions: structural components and ligands for ell surface receptors. As previously alluded to, the apolipoproteins are key structural components of lipoproteins and serve as definitive markers used to distinguish lipoproteins from each other. ApoA1 is the most abundant lipoprotein in HDL, comprising 64% of the HDL protein mass (Gillard *et al.*, 2009). ApoA1 is essential for RCT (Scott *et al.*, 2001; Temel *et al.*, 2002). ApoA1 also activates LCAT and it is postulated that much of the anti-atherogenic and cardioprotective effects of HDL may be linked to the influence of ApoA1 (Temel *et al.*, 2002).

The functional relevance of ApoA1 was first shown in studies examining administration of reconstituted HDL (rHDL) comprised of ApoA1 and phosphatidylcholine discs in cell culture models (Stein *et al.*, 1976). The presence of ApoA1 significantly improved RCT (Stein *et al.*, 1976). Further, depletion of ApoA1 in transgenic mice or anti-ApoA1 antibodies reduces LCAT activity, impairs RCT and increases vulnerability to atherosclerosis (Parks *et al.*, 1995; Temel *et al.*, 2002; Montecucco *et al.*, 2011). A study focussed on a genetic variant of ApoA1, which exists in inhabitants of a village in Italy where these individuals have very low incidence of atherosclerosis despite having low HDL-C levels (Sirtori *et al.*, 2001). A minor structural variation, produces a functionally superior ApoA molecule (ApoA1-Milano) (Nissen *et al.*, 2003). It was confirmed in a randomized trial, using recombinant ApoA1-Milano, that this genetic aberration results in an apolipoprotein whose minor structural changes endow an improved anti-atherosclerotic function (Nissen *et al.*, 2003).

### 2.2.2 Key enzymes and HDL

Although a variety of enzymes including Platelet Activating Factor Acetyl Hydrolase (PAF-AH), whose role and functions will be reviewed in section 2.4.5, LCAT and Phospholipid Transfer Protein (PLTP) have been shown to be associated with HDL, PON is considered as the most prominent enzyme in promoting key functions of HDL (Durrington *et al.*, 2001).

There are three isoforms of PON including PON1, PON2 and PON3. Whilst PON2 is located intracellularly, PON1 is the major factor contributing to the antioxidant function of HDL (James and Deakin, 2004; Mackness and Mackness, 2015). HDL provides a hydrophobic environment necessary for PON1 activity (James and Deakin, 2004). There is little association between

PON1 and other lipoproteins implying that precise interactions may be required to fulfil the antioxidant effects of PON1.

Specificity of the binding of PON1 to HDL relies on HDL heterogeneity, the peptide composition (apolipoproteins) and overall HDL composition (phospholipid components) to promote a highly specialised association (James and Deakin, 2004).

Additionally, PON1 and ApoA1 appear to be important for each other. The presence of ApoA1 in HDL structure is critical in maintaining the stability of the PON1 activity (Deakin *et al.*, 2002). Supplementary to the protein content, the lipid composition of HDL is key in regulating a number of HDL functions.

### 2.2.3 Key lipids and HDL

The three major lipid groups in HDL include triglycerides, cholesterol and phospholipids. Although the cholesterol content of HDL has long served as the measure of choice in correlating CVD risk, it is in fact phospholipids which predominate the HDL lipidome (Camont *et al.*, 2013). Of these, the negatively charged phospholipids including phosphatidylcholine are the most abundant (Larijani *et al.*, 2000). Another important phospholipid, S1P, is enriched in the smaller HDL subpopulations (Kontush *et al.*, 2007; Lee *et al.*, 2010).

S1P is a signalling molecule, capable of triggering a diverse and interconnected string of signalling pathways involved in multiple cell function including cell survival (Somers *et al.*, 2012). In the plasma, S1P is bound and transported by HDL, with which it exhibits tighter binding than other lipoproteins, such as LDL (Murata *et al.*, 2000). The diverse atheroprotective functions of HDL and the mechanisms by which these effects are achieved have been in many cases linked to the S1P content of HDL. These include, preventing ischemic injury (Theilmeier *et al.*, 2006; Frias *et al.*, 2012); reducing cytotoxicity (Kimura *et al.*, 2001; Kontush *et al.*, 2007); inducing prostacyclin release (Liu *et al.*, 2012) and preventing LDL oxidation (Kontush *et al.*, 2007).

*In vivo,* the major cellular source of S1P is the haematopoietic cells including erythrocytes, platelets and leukocytes (Pappu *et al.*, 2007). In plasma, there is a significant surplus of S1P, far exceeding the available binding sites at S1P receptors (Murata *et al.*, 2000). The majority of free S1P, however, is biologically inactive with active S1P remaining in the lipoprotein form with HDL (Sattler and Levkau, 2009).

### 2.2.4 Key proteins regulating HDL

*Phospholipid transfer protein (PLTP)*

PLTP is critical in modulating the size and composition of the HDL molecule and, along with other regulators such as cholesteryl ester transfer protein (CETP), is implicated in the generation of distinct subpopulations of HDL (Tall *et al.*, 1983; Jauhiainen *et al.*, 1993). Remodelling of HDL by PLTP is subject to the phospholipid transfer capacity as it can facilitate the transport of phospholipids between HDL and other lipoproteins (Jauhiainen *et al.*, 1993). PLTP mediated remodelling is regulated by at least three factors, including the HDL apolipoprotein composition, lipid composition and enzymatic activity of PLTP (Hattori, 2010). Remodelling by PLTP generates an unstable fusion product via two pathways, producing two populations of HDL comprised of large and small spherical HDL products (Settasatian *et al.*, 2001). Therefore PLTP, depending on the site of expression, can control the generation of a variety of HDL subclasses (Curtiss *et al.*, 2006).

*Cholesteryl ester transfer protein (CETP)*

The function of CETP is the transfer of triglycerides, cholesteryl esters and phospholipids between lipoproteins (Zilversmit *et al.*, 1975). CETP induces exchanges between lipoproteins which results in the transfer of triglycerides from VLDL to HDL and cholesteryl esters from HDL to VLDL (Hopkins and Barter, 1980). These exchanges alter HDL structure, causing changes in HDL particle size, ApoA1 composition and result in significant remodelling of HDL (Clay *et al.*, 1991).

CETP activity also causes a reduction in HDL-C concentration in plasma, evidenced in rodent models with decreased CETP activity (Inazu *et al.*, 1990). Observations such as these have prompted many large scale clinical trials which seek to selectively inhibit CETP. In addition to decreasing HDL levels, CETP induces a pro-atherosclerotic pathology, causing a shift in the lipid profile, generating VLDL and LDL at the expense of HDL (Hime, 2010). ApoA1 dissociation, caused by CETP has also been hypothesised to not only hinder removal of excess cholesterol but also lead to its accumulation (Curtiss *et al.*, 2006).

Therefore, as a result of HDL remodelling, a number of subpopulations or subclasses of HDL can be generated. Our focus will now turn to the heterogeneity of HDL itself, namely the HDL subclasses.

### 2.3 HDL heterogeneity

It was first demonstrated, using agarose gel electrophoresis, that lipoproteins can be separated on the basis of their mass: charge ratio (Asztalos et al. 2011). Non-denaturing, two dimensional gel electrophoresis as a modification of earlier methods has permitted accurate delineation of HDL subclasses (Asztalos et al. 2011). Indeed, electrophoresis techniques have undergone great refinement and now allow for not only separation of HDL into subclasses but also quantification of the relative distributions of each subclass.

The nomenclature of HDL subclasses is largely dependent on the methods used for separation which, in addition to electrophoresis, can also include immunoaffinity chromatography and ultracentrifugation (Asztalos et al. 2011).The latter utilizes differences in the protein/lipid ratio of each of the subclasses, permitting sequential floatation (Lindgren *et al.*, 1972). Combination of a number of techniques can, in principal separate HDL into 2 distinct subclasses, HDL2 and HDL3, which themselves can be further subdivided into additional subgroups (Figure 3) (Williams *et al.*, 1992; Rosenson *et al.*, 2011). HDL separation by 2D gel electrophoresis has also permitted the identification of preβ-HDL, an immature form of HDL, possibly discoidal HDL particles, which most actively participate in RCT (Asztalos and Schaefer, 2003; Rosenson *et al.*, 2011). Often preβ-HDL is grouped together with HDL3, which along with HDL2, have been studied extensively with regard to their functional roles and structural dissimilarities.

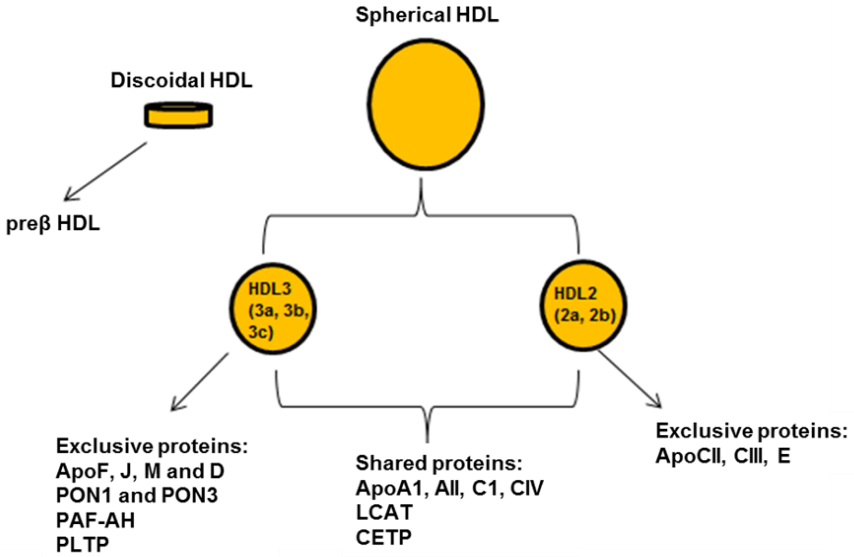



**Figure 3. Schematic diagram of HDL subclasses and protein associations. PAF-AH: Platelet activating factor acetylhydrolase; PON1: paraoxoanse; PLTP: phospholipid transfer protein; LCAT: lecithin:acetyltransferase and CETP: cholesteryl ester transfer protein.** (Davidson *et al.*, 2009)

HDL3 is more dense, protein-rich and structurally smaller and lighter than HDL2 (Chapman, 1986). HDL2, conversely, is enriched in lipids, containing greater cholesteryl ester and triglyceride molecules than HDL3 (Williams *et al.*, 1992). Proteomic analysis of the HDL subclasses indicated that distinct protein clusters were present in HDL3 (Davidson *et al.*, 2009), with S1P preferentially associated with HDL3 (Kontush *et al.*, 2007). Proteins exclusively associated with HDL3 include PON1 and PON3, PAF-AH and ApoJ while HDL2 was only associated with ApoCI/CII,CIII and ApoE (Davidson *et al.*, 2009). Therefore, HDL subclasses can be distinguished on the basis of size, density and composition.

The Lipoprint® System, presents a new approach to quantifying the relative distributions of HDL subclasses using gel electrophoresis and in this case designates them as large, intermediate and small subclasses (Hoefner *et al.*, 2001). We hypothesize that in this case HDL2 would be represented by large HDL and HDL3 by intermediate and small HDL.

### 2.4 Anti-atherogenic functions of HDL

HDL performs atherosclerotic functions through multiple ways including RCT, antioxidative, anti-inflammatory, and anti-thrombotic functions as well as regulation of endothelial homeostasis (Figure 4). The reverse cholesterol efflux pathway or RCT is the principal anti-atherogenic function of HDL and serves to prevent the onset of atherosclerosis, halting formation of atherosclerotic plaques and lesions. In order to understand the need for RCT, the pathogenesis of atherosclerosis will first need to be described.

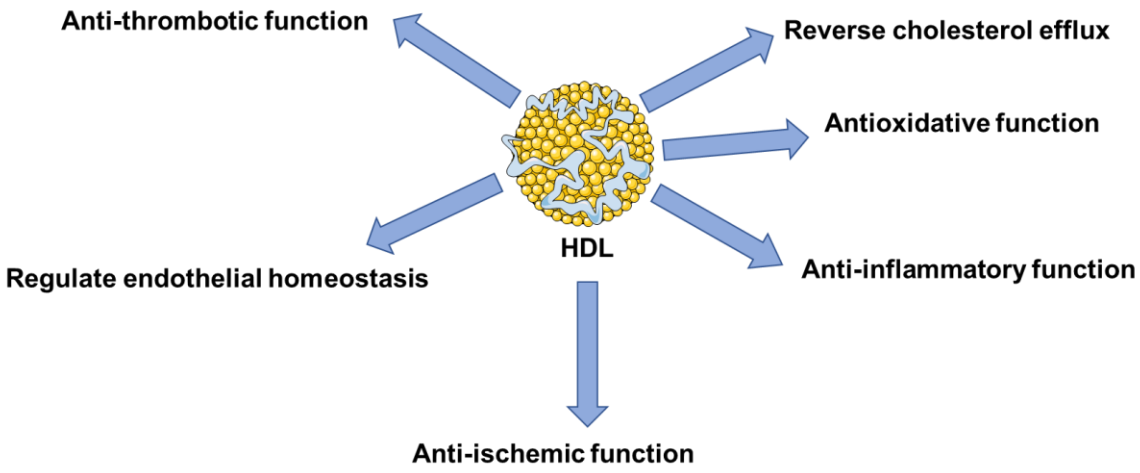
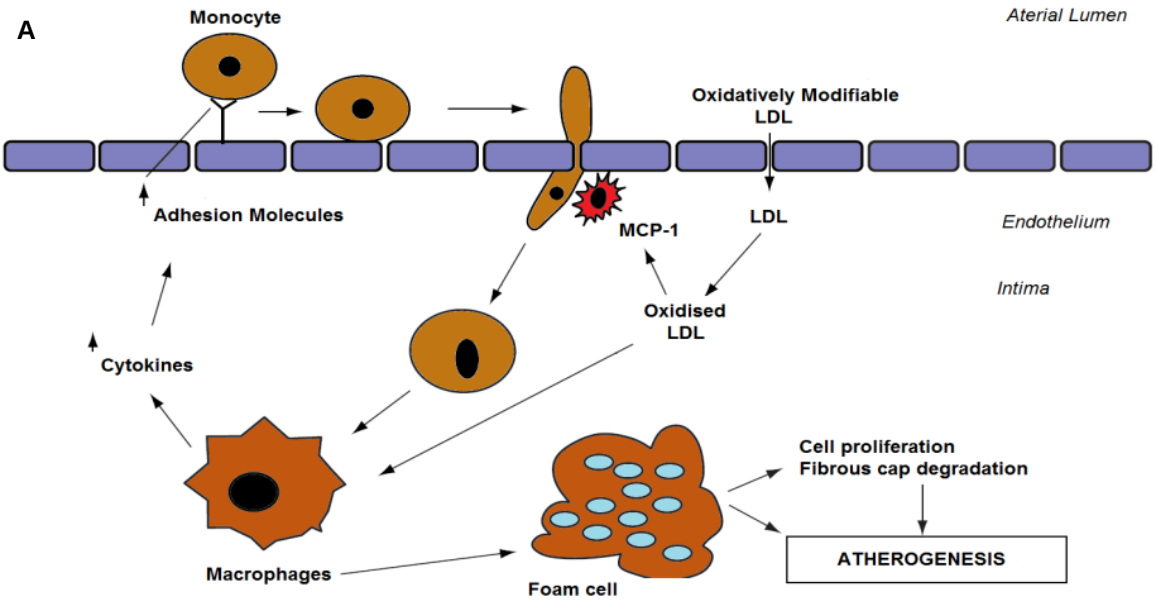



**Figure 4. Summary of the anti-atherogenic functions of HDL**

### 2.4.1 Pathogenesis of atherosclerosis

Atherosclerosis is a disease centred on chronic inflammation. Changes in the homeostasis of the endothelium, together with an increase in oxidative stress, precede the initial onset of atherosclerosis (Ross, 1999). Oxidative stress, in the form of free radicals whose generation is caused by factors such as cigarette smoking, hypertension and infection, leads to the injury of the endothelial cell layer (Ross, 1999). Oxidative injury to the endothelium results in increased endothelial permeability of monocytes and lymphocytes caused, in part, from an increase in the expression of endothelial adhesion molecules (Ginter and Simko, 2013). The resulting endothelium dysfunction promotes the influx of LDL into the intima where it undergoes oxidation.

The so called “oxidative-modification hypothesis” of atherosclerosis proposes that the primary mechanism for atherosclerosis development is through the oxidation of LDL to form oxidized LDL (OxLDL) (Diaz *et al.*, 1997). Uncontrolled uptake of OxLDL by internalized monocytes which have now differentiated into macrophages can later lead to the formation of foam cells, which form the foundation of atherosclerotic plaque (Yu *et al.*, 2013).



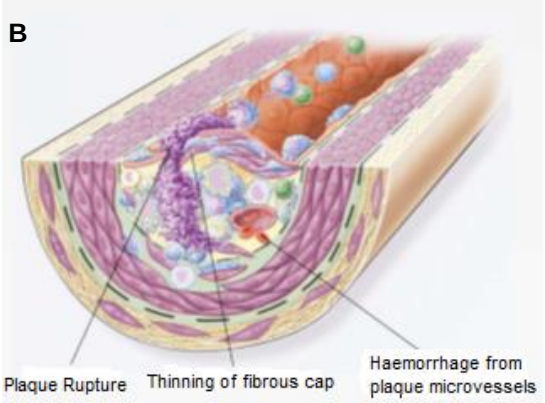




**Figure 5. Physical mechanism of atherosclerosis (A) and unstable complex plaque formation during atherosclerosis (B). Monocytes adhere to the endothelial cell layer and once infiltrated into the lumen, acquire oxidized LDL and differentiate into macrophages. Macrophages increase cytokine release promoting further monocyte adhesion and cholesteryl ester accumulation within monocytes, forming foam cells which contribute to the formation of unstable plaques (5B). Plaque rupture and disruption of the fibrous cap, as a result of macrophage related release of proteolytic enzymes, cause leakage of prothrombotic materials into the circulation prompting downstream thrombus formation. 5A adapted from** (Ginter and Simko, 2013) **and 5B from** (Ross, 1999)**.**

Foam cell formation begins with internalization of cholesterol in the form of oxidized LDL (which increases Monocyte Chemotactic Protein-1, MCP-1, expression) (Figure 5A) (Ginter and Simko, 2013; Yu *et al.*, 2013). The lipoproteins are then delivered to lysosomal bodies within the macrophages where cholesteryl esters are hydrolysed to generate free cholesterol (Yu *et al.*, 2013). To prevent free cholesterol-related cytotoxicity, the free cholesterol is re-esterified and leads to the accumulation of excess cholesteryl esters, forming foam cells (Figure 5A) (Yu *et al.*, 2013).

Macrophages and lymphocytes will continue to aggregate and multiply within a newly developing atherosclerotic lesion, continually producing cytokines and growth factors. Under chronic conditions, this leads to the recruitment of smooth muscle cells and platelets and generation of advanced lesions (Bombeli *et al.*, 1998; Ross, 1999). Continued formation of foam cells, smooth-muscle cell proliferation and platelet activation promote plaque instability, rupture of the protective fibrous cap and release of thrombogenic material into the circulation, forming a thrombus which can lead to arterial occlusion (Figure 5B) (Bombeli *et al.*, 1998; Ross, 1999; Ginter and Simko, 2013).

### 2.4.2 Reverse Cholesterol Efflux

The RCT pathway is routinely described as being the main conduit for the atheroprotective effects of HDL. The original pathway delineated by Glomset, involves the physiological removal of cholesterol from peripheral tissues and cells, to be transported by HDL, to the liver for excretion in the bile and faeces (Figure 6) (Glomset, 1968). The majority of peripheral cells are incapable of catabolizing free cholesterol and in order to circumvent cholesterol related toxicity, cholesterol is effluxed to carriers like HDL (Rader *et al.*, 2009).

There are several pathways by which cholesterol efflux occurs. These include efflux to lipid-free ApoA1 mediated by ABC transporter ABCA1, efflux to HDL particles mediated by ABCG1, efflux to mature HDL particles mediated by scavenger receptor type 1 (SRB1) and passive diffusion (Figure 6) (Castro and Fielding, 1988; Hobbs and Rader, 1999; Bortnick *et al.*, 2000; Wang *et al.*, 2004; Kennedy *et al.*, 2005; Prosser *et al.*, 2012). Passive diffusion of cholesterol involves desorption of free cholesterol molecules from the donor water-lipid interface and diffusion through the aqueous phase until absorption by a receptor (Berrougui *et al.*, 2007). Each of the other processes involves interaction with transporter/receptor molecules. These are critical in maintaining HDL functionality either through direct management of cholesterol efflux or by triggering downstream signalling pathways. These pathways can then induce antioxidant, anti-inflammatory or anti-thrombotic effects. Each of the key receptor and transporter molecules which control RCT will now be discussed.

ABCA1 interacts specifically with lipid-free ApoA1 to form immature HDL particles, however, it also interacts with caveolin-1, which mediates cholesterol homeostasis by controlling cholesterol transport (Wang *et al.*, 2001; Lin *et al.*, 2007).

ABCG1 facilitates cholesterol efflux to HDL particles, particularly in human aortic endothelial cells (Wang *et al.*, 2004; Kennedy *et al.*, 2005; Terasaka *et al.*, 2008). The SRB1 receptor is distinctive in that it can promote cholesterol efflux in both directions, promoting efflux of cholesterol from macrophages to HDL but also promoting HDL cholesterol uptake in some cases (Rothblat *et al.*, 1999). ABCA1, ABCG1 and SRB1 all control different aspects of RCT, mediating cholesterol transfer between ApoA1 and HDL (Figure 6). All three remain critical in limiting atherosclerotic plaque formation.

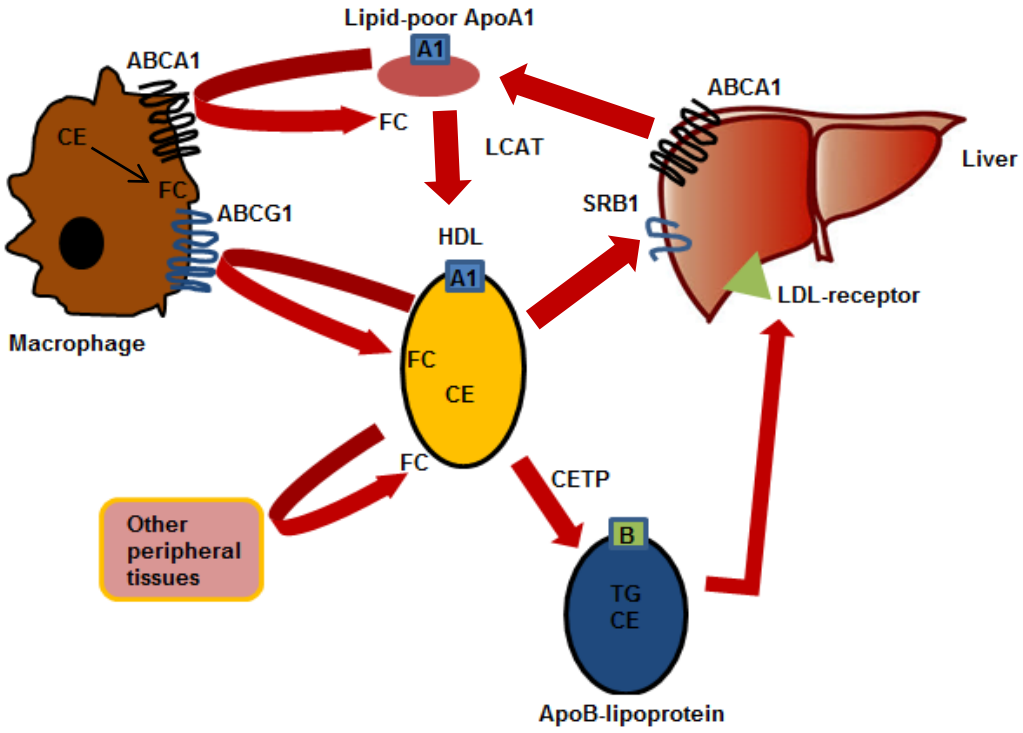



**Figure 6. Reverse cholesterol transport (RCT). Lipid-poor ApoA1 is produced by the liver and acquires free cholesterol (FC) through interaction with ABCA1 to form discoidal HDL molecules which mature into spherical HDL through the action of LCAT, whilst lipid-poor ApoA1 effluxes cholesterol accumulated in macrophages through ABCA1. Cholesterol is further effluxed and converted into cholesteryl esters (CE) in HDL through interaction with macrophage ABCG1 and can also be accumulated from peripheral tissues. HDL can deliver cholesterol directly to the liver through SRB1 interaction or indirectly via initial transfer to ApoB-lipoproteins, rich in triglycerides (TG), mediated by CETP, which then bind to the liver. Hepatic cholesterol is excreted into the bile and ultimately via the intestine, is excreted in the faeces.** (Rader *et al.*, 2009)

The relevance of CETP in cholesterol transport must not be overlooked. Some HDL-derived cholesterol delivered to the bile, is first transported to ApoB containing lipoproteins. This indicates that cholesterol effluxed to HDL can then be exchanged with LDL and VLDL to facilitate excretion (Schwartz *et al.*, 2004).

RCT serves as the primary function of HDL with many pathways being initiated by exchanges between HDL and the relevant transporters (ABCA1, ABCG1 and SRB1). These pathways may indeed trigger signalling cascades which can then perform the other anti-atherosclerotic activities usually associated with HDL. Cholesterol efflux capacity exhibits a strong, inverse correlation with prevalent coronary and peripheral atherosclerosis (Yvan-Charvet *et al.*, 2007; Out *et al.*, 2008; Tall *et al.*, 2008; Khera *et al.*, 2011; Ishikawa *et al.*, 2015), as well as the incidence of atherosclerotic cardiovascular events (Rohatgi *et al.*, 2014; Saleheen *et al.*, 2015).

However, the relationship between CVD risk and RCT is not without controversy. There are paradoxical examples of positive associations between RCT and increased risk and incidents of cardiovascular events, often as a result of compositional differences in subject HDL (Nestel *et al.*, 2012; Li *et al.*, 2013).

Key to the pathogenesis of atherosclerosis is the loss of structural viability of the endothelial cell layer. Much of the HDL function, other than RCT in principal, centres on the attenuation of endothelium dysfunction often via antioxidative effects.

### 2.4.3 Antioxidant functions of HDL

The pathogenesis of atherosclerosis can largely be attributed to atherogenic modification of LDL. These modifications include glycation, oxidation and glycoxidation (Soran and Durrington, 2011). Oxidation or glycation of LDL can render it cytotoxic to endothelial cells, smooth muscle cells and macrophages (Schwartz *et al.*, 1991). As mentioned previously, OxLDL can be more readily endocytosed by macrophages, yielding atherogenic foam cells and triggers release of MCP-1, prompting further recruitment of monocytes to the endothelial cell layer (Barter *et al.*, 2004; Yu *et al.*, 2013) Other than RCT, a principal function of HDL would involve halting LDL oxidation as a means of counteracting the deleterious downstream effects of OxLDL (Figure 7).

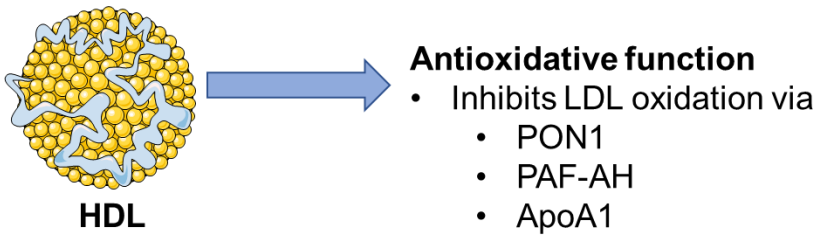



**Figure 7. Antioxidative function of HDL and proposed mechanism. PON1: Paraoxonase and PAF-AH: Platelet activating factor acetylhydrolase.**

HDL inhibits metal ion induced oxidation of LDL and lipid peroxidation (Hessler *et al.*, 1979; Parthasarathy *et al.*, 1990; Navab *et al.*, 2000). As described previously, a key structural component of HDL is PON1. It was first demonstrated that HDL protects the endothelial cell layer from OxLDL related cytotoxicity by diminishing lipid peroxide formation (Mackness *et al.*, 1991). This is accomplished by enzymatic hydrolysis of lipid hydroperoxides and oxidized phospholipids in LDL by PON1 (Mackness *et al.*, 1993a). Animal studies have confirmed the importance of PON1 in the prevention of LDL oxidation.

Transgenic mice overexpressing PON1 demonstrated an improved protection of LDL oxidation, whilst avian models where PON1 is absent from HDL structure could not protect against metal ion induced oxidation of LDL (Mackness *et al.*, 1998; She *et al.*, 2009). HDL, isolated from PON1 knockout mice lost their capacity to protect against LDL oxidation in an *in vitro* cell culture model, wherein HDL failed to inhibit monocyte recruitment with the HDL molecule rendered dysfunctional and proatherogenic (Shih *et al.*, 1998).

PON1 plays a principal role in HDL prevention of LDL oxidation (Durrington *et al.*, 2001). It is important to consider that the enzyme requires stabilisation with ApoA1, which itself has been shown as a powerful antioxidant (Navab *et al.*, 2000; Deakin *et al.*, 2002; Nofer *et al.*, 2002).

Studies on HDL functionality have utilized quantification of PON1 enzymatic activity to determine the antioxidant function of HDL (Aicher *et al.*, 2012; Kappelle *et al.*, 2012; Eroglu *et al.*, 2013; Breton *et al.*, 2014). More general assays quantifying antioxidant capacity and activity such as the dichlorofluorescein (DCF) and Oxygen Radical Absorbance Capacity (ORAC) have been applied to HDL directly (Aicher *et al.*, 2012; Breton *et al.*, 2014).

### 2.4.4 Anti-inflammatory functions of HDL

OxLDL increases monocyte recruitment by triggering the expression of MCP-1 and inflammatory responses in damaged endothelial cells (Assmann and Gotto, 2004; Barter *et al.*, 2004). Activated monocytes adhere to the endothelial layer via adhesion molecules expressed by endothelial cells. These molecules include Vascular Cell Adhesion Molecule (VCAM), Intercellular Adhesion Molecule (ICAM) and E-selectin (Kume *et al.*, 1992; Li *et al.*, 1993; Calabresi *et al.*, 2003; Barter *et al.*, 2004). ICAM is constitutively expressed by endothelial cells and interacts with leukocyte-specific integrins. VCAM and E-selectin are expressed in response to pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α) or in response to the lysophosphatidylcholine content of LDL (Kume *et al.*, 1992; Davies *et al.*, 1993; Gomaraschi *et al.*, 2008). Following monocyte binding to the endothelial cell layer, monocytes enter the intima and become trapped, with OxLDL preventing their exit

through the arterial wall through phenotypic modification of the monocytes (Quinn *et al.*, 1987). HDL exerts anti-inflammatory functions through inhibition of adhesion molecule expression and interaction with inflammatory cells.

HDL down-regulates the expression of adhesion molecules such as VCAM, ICAM and E-selectin in endothelial cells (Figure 8) (Cockerill *et al.*, 1995; Calabresi *et al.*, 2003; Gomaraschi *et al.*, 2008). The mechanism is not very well understood. It has been suggested that nitric oxide related suppression of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κβ) pathway, cyclooxygenase-2 activation and prostacyclin production are possible candidates (Cockerill *et al.*, 1999; Schmidt *et al.*, 2006; Murphy *et al.*, 2009). The lysosphingolipid component of HDL activates phosphatidyl-inositol-3 (PI-3) and Akt kinases to trigger nitric oxide synthesis by endothelial nitric oxide synthase (eNOS) which inhibits TNF-α activation (Schmidt *et al.*, 2006). Although the inhibition of the expression of adhesion molecules remains one of the anti-inflammatory actions of HDL, it also interacts directly with inflammatory cells to circumvent an inflammatory response.

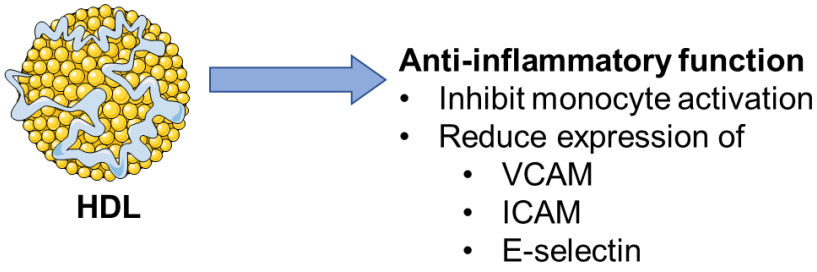



**Figure 8. Anti-inflammatory function of HDL and proposed mechanism. VCAM: Vascular cell adhesion Molecule and ICAM: Intercellular adhesion molecule**

Monocyte production of inflammatory cytokines such as TNF-α and Interleukin-1 is dependent on contact activation with T-lymphocytes (Hyka *et al.*, 2001). The ApoA1 component of HDL inhibits the production of cytokines in monocytes in a dose-dependent manner. This occurs by inhibition of contact activation of monocytes by T-cells by HDL binding to the surface of activated T-cells (Hyka *et al.*, 2001). HDL and ApoA1 reduce monocyte activation, by inhibiting expression of monocyte integrin CD11b, which binds to endothelial adhesion molecules such as ICAM (Murphy *et al.*, 2008). HDL inhibits monocyte spreading and chemotaxis (Diederich *et al.*, 2001; Kim *et al.*, 2013), implicating the actions of eNOS and SRB1 (Murphy *et al.*, 2008, 2009).

Assays seeking to quantify the anti-inflammatory function of HDL, quantify expression of adhesion molecules in endothelial cells (Calabresi et al. 2002; Cockerill et al. 1995;

Gomaraschi et al. 2008) or assess the chemotaxis of monocytes toward a gradient produced by endothelial cell culture following pre-treatment with HDL and inflammatory drugs (Kim *et al.*, 2013).

### 2.4.5 Anti-thrombotic functions of HDL

Alterations in the balance between coagulation and fibrinolysis can promote the onset of atherosclerosis (Nofer *et al.*, 2002). The aggregation and activation of platelets, are associated with arterial thrombosis (Mineo *et al.*, 2006). Thrombus formation can trigger downstream ischemic events and arterial thrombosis is inversely correlated with HDL-C levels (Gordon *et al.*, 1977; Deguchi *et al.*, 2005). HDL controls blood coagulation and inhibits platelet activation via several anti-thrombotic functions (Figure 9).

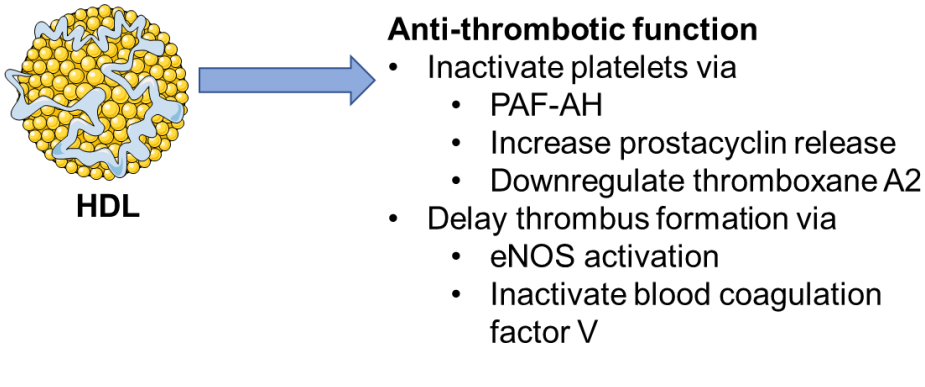



**Figure 9. Anti-thrombotic function of HDL and proposed mechanisms. PAF-AH: Platelet activating factor acetylhydrolase and eNOS: Endothelial nitric oxide synthase**

The direct link between HDL and thrombus formation can be elucidated using animal models. Using an arterial thrombosis rat model, Li et al, demonstrated reduced platelet aggregation, time to thrombus formation and thrombus weight, when animals were infused with antioxidative ApoA1 (Li et al. 1999). There are three biochemical mechanisms by which thrombosis occurs and are collectively known as Virchow's triad; dysfunction of cells within the vascular wall, disturbed blood flow and dysfunction of the blood components (Mineo *et al.*, 2006). The antiapoptotic effects and pro-angiogenic effects of HDL, largely related to HDL-mediated activation of nitric oxide pathways are mechanisms of preventing the first cause of thrombosis. The regulatory roles played by HDL in preventing blood component dysfunction and blood flow will be focussed on specifically in this section detailing anti-thrombotic HDL functions.

The blood coagulation process induces the activation and aggregation of platelets via the thrombin blood coagulation cascade (Griffin *et al.*, 1999). Using the activated protein C and

protein S anticoagulant pathways, HDL inactivates coagulation Factor V (Griffin *et al.*, 1999). HDL performs direct effects on platelets, demonstrated by the effects of administration of rHDL in diabetic patients (Calkin *et al.*, 2009). Platelet activation and aggregation was markedly reduced following administration of rHDL (Calkin *et al.*, 2009).

Down-regulation of platelet activating factor (PAF), inhibition of the release of Weibel-Palade bodies for platelet-endothelial cell interaction, prostacyclin release and down-regulation of thromboxane A2 synthesis all serve as potential mechanisms for HDL-mediated inhibition of platelet activation (Fleisher *et al.*, 1982; Stafforini *et al.*, 1987; Brill *et al.*, 2011; Camont *et al.*, 2013). PAF is a potent activator of platelets, monocytes and leukocytes (Stafforini *et al.*, 1987). The metabolism of PAF in the blood is almost completely regulated by the PAF-AH enzyme and the enzyme is a structural component of both LDL and HDL (Stafforini *et al.*, 1987). Enzymatic hydrolysis of PAF by PAF-AH renders it physiologically inactive and as such, the association of this enzyme with HDL relates to the anti-thrombotic activities of HDL (Stafforini *et al.*, 1987; Durrington *et al.*, 2001).

Another primary anti-thrombotic action of HDL is activation of prostacyclin release. Prostacyclin is an arachidonic acid-derived lipid mediator and is as a powerful inhibitor of platelet activation. Prostacyclin promotes smooth muscle relaxation and reduces the release of growth factors that promote smooth muscle cell proliferation (Vane and Botting, 1995). HDL increases prostacyclin release by endothelial cells via two mechanisms. The first involves HDL cholesteryl esters serving as arachidonic acid donors for prostacyclin production by the cyclooxygenase-2 enzyme. The second involves an increase in cyclooxygenase-2 expression (Fleisher *et al.*, 1982; Cockerill *et al.*, 1999). S1P increases the production of cyclic Adenosine Monophosphate (cAMP) in smooth muscle cells, which induces prostacyclin production by increasing cyclooxygenase-2 expression (Damirin *et al.*, 2005). PAF-AH activity and prostacyclin release mediate the anti-thrombotic effects of HDL, while HDL can also reduce thromboxane A2 production directly in platelets (Camont *et al.*, 2013). Thromboxane A2 is responsible for platelet activation.

Therefore, to assess the anti-thrombotic functions of HDL, the activity of PAF-AH (Stafforini *et al.*, 1987; Daniil *et al.*, 2011), the levels of thromboxane A2 (Camont *et al.*, 2013) or prostacyclin (Fleisher *et al.*, 1982) can be measured.

### 2.4.6 Maintenance of endothelial homeostasis

Maintenance of the homeostasis of the endothelial cell layer is imperative in the prevention of the development of atherosclerotic lesions. HDL controls proliferation and vasorelaxation of endothelial and smooth muscle cells, prevents endothelial apoptosis and has also been recently implicated as a mediator in stem cell recruitment (Chen *et al.*, 1986; De Souza *et al.*, 2010; Yvan-Charvet *et al.*, 2010).

HDL has been shown, in combination with growth factors and serum components, to stimulate the proliferation and migration of endothelial cells, as well as the proliferation of smooth muscle cells (Ross and Glomset, 1973; Libby *et al.*, 1985; Chen *et al.*, 1986; Murugesan *et al.*, 1994). Smooth muscle cell proliferation contributes to the pathogenesis of atherosclerosis. Promotion of proliferation by HDL seems counterintuitive, however, proliferation seems to only be induced by higher concentrations of HDL normally present following loss of the endothelium (Libby *et al.*, 1985). Under these conditions, smooth muscle growth may then contribute to plaque stability, replenishing cells lost from the fibrous cap (Nofer *et al.*, 2002). Aside from promoting proliferation, HDL limits vasorelaxation through modulation of eNOS.

Modulation of eNOS activity by HDL has been demonstrated in both cultured endothelial cell and animal models (Besler *et al.*, 2011). Mechanistically HDL stimulates eNOS activity through SRB1 and S1P receptor 1 and 3 (Yuhanna *et al.*, 2001; Nofer *et al.*, 2004; Igarashi *et al.*, 2007). HDL induces Akt phosphorylation and intracellular calcium ion release, which play roles in a sequence of activation steps also involving Src Tyrosine kinase, PI-3 kinase and Erk1/2 MAP kinase, leading to phosphorylation of eNOS at Ser-1177 (Mineo and Shaul, 2003; Nofer *et al.*, 2004). The activation of eNOS by HDL is hindered by OxLDL which causes displacement of eNOS and reduced activation (Blair *et al.*, 1999). Additionally, OxLDL displays endothelial cytotoxicity (Figure 10).

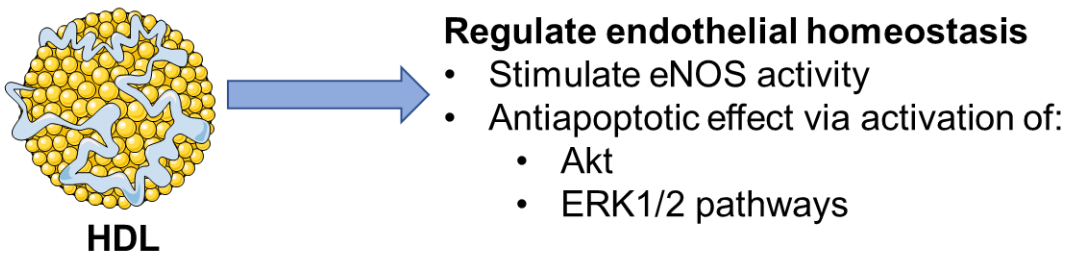



**Figure 10. HDL regulation of endothelial homeostasis and proposed mechanisms. eNOS: Endothelial nitric oxide synthase.**

OxLDL induced cytotoxicity of endothelial cells is critical in atherosclerosis development and HDL serves as a powerful antiapoptotic agent in endothelial cells. Cell death, as a result of

growth factor or serum depravation, was reduced in endothelial cells by HDL (Kimura *et al.*, 2001; Nofer *et al.*, 2001). Under inflammatory conditions, cytokines such as TNF-α, together with OxLDL are cytotoxic to endothelial cells while HDL is antiapoptotic (Sugano *et al.*, 2000; Kimura *et al.*, 2001; Frias *et al.*, 2009; De Souza *et al.*, 2010). It is well established that HDL prevents apoptosis in endothelial cells, however, maintenance of the integrity of the endothelium and the atheroprotective effects extend to diverse interactions with other cell types including cardiomyocytes. In this situation, HDL has been shown to perform an anti-ischemic function by attenuating doxorubicin induced cardiotoxicity in cardiomyocytes (Frias *et al.*, 2009). Mechanisms implicate both ApoA1 and S1P, which acts via ERK1/2 pathways (Frias *et al.*, 2009).

It is clear that HDL demonstrates a wide variety of anti-atherogenic functions that relate to reverse cholesterol efflux; the association of HDL with enzymes, apolipoproteins and sphingolipids and the inherent ability of HDL to activate eNOS, a powerful factor in triggering protective signalling pathways. Due to the functional prowess of HDL, circulating levels of HDL-C have traditionally served as a risk factor for CVD. This central focus has been a subject of great scrutiny in recent publications and brings into question the factor of quality over quantity of HDL.

**3. The validity of HDL-C as a risk factor of CVD risk: Quality versus Quantity**

It was first shown by the Framingham study that there is a direct negative association between HDL-C and CVD risk (Gordon et al. 1977). In terms of clinical relevance, it is presently the cholesterol component of HDL that is of primary importance. A wide range of retrospective and prospective epidemiological studies have consistently demonstrated its inverse correlation with the incidence of atherosclerotic disease (Gordon *et al.*, 1977; Assmann *et al.*, 1996; Barter and Rye, 1996; Goldbourt *et al.*, 1997). In the Emerging Risk Factors Collaboration, which compiled results from 68 observational studies, it was shown that an increase in 0.39 mmol/L of HDL-C translated into a reduction of 29% risk for subsequent coronary heart disease (Di Angelantonio *et al.*, 2009). HDL-C concentration is thus still incorporated in clinical guidelines as one of the primary parameters for assessing CVD risk (Piepoli *et al.*, 2016).

Perhaps surprisingly, cholesterol is a relatively minor component of HDL. It only represents 15% by weight, whilst its distribution within HDL subclasses also varies across the HDL density spectrum. It raises the question of how this asymmetrically distributed steroid can accurately reflect the protective effect of such a heterogeneous lipoprotein species. In a recent large-scale population study, composed of the CANHEART (Cardiovascular Health in Ambulatory

Care Research Team) cohort, associations between HDL-C levels and cardiovascular and non-cardiovascular deaths of individuals living in the same environment were evaluated (Ko *et al.*, 2016). The study included 631 762 individuals and concluded that complex associations exist between HDL-C levels and sociodemographic, lifestyle, comorbidity factors and mortality. As a result, HDL-C was unlikely to be a reliable cardiovascular-specific risk factor (Ko *et al.*, 2016). Whilst genetic variants causing increases in LDL-C were associated with increases in CVD risk, those associated with increasing HDL-C had weak associations with changes in CVD risk (Voight *et al.*, 2012).

Recent clinical trials that attempted to reduce cardiovascular risk by pharmacologically increasing the HDL-C have been unsuccessful (Kühnast *et al.*, 2015). This has provoked a re-think of the mechanisms by which HDL may protect the vascular system, giving weight to the argument that HDL functionality and discrete properties attributed to different HDL subclasses may better explain links between HDL and CVD risk. The following section addresses the failings of recent clinical trials in attenuating CVD risk by raising HDL-C.

### 3.1 Failures of large scale clinical trials raising HDL-C

*Statins*

Statins inhibit the hepatic synthesis of cholesterol through inhibition of 3-hydroxyl-3-methyl-glutaryl-coenzyme A reductase (Istvan and Deisenhofer, 2001). Statins have the most widespread application of the different lipid lowering agents owing to an active reduction in LDL-C levels (Opie and Gersh, 2013). While the major beneficial effect of statins is attributed to its strong capacity to decrease LDL-C, it also increases (approximately 5-10%) HDL-C levels. Despite this, it is not yet established whether statins reduce CVD risk through the increase in HDL-C levels (Jafri *et al.*, 2010).

*CETP inhibitors*

As described previously, CETP is a hydrophobic glycoprotein secreted from the liver and circulates in plasma bound to HDL. CETP reduce circulating HDL-C levels by transferring cholesteryl ester from HDL to larger lipoproteins, such as chylomicrons, VLDL and LDL, in exchange for triglycerides.

Four CETP inhibitors have reached late-stage clinical development: torcetrapib, dalcetrapib, anacetrapib and evacetrapib, all with disappointing results. Torcetrapib was the first tested on a large scale in patients with a history of CVD and was administered in combination with statin therapy (Barter *et al.*, 2007). Lipid profile results were very promising with a 72% rise in HDL-

C, however, despite this substantial rise, there was an increased risk of death for patients in the treatment group (Barter *et al.*, 2007). It was postulated that torcetrapib has several off-target effects including raising systolic blood pressure (Barter et al. 2007). In the Stable Coronary Heart Disease Patients With Recent Acute Coronary Syndrome (dal-OUTCOMES) trial, dalcetrapib was tested in patients with acute coronary syndrome (Schwartz *et al.*, 2012). Treatment had no significant effect on cardiovascular outcomes, and concerns were highlighted over CETP inhibitor related side-effects in patients (Schwartz *et al.*, 2012). Of the remaining two trials, the Assessment of Clinical Effects of Cholesteryl Ester Transfer Protein Inhibition With Evacetrapib in Patients at a High Risk for Vascular Outcomes (ACCELERATE) trial was terminated by the pharmaceutical company as non-effective (Lincoff *et al.*, 2017), and the results of the Randomized Evaluation of the Effects of Anacetrapib Through Lipid-modification (REVEAL) trial are expected late 2017.

*Niacin*

Niacin is the most efficient HDL-C raising drug. Mechanisms of action include non-competitive inhibition of hepatocyte microsomal diacylglycerol acyltransferase-2 (DGAT2), an enzyme which catalyses the final reaction involved in triglyceride synthesis (Ganji *et al.*, 2002) and selective inhibition of ApoA1 uptake without influencing de novo synthesis (Jin *et al.*, 1997). Widespread application of niacin treatment has been limited by adverse side-effects.

In the Atherothrombosis Intervention in Metabolic Syndrome with Low HDL/High Triglycerides: Impact on Global Health Outcomes (AIM-HIGH) trial, patients with established CVD along with low HDL-C were maintained on statin or ezetimibe treatment prior to treatment with niacin (Boden *et al.*, 2011). While a substantial 25% increase in HDL-C was noted, niacin therapy had no influence on primary CVD events or secondary endpoints indicating no clinical benefit of the therapy (Boden *et al.*, 2011). The Heart Protection Study 2 - Treatment of HDL to Reduce the Incidence of Vascular Events (HPS2-THRIVE) produced similarly futile results (HPS2-THRIVE Collaborative Group, 2013). The combination of slow release niacin treatment and simvastatin in patients with a history of CVD, resulted in an increase in myopathies and non-fatal side-effects, despite a rise in HDL-C (HPS2-THRIVE Collaborative Group, 2013). In contrast, however, a meta-analysis including 11 trials using niacin treatment indicated a significant reduction in CVD events (Lavigne and Karas, 2013). There is therefore, however, no conclusive evidence supporting niacin treatment as effective as reducing CVD risk by raising HDL-C levels.

*Fibrates*

Fibrates do not reduce LDL-C to the same extent as statins, however, they are still widely prescribed, in many cases as a secondary treatment in combination with statins (Moutzouri *et al.*, 2010; Katsiki *et al.*, 2013; Opie and Gersh, 2013). The mechanism of action of fibrate activity is broad and can be summarised as: induction of lipoprotein lipolysis; induction of hepatic fatty acid uptake; increased removal of LDL particles; inhibition of cholesterol and triglyceride exchange between HDL and VLDL; stimulation of HDL production via induction of hepatic synthesis of ApoA1 and ApoAII and reduced production of VLDL due to reduction of free fatty acid to the liver (Vu-Dac *et al.*, 1995; Berthou *et al.*, 1996; Staels *et al.*, 1998).

Clinical trials which assessed the influence of fibrates on HDL-C in patients were the Helsinki Heart study (Frick *et al.*, 1987) and the Veterans Affair High-density lipoprotein Intervention (VA-HIT) trial (Rubins *et al.*, 1999). In both trials, a moderate rise in HDL-C was accompanied by a reduction in the incidence of coronary heart disease (Frick *et al.*, 1987; Rubins *et al.*, 1999). Similar findings were made using benzafibrate in the Benzafibrate Infarction Prevention (BIP) trial (BIP Study Group, 2000). In contrast to this, were the results from the Action to Control Cardiovascular Risk in Diabetes (ACCORD) lipid trial (ACCORD Lipid Group, 2010). Fenofibrate used in combination with simvastatin therapy failed to significantly increase HDL-C or lower risk overall, however, in specific sub-set of patients, such as those with high triglyceride and low HDL-C, patients did benefit from the treatment with reduction in risk (ACCORD Lipid Group, 2010).

A possible explanation of the failure of the majority of these clinical trials relates to the concept of dysfunctional HDL. Under these conditions, even if a particular individual may possess a higher HDL-C, functional impairment of HDL may have consequences for risk of CVD.

### 3.2 Dysfunctional HDL associated with disease

The concept of dysfunctional HDL relates to a total loss of HDL function wherein the normal anti-atherogenic lipoprotein starts displaying pro-atherogenic properties, often as a result of structural changes (Kontush *et al.*, 2013; Serban *et al.*, 2014; Rosenson *et al.*, 2016). Dysfunctional HDL was first demonstrated during acute phase response under inflammatory conditions in human patients (Van Lenten *et al.*, 1995). Dysfunctional HDL had a loss in PON1 and PAF-AH activities, along with a loss in the ApoA1 content, rendering it pro-inflammatory (Van Lenten *et al.*, 1995). Similarly, during acute phase response, acute phase proteins such as serum amyloid A (SAA), a pro-inflammatory protein, replaces ApoA1 in HDL structure (Cabana *et al.*, 1996). Binding of SAA to proteoglycans can immobilize HDL in the arterial wall, preventing it from performing anti-atherogenic functions (Lewis *et al.*, 2004). Increased SAA content in HDL increases CVD risk due to SAA modifying vascular properties of HDL

(Zewinger *et al.*, 2015). Triglyceride enrichment in the HDL core can cause inhibition of HDL function (Cabana *et al.*, 1996; Brites *et al.*, 2000). Triglyceride content may also alter ApoA1 conformation causing inhibition of ApoA1 and HDL functions (Curtiss *et al.*, 2000). Subsequent to findings from acute phase response, a number of other pathologies and conditions have elucidated the phenomenon of dysfunctional HDL.

HDL is the major carrier of lipid hydroperoxides *in vivo*, which have increased susceptibility to oxidation and therefore causes loss of function of HDL (Bowry *et al.*, 1992; Shao and Heinecke, 2009). Myeloperoxidase can interact with ApoA1, causing nitration and chlorination, resulting in generation of pro-atherogenic HDL with loss in ABCA1-mediated RCT (Pennathur *et al.*, 2004; Zheng *et al.*, 2004; Huang *et al.*, 2014). Myeloperoxidase also inactivates paraoxonase through oxidation of Tyr171 on PON1 (Huang *et al.*, 2013).

Dysfunctional HDL is characteristic in patients with coronary artery disease, presenting with a pro-inflammatory HDL phenotype when compared to controls (Ansell *et al.*, 2003; Besler *et al.*, 2011; Sattler *et al.*, 2015). In chronic renal disease, the HDL functions are impaired and the capacity to promote cholesterol efflux, the antioxidant and anti-inflammatory effects are diminished compared to HDL from healthy subjects (Vaziri, 2015). Recent data suggests the role of carbamylation in this process (Sun *et al.*, 2016). HDL can become dysfunctional in patients with diabetes mellitus as a result of glycation of HDL (Nobécourt *et al.*, 2010; Brinck *et al.*, 2016). This has also been shown in patients with insulin resistance, whilst smoking has been linked with producing dysfunctional HDL subclasses due to an increased susceptibility to glycation (McMillen *et al.*, 2005; Song *et al.*, 2015). Additionally, platelets can modify native HDL, resulting in a dysfunctional and pro-thrombotic form of HDL (Blache *et al.*, 2012).

An important consideration when examining the link between HDL levels, CVD risk and overall HDL functionality is the subclass distribution of HDL.

Each HDL subclass, as a result of their differences in composition, can perform different functions. The relevance of each subclass as independent predictors of CVD risk as well as functional superiority between subclasses will now be discussed.

### 3.3 Anti-atherosclerotic functions of HDL2 and HDL3

The contribution of each of the HDL subtypes to overall HDL functionality has been a subject of significant debate in the recent literature. From early mechanistic studies in fibroblasts which reported a difference in the capacity of different HDL subclasses to efflux cholesterol, there seems to indicate a possibility of a functionally superior HDL subclass (Oram *et al.*, 1981).

Linking a specific HDL subclass as a more accurate predictor of risk is challenging due to the results of mechanistic studies and taking into account the results of epidemiological studies.

Early reports suggest HDL3 has increased cholesterol efflux capacity compared to HDL2 (Oram *et al.*, 1981). Improved cholesterol efflux by HDL3, was attributed to enrichment with negatively charged phospholipids (Camont *et al.*, 2013). The vast majority of mechanistic studies into HDL subclass functionality, point toward HDL3 as the functionally superior subclass. The outcomes of pre-clinical studies, which have compared HDL3 and HDL2 are summarized in Table 1.

**Table 1. Data from pre-clinical studies distinguishing HDL subclasses on the basis of functionality.**

| Pro-HDL3 | | Pro-HDL2 | |
|---|---|---|---|
| Main finding | Reference(s) | Main finding | Reference(s) |
| Improved cholesterol efflux overall<br>Improved ABCA1-mediated cholesterol efflux | (Oram *et al.*, 1981) (Camont *et al.*, 2013)<br>(Asztalos *et al.*, 2005) (Favari *et al.*, 2009) | Improved SRB1-mediated cholesterol efflux | (Asztalos *et al.*, 2005) (Favari *et al.*, 2009) (Julia *et al.*, 2010) (Yancey *et al.*, 2000) |
| Increased antioxidant function | (Camont *et al.*, 2013) (Curtiss *et al.*, 2000) (Davidson *et al.*, 2009) (Kontush *et al.*, 2003) (Kontush *et al.*, 2007) | Reduction in LDL binding to proteoglycans | (Umaerus *et al.*, 2012) |
| Increased anti-inflammatory function | (Ashby *et al.*, 1998) (Camont *et al.*, 2013) | | |
| Increased antiapoptotic function | (Camont *et al.*, 2013) (De Souza *et al.*, 2010) (Kontush *et al.*, 2007) | | |
| Increased anti-thrombotic function | (Camont *et al.*, 2013) | | |

A recent study sequentially isolated each HDL subfraction from healthy human subjects, and quantified functionality in five assays (Camont *et al.*, 2013). These included the cholesterol efflux capacity, antioxidative, antithrombotic, antiapoptotic and anti-inflammatory functions of HDL2 and HDL3. In all of the assays, HDL3 proved significantly superior to HDL2 regarding basic HDL functionality (Camont *et al.*, 2013). The authors postulated that a difference in lipid

composition of HDL3 could be a possible explanation for these observations and indeed, structural analysis of rHDL mimicking HDL2 and HDL3 has proven useful in illustrating how these differences may affect functionality (Camont et al. 2013; Curtiss et al. 2000). Conformational changes in the HDL3 lipidome allow enhancement of the mobility of the active region of ApoA1, permitting improved surface access, increasing ApoA1 related functionality (Curtiss et al. 2000; Kontush & Chapman 2010). Another important lipid component of HDL3 is S1P which is almost exclusively associated with the smaller HDL subclass (Kontush *et al.*, 2007; Lee *et al.*, 2010).

As discussed in section 4.3, the anti-atherogenic function of S1P as a signalling molecule may often relate directly to HDL functionality (Mineo *et al.*, 2006). The S1P component of HDL has been shown to induce nitric oxide release (Nofer *et al.*, 2004); prevent ischemic injury and trigger cardioprotective signalling pathways (Theilmeier *et al.*, 2006; Frias *et al.*, 2012); reduce OxLDL related cytotoxicity (Kimura *et al.*, 2001; Kontush *et al.*, 2007); induce prostacyclin release via activation of cyclooxygenase-2 (Liu *et al.*, 2012) and together with HDL3, prevent LDL oxidation (Kontush *et al.*, 2007). Additionally, the S1P component of HDL3 increases the release of platelet activator inhibitor, which negatively modulates fibrinolysis *in vivo*, contributing to an increase in CVD risk (Lee *et al.*, 2010).

Patients with coronary artery disease had a lower S1P concentration in HDL which could be raised using *in vitro* S1P loading (Sattler *et al.*, 2010, 2015). This observation was extended to patients with coronary in stent restenosis (Jing *et al.*, 2015) and in type 2 diabetic patients (Brinck *et al.*, 2016). S1P is inversely correlated with glycated haemoglobin (HbA1c) in type 2 diabetic patients and the concentration of S1P is directly correlated with its cardiac specific anti-apoptotic capacity (Brinck *et al.*, 2016). Other than the benefits of association with S1P, HDL3 has been distinguished from HDL2 as a superior HDL subclass in additional mechanistic studies.

Regarding the anti-inflammatory and antiapoptotic functions of HDL, HDL3 was shown to inhibit TNF-α induced expression of VCAM in HUVEC cells to a greater extent than HDL2 (Ashby *et al.*, 1998). HDL3 also showed improved cytoprotection of HMEC-1 cells from OxLDL (De Souza *et al.*, 2010). HDL3 demonstrates a more potent antioxidant capacity than HDL2 and is itself, less prone to oxidation than the larger subclass (Kontush *et al.*, 2003; Shuhei *et al.*, 2010). Mechanistically, HDL3 prevents LDL oxidation and inhibits generation of lipid hydroperoxides, which are transferred from LDL to HDL3 (Zerrad-Saadi *et al.*, 2009). In addition to this mechanism, the superior antioxidant function of HDL3 can relate to selective association with antioxidant enzymes such as PON1, LCAT and PAF-AH (Davidson *et al.*, 2009).

In apparent contrast, HDL2 was shown to elicit improved free cholesterol efflux when compared to HDL3, owing to an improved phospholipid content (Yancey *et al.*, 2000). However, the difference between HDL2 and HDL3 in cholesterol efflux observed by Yancey et al., was pronounced in SRB1 expressing cells. HDL2 has been shown to exclusively promote cholesterol efflux by SRB1 (Yancey *et al.*, 2000; Asztalos *et al.*, 2005; Favari *et al.*, 2009; Julia *et al.*, 2010). Using rHDL as analogues, it was shown that preβ-HDL and HDL3 promote ABCA1 mediated cholesterol efflux (Asztalos *et al.*, 2005; Favari *et al.*, 2009). This indicates that each HDL subclass may promote cholesterol efflux via different receptors with smaller HDL molecules promoting ABCA1 mediated efflux and larger molecules promoting SRB1 mediated efflux. Both HDL3 and HDL2 equally promote ABCG1 mediated transport (Favari *et al.*, 2009; Rothblat and Phillips, 2010). Regarding further functionality based studies, HDL2 was shown, in a rather novel mechanism, to more actively prevent LDL associations with proteoglycans, thereby inhibiting LDL entrapment and atherosclerosis (Umaerus *et al.*, 2012).

### 3.4 HDL2 and HDL3 as a better index of CVD risk

Early epidemiological studies describe HDL2 as more accurate risk indicator for CVD. Indeed, myocardial infarction survivors had low levels in HDL2 (Brugger *et al.*, 1986), and HDL2 was inversely correlated with coronary heart disease risk (Johansson *et al.*, 1991). Modern studies continue to argue for HDL2 as a risk factor. A large study of 4594 healthy patients demonstrated that a decrease in HDL2 was associated with increased CVD risk (Musunuru *et al.*, 2009). Similarly patients with acute coronary syndrome displayed lower levels of HDL2 and higher levels of HDL3 (Tian *et al.*, 2014).

In contrast, post-hoc analysis of two prospective studies, the IDEAL (Incremental Decrease in End Points through Aggressive Lipid Lowering) trial and the EPIC (European Prospective Investigation into Cancer and Nutrition)-Norfolk case-control study, showed that a very high concentration of HDL2 particles, may be associated with increased rather than decreased CVD risk (van der Steeg *et al.*, 2008). Kavo et al., studied HDL from patients who survived a myocardial infarction (MI) at a young age (≤35 years) and healthy control subjects and showed that MI patients had reduced preβ-1 and HDL3 and elevated HDL2 (Kavo *et al.*, 2012). Martin and colleagues, analysed the data from two cohorts, the Translational Research Investigating Underlying disparities in acute Myocardial infarction Patient's Health Status (TRIUMPH) and Intermountain Heart Collaborative Study (IHCS), which indicated that lower HDL3, rather than lower HDL2 and lower total cholesterol, was an improved predictor of mortality in myocardial infarction patients (Martin *et al.*, 2015). These data are confirmed by the recent results of the

secondary analysis of the AIM-HIGH Study which indicate that the levels of HDL3 and no other lipoprotein fractions, were predictive of cardiovascular events (Albers *et al.*, 2016).

Pre-clinical and new epidemiological data seem to indicate that HDL3 may be an improved predictor of CVD risk suggesting that HDL heterogeneity is an important consideration of HDL quality vs quantity. Large scale clinical trials have failed to show an attenuation in CVD risk, despite significant increases in HDL-C. Additionally, dysfunctional HDL may lead to increased risk and the heterogeneity of the HDL population itself, have clearly shown that a routine measurement of HDL-C concentration does not adequately predict risk (Chang *et al.*, 2017). Having outlined the diverse functions performed by HDL, biochemical measurement of these functions as well as subclass distribution may be more relevant means to assess CVD risk. For example, HDL function was impaired in patients with myocardial infarction independent of the HDL-C levels (Annema *et al.*, 2016).

To summarize, the quality vs quantity debate of HDL; two individuals may have equivalent HDL-C values, however, an individual with better HDL functionality may have lower risk of CVD. This may be due to dysfunctional HDL in the other individual linked to structural changes in their HDL particle and a reduction in S1P content (Figure 11).

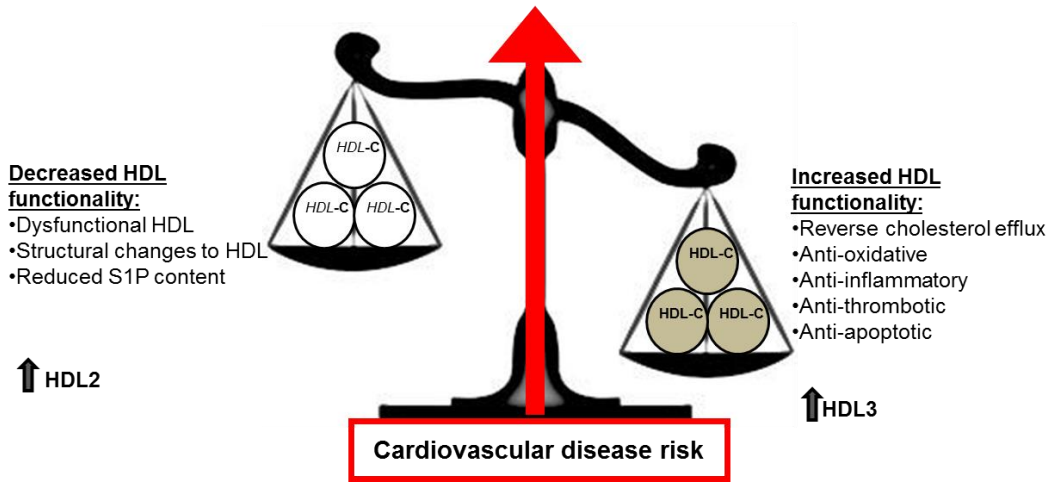



**Figure 11. Quality vs quantity of HDL in relation to cardiovascular disease risk. Adapted from (Woudberg et al. 2016). Literature suggests that HDL3 is associated with improved HDL function.**

Measurement of HDL function and subclass in patients at risk of CVD due to traditional risk factors will be important in illustrating this. Therefore, the concluding section considers such risk factors and how these may have implications for HDL function and subclass.

## 4. Cardiovascular risk factors and HDL function and subclass

There are multiple contributors or risk factors for CVD, however, few have a greater negative impact on disease outcomes than dietary intake, smoking, obesity, ethnicity and hypertension. These 5 examples will be focussed on owing to their potential impact, not only on disease risk but also on HDL functionality, composition and subclass distribution.

### 4.1 Diet

#### 4.1.1 Diet heart hypothesis

The earliest study that examined the influence of diet on atherosclerosis was conducted in 1908, where high dietary intake of cholesterol in rabbits promoted the onset of atherosclerosis (Ignatowski, 1908). Studies in human populations carried out by Keys began to show how high dietary fat and carbohydrates increase the risk of CVD (Keys, 1957). Populations with the greatest intake of saturated fat had the highest serum cholesterol whilst follow-up indicated that these groups also had the highest incidence of coronary artery disease (Keys, 1980). The so-called “diet-heart hypothesis” originally detailed how dietary fats, carbohydrates and salt increased LDL-C, lead to obesity and contributed to hypertension (Figure 12) (Rossouw, 1983).

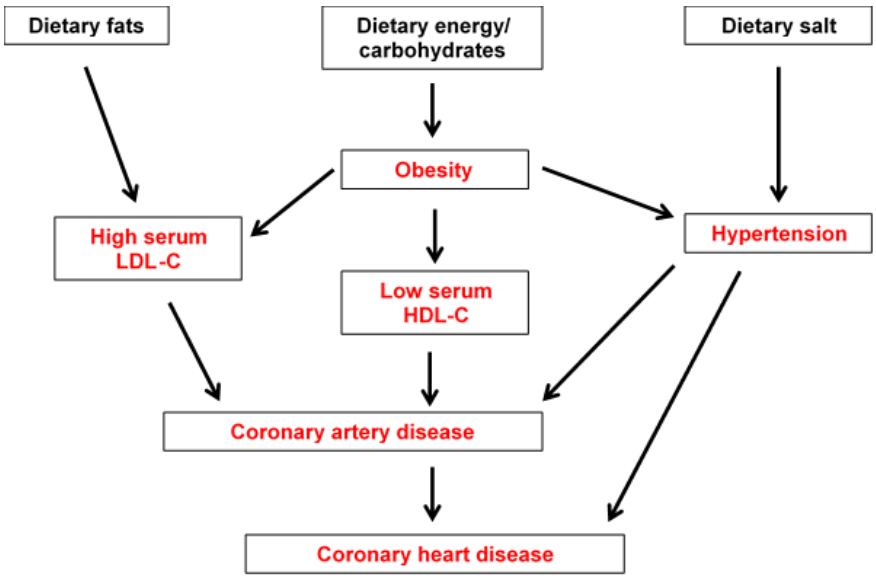



**Figure 12. Summary of the “diet-heart hypothesis”. Adapted from** (Rossouw, 1983)**.**

The relationship between diet and CVD has been extensively reviewed by Dalen and Devries, who concluded that, whilst the majority of dietary interventions (low-fat, low-cholesterol) reduced serum cholesterol concentrations, they did not reduce CVD risk (Dalen and Devries,

2014). In contrast, the Mediterranean diet is cardioprotective despite unchanged lipid content of LDL-C and total cholesterol  A meta-analysis of observational and cross-sectional studies on adherence to the Mediterranean diet showed decreased risk of metabolic syndrome and inverse associations with waist circumference, blood pressure and low HDL-C concentrations (de Lorgeril *et al.*, 1999; Dalen and Devries, 2014; Godos *et al.*, 2016). As reviewed below, the Mediterranean diet is the only diet which has demonstrated a beneficial effect on HDL function and subclass.

### 4.1.2 The Mediterranean diet

*What is the Mediterranean diet?*

The diet is characterised by key features including: use of olive oil, fruit and nuts, vegetables, non-refined cereals and legumes as main sources of fibre, and plant-derived antioxidants such as vitamins and polyphenols (Zamora-Ros *et al.*, 2012, 2013); frequent consumption of fish as the primary source of protein and poly-unsaturated fatty acids (Marventano *et al.*, 2015); moderate consumption of red wine (Giacosa *et al.*, 2013) and low consumption of meats and sweets (Di Daniele *et al.*, 2014).

*The Mediterranean diet and HDL subclass and function*

Mechanisms of action for the Mediterranean diet include: prevention of oxidative modification of lipids and DNA (Mitjavila *et al.*, 2013); anti-inflammatory effects including decreasing the expression of monocyte adhesion molecules (Mena *et al.*, 2009); modulation of the metabolomics profile (Martínez-González *et al.*, 2016) and modulation of gene expression related to CVD (Castaner *et al.*, 2013). Antioxidant and anti-inflammatory effects have functional implications for HDL and indeed, olive oil, a principal component of the diet, improved HDL cholesterol efflux (Hernáez *et al.*, 2014), increased PON1 activity (Loued *et al.*, 2013) and reduced endothelial ICAM expression (Hernáez *et al.*, 2016). Olive oil consumption also lead to changes in HDL subclass distribution, increasing HDL2 levels, as well as increasing LCAT activity and HDL antioxidative capacity (Farràs *et al.*, 2015). Supplementation with olive oil improved HDL expression of ApoA1, PON1 and other proteins related to HDL-induced cardioprotection (Pedret *et al.*, 2015). Similarly to olive oil, other components of the Mediterranean diet have been individually shown to improve HDL function. This includes the mono-unsaturated fatty acid component of the diet, which improved circulatory endothelial function, reduced adhesion molecule expression (including ICAM and VCAM), expression of von Willenbrand factor and tissue factor pathway inhibitor, which are responsible for coagulation (Pérez-Jiménez *et al.*, 1999).

In the first study of its kind, Hernáez et al, showed that adherence to the Mediterranean diet improved HDL function and altered HDL particle size versus low-fat diets (Hernáez *et al.*, 2017). Adherence to the diet increased cholesterol efflux, improved HDL induced cholesterol esterification and decreased CETP activity. Compared to a low-fat diet, the Mediterranean diet increased PON1 activity, increased HDL capacity to prevent LDL oxidation and increased eNOS activity (Hernáez *et al.*, 2017). Increases in HDL particle size were however equivalent to the effect resulting from the low-fat diet (Hernáez *et al.*, 2017). Examining HDL subclass distribution itself, analysis of dietary intake of young adults showed significant correlations between increased sugar and saturated fat consumption and shifts in HDL and LDL subclass distribution, favouring decreases in size in both HDL and LDL (Bogl *et al.*, 2013). Analysis of the lifestyle habits of 290 healthy men indicated that physical activity, moderate alcohol intake and consumption of mono-unsaturated fatty acids in their diet favourably increased HDL2 concentrations (Parlesak *et al.*, 2014).

### 4.2 Smoking

Cigarette smoking is the second most common cause of death worldwide and is responsible for approximately 6 million worldwide deaths annually (World Health Organization, 2015). Cigarette smoking is therefore also an independent risk factor for CVD (Pearl, 1938; Doll and Hill, 1956; Hammond and Horn, 1958; Bazzano *et al.*, 2003). The harmful mechanisms of action from smoking include endothelial dysfunction (Celermajer et al. 1993; Ijzerman et al. 2003); increased oxidative stress (Tangiwa *et al.*, 1994); inflammation in the form of increased expression of VCAM, ICAM and E-selectin (Bermudez *et al.*, 2002; Mazzone *et al.*, 2002), increased monocyte adhesion (Kalra *et al.*, 1994) and changes in the lipid profiles of the smoker. Smokers have higher total cholesterol, LDL-C and triglycerides with lower HDL-C compared to non-smokers (Craig *et al.*, 1989; Nakamura *et al.*, 2009). Additionally, smoking is associated with increases in LDL oxidation (Heitzer *et al.*, 1996; Pech-Amsellem *et al.*, 1996). With deleterious consequences for the smoker's lipid profile together with biochemical effects relating to the endothelium, it is intuitive that smoking will also have negative consequences for HDL function and subclass (Figure 13).

Notably, smoking reduces PON1 activity (Nishio and Watanabe, 1997; James *et al.*, 2000); ApoA1 content and synthesis (Sigurdsson *et al.*, 1992; Richard *et al.*, 1997) and LCAT activity (McCall *et al.*, 1994). Although still debated, there may be smoking-related alterations in the activities of CETP and hepatic lipase (He *et al.*, 2013). HDL apolipoproteins are also susceptible to increased oxidative modification due to cigarette smoking whilst HDL treated with cigarette smoke extracts had reduced cholesterol efflux capacity (McCall *et al.*, 1994; Ueyama *et al.*, 1998). Regarding HDL subclass distribution, smoking was associated with

significant decreases in HDL particle size, although the latter effect was significant in women only (Moriguchi *et al.*, 1991; Freeman *et al.*, 1998; Imamura *et al.*, 2002; Beauchamp *et al.*, 2010).

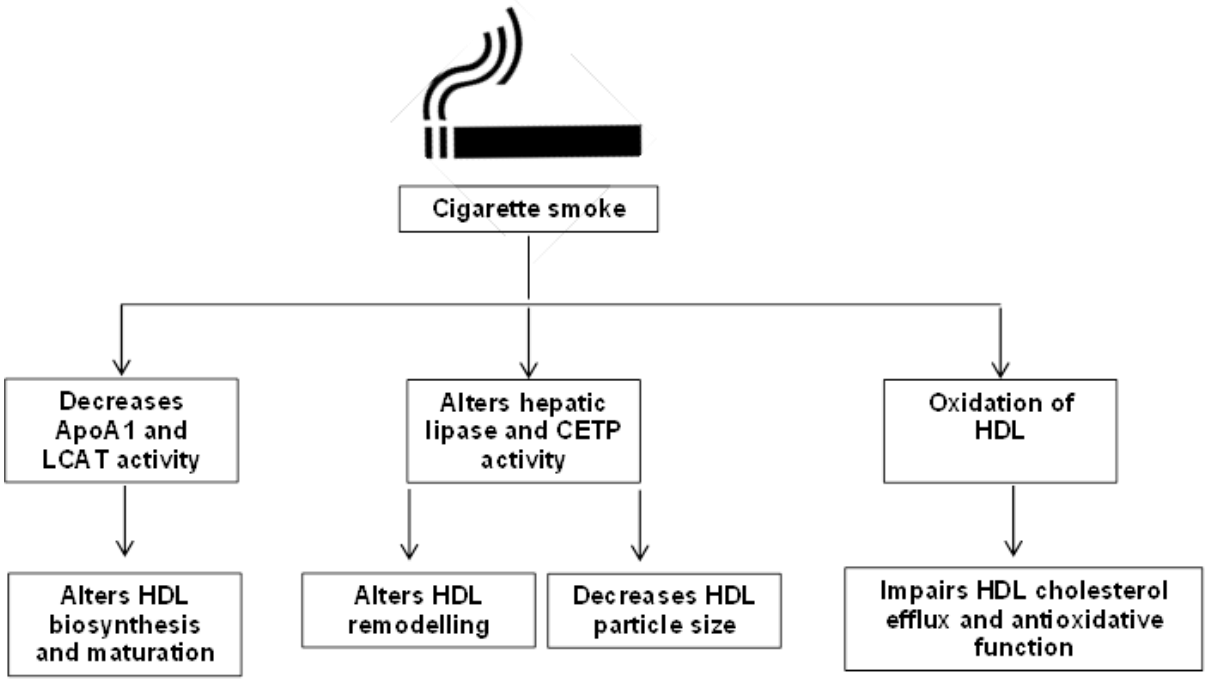



**Figure 13. Negative effects of cigarette smoking on HDL. CETP: Cholesteryl ester transfer proteinand LCAT: Lecithin cholesterol acyltransferase.**

### 4.3 Obesity

Approximately 23% of the worldwide burden of ischemic heart disease can be attributed to obesity, with obesity prevalence doubling since 1980 (World Health Organization, 2014). The deposition of adipose tissue in obesity contributes to development of hypertension, insulin resistance, hyperglycaemia and metabolic syndrome (Kopelman, 2007). Volume expansion and an increase in cardiac output causes structural changes in the heart, increasing wall stress and resulting in wall thickening and eventually hypertrophy (Pascual *et al.*, 2003; Kopelman, 2007). There is a 3.6x greater risk for coronary artery disease for each unit increase in body mass index (BMI) whilst obesity is a contributory factor for cardiac failure in more than 10% of patients (Kopelman, 2007). Accordingly, BMI serves as an independent predictor of risk for CVD (DeVallance *et al.*, 2015).

#### 4.3.1 Obesity is associated with changes in serum lipids

Dyslipidaemia progressively develops with increasing degrees of abdominal fatness, resulting in an increase in LDL-C and triglyceride concentrations (Terry *et al.*, 1989; Williams *et al.*, 1995; Nieves *et al.*, 2003; Goff Jr, 2005). However, longitudinal data suggest that, in a South

African cohort, HDL-C concentrations are maintained over time despite increases in body weight (Chantler *et al.*, 2015). With little effect on HDL-C concentrations, functional impairment of HDL has been shown in obese adolescents as a result of reduced HDL-induced activation of eNOS (Matsuo *et al.*, 2013). Smaller HDL particle size has been observed in obese European populations compared to normal-weight individuals (James et al. 1997; Magkos et al. 2012; Tian et al. 2006). A primary means of intervention includes a combination of exercise and dietary control. The effect of exercise on HDL will now be discussed.

### 4.3.2 Exercise training interventions restore lipid profiles in obese subjects

A systematic review of dietary and exercise interventions in obesity concluded that inclusion of an exercise in the intervention improved weight loss, reduced dyslipidemia and insulin resistance (Wood *et al.*, 1988; Lavie and Milani, 1997; Hawley, 2004; Curioni and Lourenco, 2005; Ohta *et al.*, 2005). Expectantly, exercise training reduced CVD risk in the Health professionals Follow-up study (Tanasescu *et al.*, 2002). Exercise interventions are either aerobic, or resistance based interventions or a combination.

There are, however, a number of conflicting reports regarding the effects of aerobic exercise training on blood lipids in overweight adults (Klancic *et al.*, 2016). A recent meta-analysis aimed to clarify the extent of aerobic exercise interventions on blood lipids. Analysis of studies from North America, Europe, Asia and Australia indicated that exercise training was associated with decrease in triglycerides, but it did not influence total cholesterol, LDL-C or HDL-C (Cai and Zou, 2016). However, in another study including type 2 diabetic patients, as little as 30 minutes of moderate intensity, daily exercise training increased HDL-C (Argani *et al.*, 2014). The results of studies examining the effects of exercise training on lipid parameters have been confounded by several variables which influence lipid biology including genetics, diet and environmental factors. Further, it is therefore difficult to delineate the hemodynamic effects of exercise training from its effects on body fat composition and weight loss (Blazek *et al.*, 2013). Meta-analysis of trials between 1955 and 2003 indicated a marginal, 2% increase in HDL-C (Kelley and Kelley, 2006). Further, analysis of trials involving aerobic exercise training alone concluded that a mean increase in HDL-C of 2.5 mg/dl was associated with exercise (Kodama *et al.*, 2007).

#### 4.3.2.1 Exercise interventions alter HDL subclass and function

Whilst some exercise intervention studies failed to show changes in HDL-C, there has been demonstrations of improvements in HDL function (Blazek *et al.*, 2013). An American study of mainly obese black individuals showed that a mild walking intervention (6 months) resulted in

weight loss, decrease in HDL-C, decrease in cholesterol efflux and antioxidant capacity, but with no effect on PON1 activity (Aicher *et al.*, 2012). A 3 month aerobic exercise intervention in Brazilian metabolic syndrome patients increased PON1 activity and RCT, with no change in HDL-C (Casella-Filho *et al.*, 2011). Cycle ergometer training for 6 weeks in elderly Japanese patients was associated with an increase in HDL-C and LCAT activity (Riedl *et al.*, 2010). After a 9 week aerobic intervention in a population from the Czech Republic, participants had a weight reduction, with no change in HDL-C (Lesna *et al.*, 2009), while an increase in RCT was only significant in individuals with substantial weight loss (Lesna *et al.*, 2009). Finally, type 2 diabetic Brazilian patients in a 4 month program had improved HDL antioxidant function and an altered subclass distribution (Ribeiro *et al.*, 2008). Other than the aforementioned studies, little is known regarding the benefits of an exercise intervention on the functional and structural properties of HDL, particularly in ethnic populations at high risk of type 2 diabetes (Sukala *et al.*, 2012).

### 4.4 Ethnicity

There has been an increase in non-communicable diseases throughout Africa (Mayosi *et al.*, 2009), largely driven by an aging population, as well as Westernization of dietary and exercise trends (Akinboboye *et al.*, 2003; Mayosi *et al.*, 2009). This can be demonstrated by the "immigrant effect". For example, the prevalence of type 2 diabetes shows a rising gradient from black Africans to African American and African migrants (JC *et al.*, 1999). A comparison of local Cameroonians and migrants in Paris showed a younger age of diagnosis of type 2 diabetes in the migrant patients (Choukem *et al.*, 2014). This may be attributed to earlier diagnosis of metabolic disorders, due to an improved access to health care, or may be due to marked changes in the socioeconomic environment, including shifts in dietary intake toward a Westernized diet (Choukem *et al.*, 2014). Conversely, access to drugs might improve some health outcomes. Notably, African diabetic migrants displayed greater HDL-C concentrations than matched African diabetic patients, most likely due to access to statin therapy, which is available in more developed countries (Choukem *et al.*, 2014). It is clear that large parts of Africa are under-going an epidemiological transition. Before attributing these changes to all individuals in an African setting, it is important to consider how different ethnic groups display distinct risk profiles of CVD.

#### 4.4.1 Ethnicity specific CVD risk

A number of studies have characterised ethnicity-specific differences in CVD risk in non-African populations, particularly focusing on minority groups (Castelli *et al.*, 1977; Cappuccio, 1997; Frank *et al.*, 2014). A systematic review of cardiovascular risk factors in North

Americans concluded that African American populations exhibit higher obesity, diabetes and high blood pressure than white American populations (Gasevic *et al.*, 2015), resulting in a greater risk for coronary artery disease, stroke and cardiovascular outcomes than their white American counterparts (Lloyd-Jones *et al.*, 2009; Duke *et al.*, 2012; McTigue *et al.*, 2014). The review attributes the higher risk in African Americans to a poor socioeconomic status, lack of education and lack of access to adequate health care system compared to white Americans (Mensah *et al.*, 2005). Metabolic profiling has indicated a unique set of risk factors, relating to prevalence of different CVD within black or African populations.

Despite possessing similar risk factors to African Americans, black Africans present with different CVD compared to African Americans, most likely due to a difference in epidemiological transition between Africa and America. Comparing black Africans to white Africans, black Africans present with higher blood pressure, diabetes mellitus, hypertension and cerebrovascular disease (Chaturvedi *et al.*, 1993; Cappuccio *et al.*, 1997; Fowkes *et al.*, 2006; Mayosi *et al.*, 2009; Golden *et al.*, 2012). In contrast, coronary heart disease remains less common in black African populations. Less than 10% of black African patients present with ischemic heart disease, a CVD much more routinely associated with white population and patients in developed countries (Rossouw, 1983; Akinboboye *et al.*, 2003; Mayosi, 2007; Sliwa *et al.*, 2008; Agyemang *et al.*, 2009). Within South Africa, cholesterol-attributable mortality was higher in white compared to black populations, with only 1.8% mortality attributable to 'sub-optimal' cholesterol levels in the South African black population (Norman *et al.*, 2007).

### 4.4.2 Ethnicity-specific lipid profiles

Differences in the susceptibility to CVD in Africa may be a result of distinct ethnic-specific lipid profiles. Populations of African descent have lower total-cholesterol, LDL-C and triglycerides than their white counterparts (Sundquist *et al.*, 2001; Agyemang *et al.*, 2009; Frank *et al.*, 2014). It was generally thought that the lower incidence of coronary heart disease in black African populations may be attributed to their greater HDL-C, which has been consistently shown in African American, compared to white Americans (Castelli *et al.*, 1977; Chaturvedi *et al.*, 1994; Grundy *et al.*, 2014). Within Africa, high HDL-C concentrations were associated almost exclusively with black African populations (Steyn *et al.*, 1991; Seedat *et al.*, 1992; Akinboboye *et al.*, 2003; Alberts *et al.*, 2005; Sumner *et al.*, 2011). Studies from the early 1990's in South Africa indicated that the majority of the black population presented with high protective HDL-C/triglyceride ratios (Steyn *et al.*, 1991; Seedat *et al.*, 1992). However, recent South African studies have shown that HDL-C does not differ in black population, compared to white and other ethnic groups (Table 2).

**Table 2. Findings of South African population studies comparing HDL-C levels**

| Population | Population age | Gender (%) | Finding | Reference |
|---|---|---|---|---|
| Black (n=458) | 16-69 years | Men (52%) and women (48%) | Protective high HDL/triglyceride ratio in the majority of patients | (Seedat *et al.*, 1992) |
| Black (n=15) White (n=14) | Pre-menopausal | Women | No significant difference across groups | (Punyadeera *et al.*, 2001) |
| Black (n=1823) White (n=142) Mixed race (n=87) Indian (n=133) | 40-75 years | Men (53%) and women (47%) | | (Sliwa *et al.*, 2008) |
| Black (n=28) White (n=28) | 18-45 years (pre-menopausal) | Women | Lower HDL-C in black compared to white | (Goedecke *et al.*, 2010) |
| Black (n=209) White (n=234) | 18-45 years (pre-menopausal) | Women | | (Ellman *et al.*, 2015) |

In the Heart of Soweto study, black African patients had lower total cholesterol, LDL-C and triglycerides compared to Indian, white and mixed ethnic groups, however HDL-C did not differ between ethnic groups (Sliwa *et al.*, 2012). Similarly, South African white women presented with higher total cholesterol, triglycerides and LDL-C concentrations than black women, but HDL-C concentrations did not differ by ethnicity (Punyadeera *et al.*, 2001). In contrast, Goedecke et al, showed that black women from the Western Cape region in South Africa presented with lower HDL-C, than their white counterparts, despite lower triglycerides and total cholesterol (Goedecke *et al.*, 2010). This was further confirmed by Ellman et al, even after adjusting for differences in total fat mass and visceral adipose tissue mass (Ellman *et al.*,

2015). These studies consistently showed that HDL-C concentrations are similar or lower in black African women compared to white pre-menopausal women. There is an emerging body of evidence from population studies suggesting that any protective lipid profile in black populations cannot be linked to a higher HDL-C, as previously shown in American populations. This may then have implications for HDL function.

### 4.4.3 Ethnicity-specific effects on HDL function

African Americans had equivalent activity levels of PON1 despite having similar concentrations of HDL-C compared to white Americans (Gaillard *et al.*, 2011). PON1 polymorphisms, such as L-55M, which results in reduced serum PON1 activity, are less frequent in Asian and black populations (Phuntuwate *et al.*, 2005; Healy *et al.*, 2015; Mackness and Mackness, 2015). There is further evidence to suggest that there are ethnic-specific differences in HDL function. Functional impairment of HDL in South Asians was postulated as contributing to an increased risk of coronary artery disease (Dodani *et al.*, 2008). Compared to Caucasians, South Asians had impaired HDL antioxidative and anti-inflammatory function (Bakker *et al.*, 2016). African Americans had impaired endothelial vasodilation response compared to white American populations, whilst endothelium dysfunction in metabolic syndrome patients was ethnicity-specific with African Americans having a greater endothelial dysfunction (Stein *et al.*, 1997; Gainer *et al.*, 2001; Rosenbaum *et al.*, 2002; Lteif *et al.*, 2005). These studies therefore suggest that ethnicity as a risk factor for CVD not only results in specific risk profiles for different populations but also is associated with differences in HDL functionality.

## 4.5 Hypertension and heart failure

*The burden of hypertension*

Hypertension related complications account for 9.4 million deaths worldwide annually (World Health Organization, 2013). Increased burden of hypertension is as a result of an aging population, increased inactivity and poor dietary habits (Bello, 2013). Hypertension, commonly referred to as “high blood pressure” is defined by the Seventh Joint National Committee Report (JNC7) in two stages pertaining to systolic blood pressure between 140 and 159 mmHg (stage 1) and systolic blood pressure exceeding 160 mmHg (stage 2). Raised systolic blood pressure is associated with several cardiovascular complications including ischemic heart disease and stroke (Forouzanfar *et al.*, 2017). Chronic increased systolic blood pressure is also the principal cause of left ventricular hypertrophy (Verdecchia *et al.*, 1990). Transitioning to HF, the left ventricle is dilated with reduced ejection fraction, often as a result of coronary artery

disease or myocardial infarction, although this is still debated (Izzo, 2004; Drazner, 2005). HF prevalence is increasing worldwide (Go *et al.*, 2013). Hypertension is indeed a major cause of HF in developing countries and the burden of congestive cardiac failure is high in Nigerian hospitals (Mendez and Cowie, 2001; Onwuchekwa and Asekomeh, 2009). In a cohort of 1 515 patients, hypertensive HF was the most common form of HF in Abuja, Nigeria (Ojji *et al.*, 2013).

Between 1990 and 2015, deaths due to raised systolic blood pressure increased, and remains the highest contributor to disability adjusted life years (DALY's) (GBD 2015 Disease and Injury Incidence and Prevalence Collaborators, 2016; Forouzanfar *et al.*, 2017).

Age standardized DALY's are highest in Oceania and central and sub-Saharan Africa with up to 20-25% adult Nigerians, for example, being hypertensive (Ogah, 2006; Forouzanfar *et al.*, 2017). Recent studies, however, speculate that the prevalence of hypertension may be as high as 42% in Nigerian populations with many individuals being unaware of their illness (Ulasi *et al.*, 2011). American Heart Association statistics indicate that black populations are at substantially higher risk than Caucasian or white populations (Mozaffarian *et al.*, 2015). Additional risk in black populations can be attributed to a diet higher in sodium and lower in potassium, low in-takes of fruits and vegetables and lack of physical activity (Bello, 2013). An additional factor is a heightened sensitivity and production of aldosterone, the primary sodium retaining hormone (Tu *et al.*, 2014). Genetic aberrations in epithelial sodium channels, specific to the ENaC gene, are also likely contributors to higher hypertension in black populations (Rayner and Spence, 2017).

### 4.5.1 Hypertension, heart failure and HDL subclass and function

The pathogenesis of hypertension proposes a number of causal factors relating to diet, renal function and stress (Oparil *et al.*, 2003). Endothelial dysfunction has been a recent important consideration, particularly in light of HDL functionality research. Nitric oxide, as previously stated, is a potent vasodilator, inhibits platelet adhesion and inhibits the migration and proliferation of smooth muscle cells (Oparil *et al.*, 2003). Nitric oxide related vasodilation is reduced in hypertensive patients (Oparil *et al.*, 2003). HDL stimulates eNOS activity through the binding of S1P receptors (Nofer *et al.*, 2004; Igarashi *et al.*, 2007; Besler *et al.*, 2011) whilst polymorphisms in the eNOS gene have also been associated with hypertension (Miyamoto *et al.*, 1998; Kishimoto *et al.*, 2004). In addition, much of the functional impairment of eNOS has been attributed to increased oxidative stress in the endothelium (Cai and Harrison, 2000). Considering the functional link to eNOS as well as the antioxidative properties of HDL, it is intuitive to postulate that impaired HDL function may be associated with hypertension. Indeed,

recent literature suggests that dysfunctional HDL is characteristic in pulmonary hypertension patients (Ross *et al.*, 2015), while HDL subclass distribution may be associated with changes in the hypertensive disease state (Zhang *et al.*, 2015).

Diagnosis of HF is primarily performed using echocardiography. However, echocardiography is not easily accessible in low-resource environments, particularly in Africa (Ogah *et al.*, 2006). Echocardiography requires expensive equipment and skilled staff. As a result, there is a need to establish novel biomarkers of heart disease which can distinguish HF patients from those with hypertension (Ojji *et al.*, 2013). Whilst brain natriuretic peptide (BNP) is currently recognised as the most sensitive biomarker for HF, preliminary data may suggest that HDL and HDL subclass may be possible biomarkers for use in this regard (Zhang et al., 2015). The Lipoprint® System does not require specialised training and can be readily established in a diagnostic laboratory. Similarly to hypertension, increased oxidative stress has been shown as a key mediator in the pathogenesis of HF (Okonko and Shah, 2014), implying that HDL function may be related to HF. Studies have shown that there was a notable reduction in HDL-associated PON1 activity in patients with systolic HF (Tang *et al.*, 2011). In patients with chronic HF, HDL antioxidative function was a strong and independent predictor of mortality (Schrutka *et al.*, 2016). An observational study in an Arab population at high risk of HF showed low levels of PON1 activity (Gugliucci *et al.*, 2015). In addition to PON1 activity and HDL antioxidative function, in acute HF patients, cholesterol efflux capacity contributes to, but is not a significant independent risk factor, for hospital mortality (Potocnjak *et al.*, 2016).

**Conclusion**

CVD is the leading cause of preventable death worldwide. The relationship between HDL and CVD risk has been well established. Biogenic synthesis of the HDL particle is a complex process, which can be modulated at multiple points, and together with remodelling processes can generate distinct HDL populations capable in many cases of eliciting functions with different degrees of efficacy. Functions include the reverse cholesterol efflux pathway, which removes cholesterol from foam cells, preventing the development of atherosclerotic lesions. In addition, HDL demonstrates antioxidative, anti-inflammatory, antithrombotic and antiapoptotic functions. This wide range of anti-atherosclerotic functions has often been the reason for several large-scale trials aimed at increased HDL levels to reduce risk. The outcomes of these trials have failed to reduce CVD risk. Special consideration of the HDL “quality” rather than “quantity” is required to more accurately determine CVD risk. The concentrations and individual functions of HDL subclasses, including HDL2 and HDL3 could therefore be more useful in determining risk of particular CVD. CVD risk factors have varied influence over the functionality and composition of HDL sub-populations. Measurement of

HDL ”quality” in populations at risk of CVD may therefore serve as a more accurate indicator of disease progression or risk than rudimentary measurement of HDL-C concentrations.

# CHAPTER TWO: AIMS AND HYPOTHESIS

## CHAPTER TWO: AIMS AND HYPOTHESIS

### 1. SUMMARY

CVD is the leading cause of death worldwide and is becoming an increasing burden in African countries due, at least in the past, to rapid urbanization and a rapid shift to Westernized lifestyle trends (Mayosi *et al.*, 2009). HDL is the smallest of the circulating lipoproteins and displays a number of anti-atherogenic functions including reverse cholesterol transport, antioxidative, anti-inflammatory and anti-thrombotic activities (Nofer *et al.*, 2002). Epidemiological studies have shown strong negative associations between the concentration of HDL-C and the risk of CVD (Gordon *et al.*, 1977). The cholesterol content of HDL is, however, only a minor component of a lipoprotein, whose complexity only increases with continuing research. The HDL particle is made of a number of enzymes including PON1 and PAF-AH, as well as structural proteins and S1P (D. Stafforini *et al.*, 1987; Durrington *et al.*, 2001; Kontush *et al.*, 2007; Gillard *et al.*, 2009). All of these structural components contribute to HDL function. Accurate quantification of HDL functionality and the elucidation of distinct HDL subclasses, reportedly with unique functional properties themselves, have brought into question the validity of simply quantifying HDL-C as means of assessing CVD risk (Camont *et al.*, 2013). Although HDL-C concentration is still included in the clinical guidelines as one of the primary parameters for assessing cardiovascular risk, failures of large-scale clinical trials, the discovery of so-called “dysfunctional HDL” and the findings of population studies have suggested that the quality of the HDL, the functionality and distribution of individual HDL subclasses, rather than the overall quantity of HDL should be considered to better define the risk for CVD.

Traditional CVD risk factors include obesity, ethnicity and hypertension. Obesity prevalence continues to increase worldwide and has deleterious consequences for the lipid profile of patients, including changes in HDL function and subclass (James *et al.*, 1997; Goff Jr, 2005). A proposed intervention to limit CVD risk associated with obesity is exercise training and early data in the literature suggest that, in addition to improving cardiovascular health, exercise training can alter HDL function and composition (Argani *et al.*, 2014). Very little is known about the differences in lipid profiles function to ethnicity and whether changes in lipid profiles are associated with differences in CVD risk factors. Hypertension is the most prevalent risk factor for CVD in central and sub-Saharan Africa and often progresses to hypertensive HF (Mendez and Cowie, 2001; Sliwa *et al.*, 2008; Onwuchekwa and Asekomeh, 2009). Pathophysiological contributions to hypertension include endothelial dysfunction, whose structural integrity *in vivo* is associated with normal HDL composition and function. However, whether hypertension may be associated with early changes in HDL subclass and function is unknown.

## 2. STUDY AIM

The current project aims to explore whether specific cardiovascular risk factors such as hypertension, ethnicity and obesity may be associated with differences in HDL subclass distribution and functionality. Exercise, as an intervention in obese black South African women, will also be assessed for its possible effectiveness at improving HDL function and modifying HDL subclass distribution.

## 3. HYPOTHESIS

We hypothesize that cardiovascular risk factors such as obesity and hypertension will be associated with a shift in HDL subclass distribution and will adversely impact on HDL functionality. We also hypothesize that this effect may be ethnicity-dependent. Furthermore, we hypothesize that an exercise intervention will attenuate the negative effects of obesity on HDL subclass distribution and function.

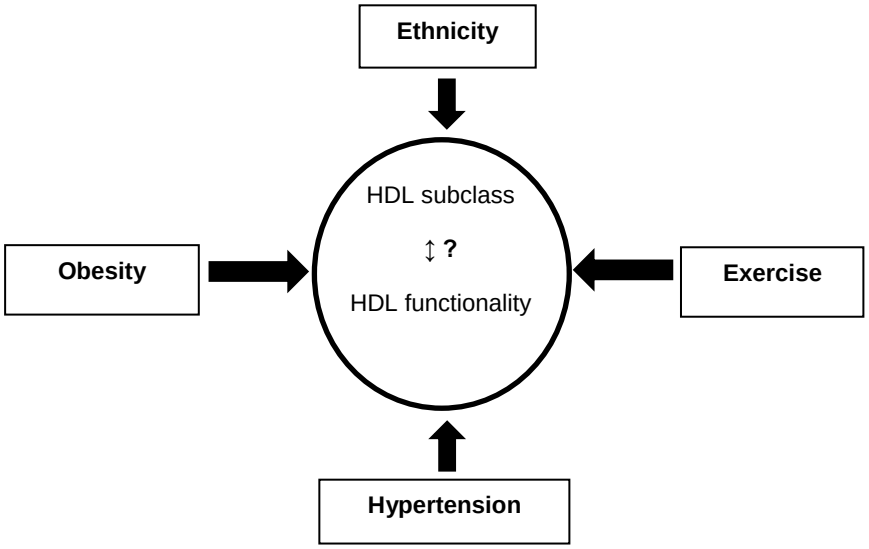



**Figure 14. Hypothesis of the PhD study**

## 4. STUDY OBJECTIVES

To explore the above hypothesis, the following objectives will be addressed:

➡ To assess HDL subclass and functionality in normal-weight and obese black and white South African women (Chapter 4)

HDL subclass will be assessed in blood sera using the Lipoprint® system.

HDL functionality will be assessed by measuring HDL antioxidative activity (PON1 activity and ORAC assay), the anti-thrombotic activity (PAF-AH activity) and HDL anti-inflammatory properties (reduction of VCAM expression in human umbilical vein endothelial cells, HUVEC).

➡ To assess whether a 12- week exercise intervention in obese black women may be associated with a change in HDL subclass and functionality (Chapter 5).

HDL subclass will be assessed in blood sera using the Lipoprint® system. HDL functionality will be assessed by measuring HDL antioxidative activity (PON1 activity and ORAC assay), the anti-thrombotic activity (PAF-AH activity) and HDL anti-inflammatory properties (reduction of VCAM expression in HUVEC) and reverse cholesterol efflux.

➡ To assess whether hypertension with/without HF in Nigerian black men and women is associated with differences in HDL composition, subclass distribution and function (Chapter 6).

HDL subclass will be assessed in blood sera using the Lipoprint® system.

HDL composition will be assessed by measuring HDL content in ApoA1, ApoM and S1P.

HDL functionality will be assessed by measuring HDL antioxidative activity (PON1 activity), anti-thrombotic activity (PAF-AH activity), HDL anti-inflammatory properties (reduction of VCAM expression in HUVEC) and HDL vasodilatory properties (activation of eNOS).

Prior to analysis of patient samples, a method for isolation of pure HDL-C from low volume serum samples will be optimized (Chapter 3).

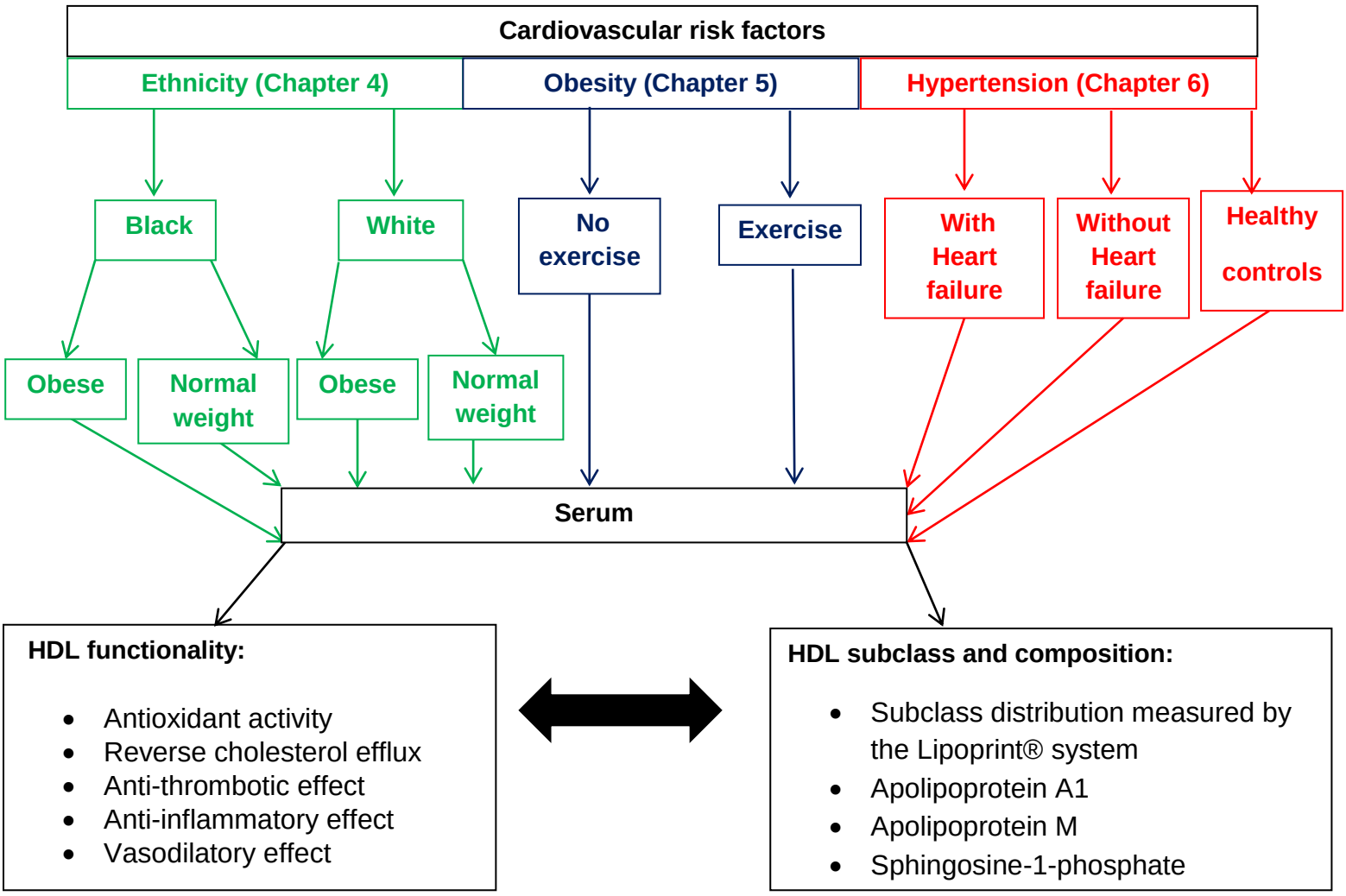



**Figure 15. General design of the PhD thesis**

# CHAPTER THREE: OPTIMIZATION OF THE ISOLATION OF PURE HDL FROM LOW VOLUME SERUM SAMPLES TO CRITICALLY ASSESS THE FUNCTIONALITY OF HDL

## CHAPTER THREE: OPTIMIZATION OF THE ISOLATION OF PURE HDL FROM LOW VOLUME SERUM SAMPLES TO CRITICALLY ASSESS THE FUNCTIONALITY OF HDL

### 1. INTRODUCTION

Experimental models are available to assess the functionality of HDL such as the anti-atherosclerotic functions including antioxidative, anti-inflammatory and anti-apoptotic effects. Most of these models require a primary isolation of HDL from serum. This step is critical as it is important to attain a pure sample devoid of albumin contamination or other lipids, which would affect analysis and conclusions of any study. Classical HDL isolation methods utilize density shift ultracentrifugation alone and are performed using large volumes (5 ml) of serum (Bronzert and Brewer, 1977). These techniques often fail to remove contaminating concentrations of albumin. For functionality assays of HDL involving the use of animal samples or when only limited volumes of serum are available (<1 ml), a technique for efficient isolation of pure HDL is essential.

There are a number of techniques available for HDL isolation including the traditional method described by Bronzert & Brewer (Bronzert and Brewer, 1977). As an alternative, Wiebe & Smith assessed six different methods of HDL isolation, all of which employed an initial precipitation step to remove ApoB-containing lipoproteins including LDL and VLDL (Wiebe and Smith, 1985). Indeed, inclusion of this first step is important to obtain isolated functional HDL with adequate yields (Wiebe and Smith, 1985). An example of a method employing initial removal of LDL and VLDL is the Gidez method (Gidez *et al.*, 1982). Prior removal of other lipoproteins allows HDL to be differentially floated above the albumin layer during ultracentrifugation. The first isolation technique tested was a “scaled-down” version of traditional methods (Bronzert and Brewer, 1977). The protocol was adapted to low-volume serum samples (200 µl) and the samples were centrifuged at different time intervals to optimize HDL isolation. Ethanol precipitation was incorporated as means of better purifying the final sample. The Gidez method was then compared to traditional methods.

**The aim of this study was to optimize a technique for the isolation of pure HDL from low volume serum samples. Requirements included isolation of a sample devoid of albumin and LDL contamination, with adequate yields for future HDL functionality assays.**

2. MATERIALS AND METHODS:

### 2.1 HDL isolation using ethanol precipitation

For the isolation of HDL from serum samples with minimal volume, the method described by Bronzert & Brewer, was first considered (Bronzert and Brewer, 1977). HDL was isolated by density shift centrifugation with 200 µl of serum dissolved in potassium bromide to adjust the density to 1.060 g/ml. Sera were transferred to thick-wall polycarbonate ultracentrifuge tubes (Beckman, cat 343775) and centrifuged at 200 000g at 15°C for 2.5, 5, 7, 9 and 16 hours. Following centrifugation, the upper, middle and lower fractions within each tube were collected in 50 µl aliquots. Fraction samples were dialysed against PBS (Phosphate buffered saline, pH 7.4) in Spectra/Por 2 RC membrane (12 000-14 000 kDa) (GIC Scientific, cat 132676) overnight at 4°C with a single buffer change after at least 90 minutes. Following dialysis, aliquots of each sample were added to two volumes of ice cold ethanol and centrifuged at 10 000g for 5 minutes at 4ºC in a Microcentaur microcentrifuge (MSE). This yielded "precipitated fractions" and was performed as a means of removing contaminating proteins in a modification to the method proposed by (Li *et al.*, 2012). Protein concentration was determined by the method described by Markwell et al (Markwell et al. 1978).

### 2.2 HDL isolation using the Gidez method

A second method was proposed wherein the primary step involved removal of ApoB containing lipoproteins (including LDL) (Gidez et al., 1982). Briefly, serum samples were gently mixed with 1/10 volume of a mixture containing 1 part heparin (Mucosal, Fresenius, 500 iu/ml) and 2 parts 1.12 M Manganese Chloride solution. Samples were centrifuged at 10 000g for 1 hour at 4°C. The supernatant was dialysed against PBS in Spectra/Por 2 RC membrane (12 000-14 000 kDa) (GIC Scientific, cat 132676) for 1 hour to affect salt removal. 200 µl aliquots were then dissolved in sodium bromide (275.5 mg/ml of supernatant), transferred to thick-wall polycarbonate ultracentrifuge tubes (Beckman, cat 343775) and centrifuged at 223 000g at 4°C for 5, 16, 18 and 20 hours in independent experiments performed in triplicate. The upper 70 µl and lower fraction samples were dialysed against PBS (pH 7.4) in Spectra/Por 2 RC membrane (12 000-14 000 kDa) (GIC Scientific, cat 132676) overnight at 4°C with a single buffer change after at least 90 minutes. Protein concentration was determined by the method described by Markwell et al (Markwell et al. 1978).

### 2.3 SDS-PAGE analysis of HDL purity

In order to analyse the efficiency of each of the isolation protocols, samples were equally loaded (10 µg of protein) onto a 12% reducing SDS-PAGE (Sodium dodecyl-sulfate polyacrylamide gel electrophoresis) gel (Laemmli, 1970). Gels were stained with Coomassie

Blue and photographed using Gel Doc™ EZ Imager (Bio-Rad). For quantification of HDL, LDL and albumin levels in each fraction, densitometry was determined using Quantity One software. In this case, relative intensity is calculated by dividing intensity of the approximately 500 kDa band (corresponding to ApoB) and the 28 kDa band (corresponding to ApoA1) by the same band in the serum reference lane. For quantification of HDL purity relative to albumin, relative intensity is calculated by dividing intensity of the approximately 28 kDa band by the intensity of the albumin band (at approximately 70 kDa) to quantify ApoA1/Albumin ratio.

### 2.4 Oxygen Radical Absorbance Capacity (ORAC) Assay

The ORAC assay measured total antioxidant capacity and was used in this case to assess the functionality of HDL following isolation. Isolated HDL fractions were diluted 1:100 and precipitated samples diluted 1:40 in phosphate buffer (pH 7.4). For analysis of whole serum, samples were added in two volumes of ice cold ethanol and centrifuged at 10 000g for 5 minutes at 4°C in a Microcentuar microcentrifuge (MSE). Supernatants were diluted 1:363 in phosphate buffer. All sample antioxidant capacity were analysed using the ORAC assay described by (Cao *et al.*, 1993) . Briefly, Trolox standards by diluted the stock solution (100 mM) in phosphate buffer to give a dilution range of 10 nM to 0.078 nM. Fluorescein (3', 6' – dihydroxyspiro[isoberyofuran – 1[3H], 9'[9H] – xanthen] – 3-one) and AAPH (2,2' – azobis (2-amidinopropane) dihydrochloride) were prepared fresh in phosphate buffer. The working fluorescein solution of fluorescein was 95.7 nM and AAPH equated to 32.1 µM per well. Trolox standards as well as HDL samples (50 µl) were added to wells in 96-well plates (AEC-Amersham) along with AAPH and fluorescein. Fluorescence was measured over time using (plate reader) (Excitation 485nm, Emission 520nm).

### 2.5 Chemicals

Unless otherwise stated, all chemicals were of analytical grade, supplied by Sigma Aldrich (St. Louis, Missouri, United States). Purified HDL was a generous gift from Dr Miguel Frias from the University of Geneva.

### 2.6 Statistical analysis

All results represent mean ± standard error of mean (SEM). For comparisons of relative intensity of ApoB, ApoA1 and the ApoA1/albumin ratio, one or two-tailed student's t-tests were used.

3. RESULTS:

### 3.1 Traditional HDL isolation utilizing ethanol precipitation

The electrophoresis gel of whole serum, upper, middle and lower ultracentrifugation fractions, both precipitated and non-precipitated is presented in Figure 16. None of the upper, middle and lower fractions produce pure samples of HDL or other lipoproteins. Indeed, density shift ultracentrifugation employed in this assay produced an upper fraction characterised by large protein bands (>170 kDa), corresponding to ApoB, present in LDL Albumin contamination, evidenced by a 70 kDa band was present predominantly in middle and lower fractions. A protein band of 26 kDa was present in all samples and corresponded to ApoA1 of HDL. Addition of ethanol removes, to a large extent, the 70 kDa albumin band present in all lanes, yielding a more concentrated 26 kDa ApoA1 in each of the precipitated samples. This was particularly the case in the middle fraction.

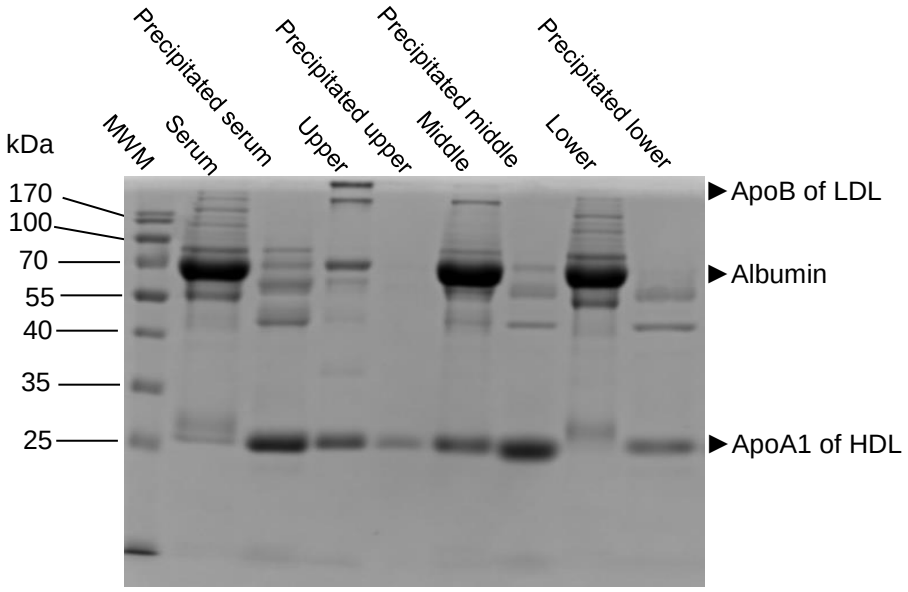



**Figure 16. 12.5% Reducing SDS-PAGE gel of upper, middle and lower serum fractions following centrifugation for 5 hours. Density-shift ultracentrifugation was carried out on serum aliquots using potassium bromide. Samples were centrifuged at 200 000g for 5 hours at 15°C and upper, middle and lower fractions were extracted in 50 µl aliquots. Aliquots of these samples were added to two volumes of ice cold ethanol and centrifuged at 10 000g for 5 minutes. The resulting supernatants as well as upper, middle and lower fractions were run on the electrophoresis gel.**

*Testing the antioxidant function of isolated HDL*

Using the ORAC assay, the antioxidant capacity of HDL from lower fractions, contaminated with serum albumin, indicated a significant decrease in antioxidant capacity in isolated fractions following ethanol precipitation (3 474 ± 205 vs 16 317 ± 3 947 nmol/ml Trolox equivalents (TE), $p < 0.05$) (Figure 17).

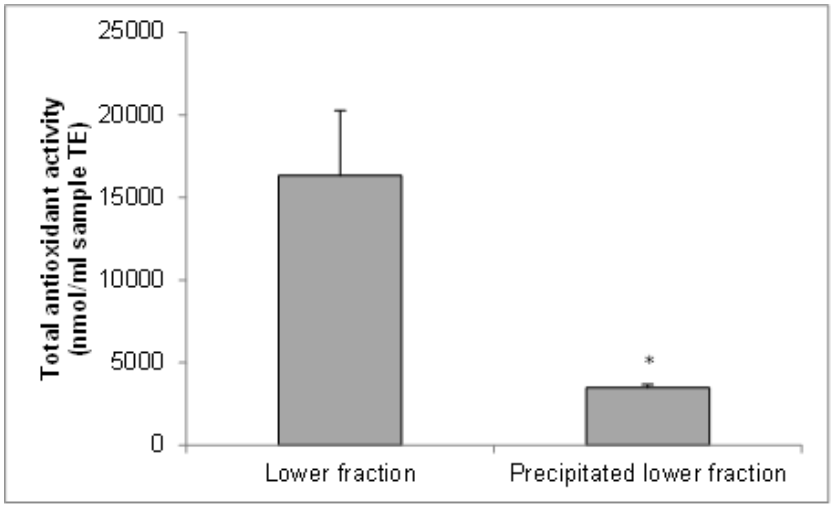



**Figure 17. Effect of ethanol precipitation on antioxidant function in lower fractions. The lower fractions, following ultracentrifugation for 5 hours, were precipitated with two volumes of ethanol and analysed using the Oxygen radical Absorbance Capacity (ORAC) assay as a measure of total antioxidant capacity. TE: Trolox equivalent (n=4). Results are represented as means ± SEM. * $p < 0.05$.**

*Testing the effect of ethanol precipitation on HDL antioxidant function*

In order to clarify whether loss of antioxidant function was due to removal of serum antioxidants such as albumin or as a result of ethanol effects on HDL; purified HDL was precipitated in the same manner and ORAC analysis was carried out (Figure 18).

Commercial HDL, was precipitated by ethanol using the same method as described previously. Figure 18 indicates that the total antioxidant functions of nascent and precipitated HDL were not significantly different from each other.

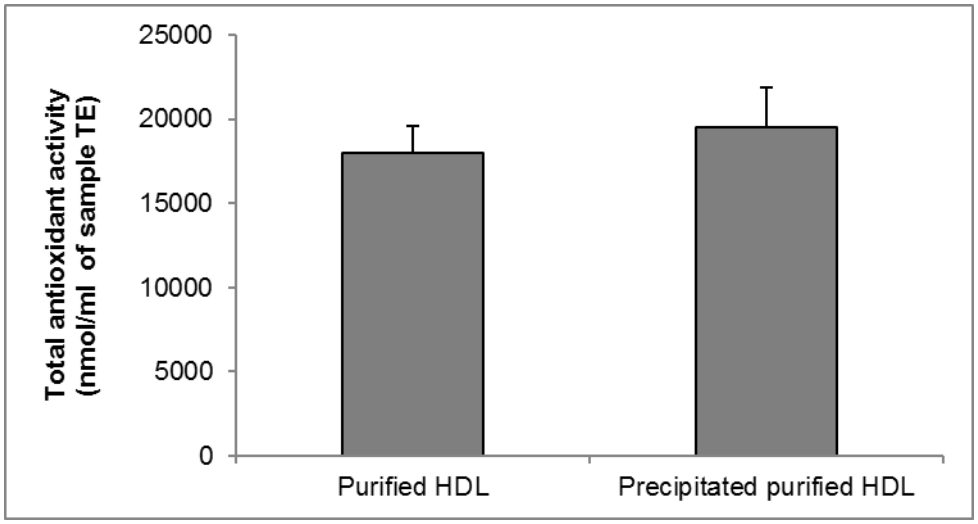



**Figure 18. Effect of ethanol precipitation on antioxidant functionality of purified HDL. Previously isolated HDL was added to two volumes of ethanol and was centrifuged at 10 000g for 5 minutes. The resulting supernatant was assayed using the ORAC assay as a measure of total antioxidant capacity. TE: Trolox equivalents (n=3). Results are represented as means ± SEM.**

*Optimization of traditional methods using difference centrifugation times*

In order to further optimize the method, isolation by centrifugation, was carried out at different time points and upper, middle and lower fractions were analysed by SDS-PAGE, as previously described. Densitometry allowed relative quantification of HDL and LDL by measuring the bands intensity corresponding to ApoA1 and ApoB, respectively.

Figure 19 indicates a time dependent relationship between HDL content, albumin contamination and LDL content. In the upper fraction, the electrophoresis gel (Figure 19A) indicates that the albumin contamination was evident in the shorter centrifugation time of 2.5 hours. When time was extended to 5 hours and longer, the albumin level decreased. Densitometry, shown in Figure 19B, quantified “LDL” and “HDL” using band intensity of the ApoB (>170 kDa) and ApoA1 (26 kDa) bands. There were no significant differences between

LDL and HDL content for each of the centrifugation times in the upper fractions. Unexpectedly, HDL was present in the upper fraction.

The electrophoresis gel pattern and densitometric data of the middle fraction is represented in Figure 19C and D. Whilst, according to the gel pattern alone, it appeared that albumin contamination was minimal for the 16 hour centrifugation time, this was still present to an unsatisfactory level. A time dependent relationship between HDL content and centrifugation time was present for the middle fraction. Centrifugation of serum for 7 hours produced significantly greater HDL levels of 1.89 AU ± 0.09 compared to the 2.5, 5 and 16 hour intervals (1.06 ± 0.06; 1.41 ± 0.18 and 1.37 ± 0.05, respectively). Since the 7 and 9 hour centrifugation times were not significantly different from each other, either duration would be appropriate for more efficient HDL isolation in the middle fraction. Of concern, was that along with albumin contamination, LDL was also present in all middle fractions for the 7 and 9 hour centrifugation times.

As expected, no LDL was present in the lower fraction, however, no time dependent relationship between HDL content and centrifugation time was evident (Figure 19E and F). HDL levels in the lower fraction were lower than the middle fraction indicating that HDL was localised in the middle and upper fractions, and not in the lower fraction. Albumin was again present in the lower fraction regardless of centrifugation time.

In summary, using this initial methodology based on traditional HDL isolation techniques, isolation of HDL by density-shift ultracentrifugation was unable to isolate pure HDL. The addition of ethanol led to a sample devoid of albumin contamination, but the concentration of isolated HDL was not sufficient. We, therefore, aimed to establish an alternative technique for HDL isolation using the method described by Gidez et al which involves prior precipitation of VLDL and LDL (Gidez *et al.*, 1982).

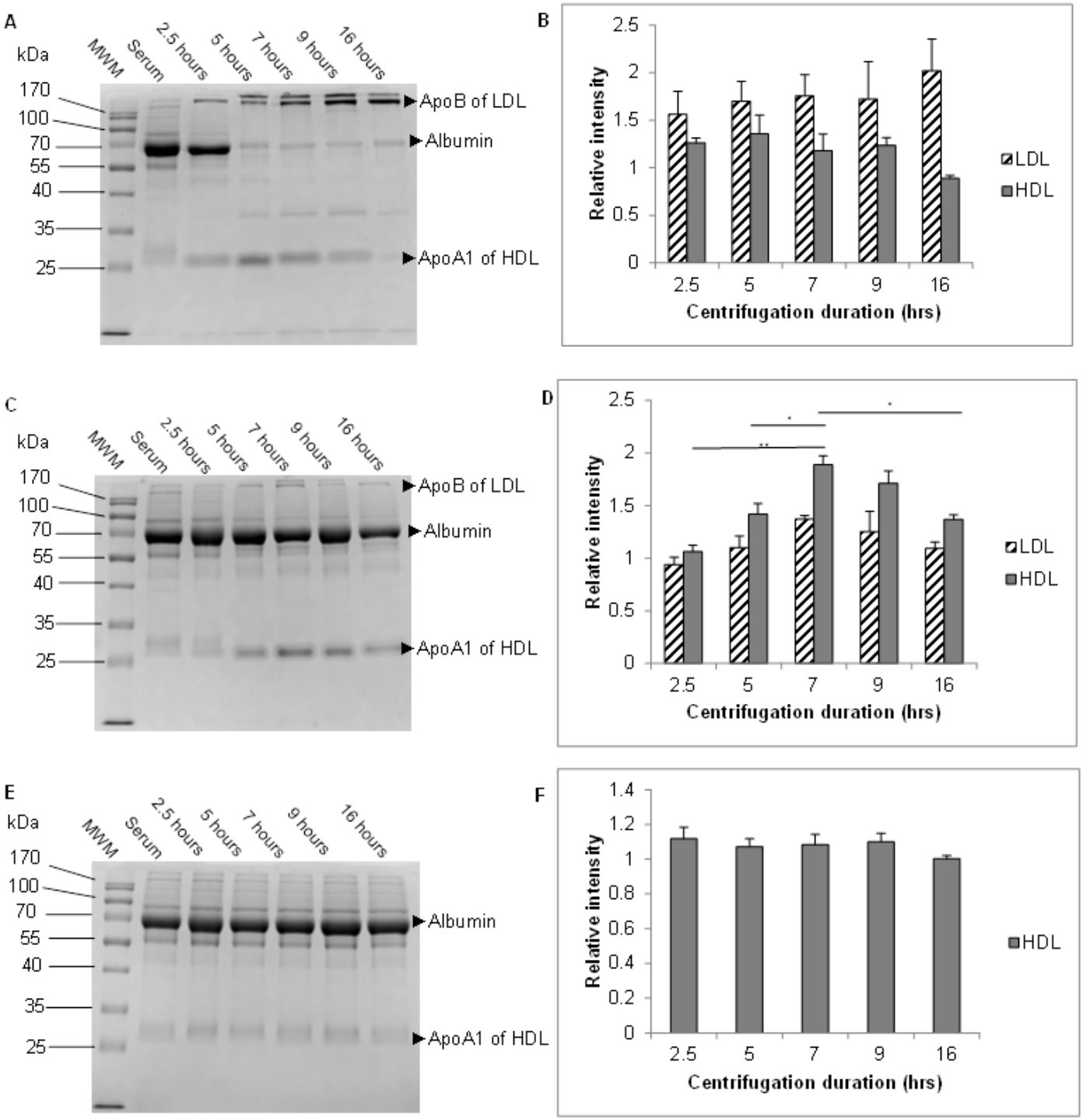



**Figure 19. Optimization of centrifugation duration for HDL isolation. Density of serum samples was adjusted using potassium bromide before samples were centrifuged at 200 000g at 15°C for 2.5, 5, 7, 9 and 16 hours. Upper (A), middle (C) and lower samples (E) were collected in 50 µl aliquots and were loaded onto 12.5% reducing SDS PAGE gels. Densitometry quantified LDL and HDL levels for each time point by measuring ApoB (>170 kDa) and ApoA1 (26 kDa) bands respectively. Densitometry of upper (B), middle (D) and lower (F) fractions at each duration of centrifugation. (n=3) Data represented as means ± SEM. ** $p < 0.01$, * $p < 0.05$.**

### 3.2 HDL isolation via the Gidez method

The Gidez method permitted effective removal of ApoB lipoproteins. Following HDL isolation, using the Gidez method, upper and lower fractions for each centrifugation time were run on SDS-PAGE gels and densitometry of ApoA1 and albumin was determined. The ratio of ApoA1 to albumin in the upper layer for each time period served as a measure of HDL purity.

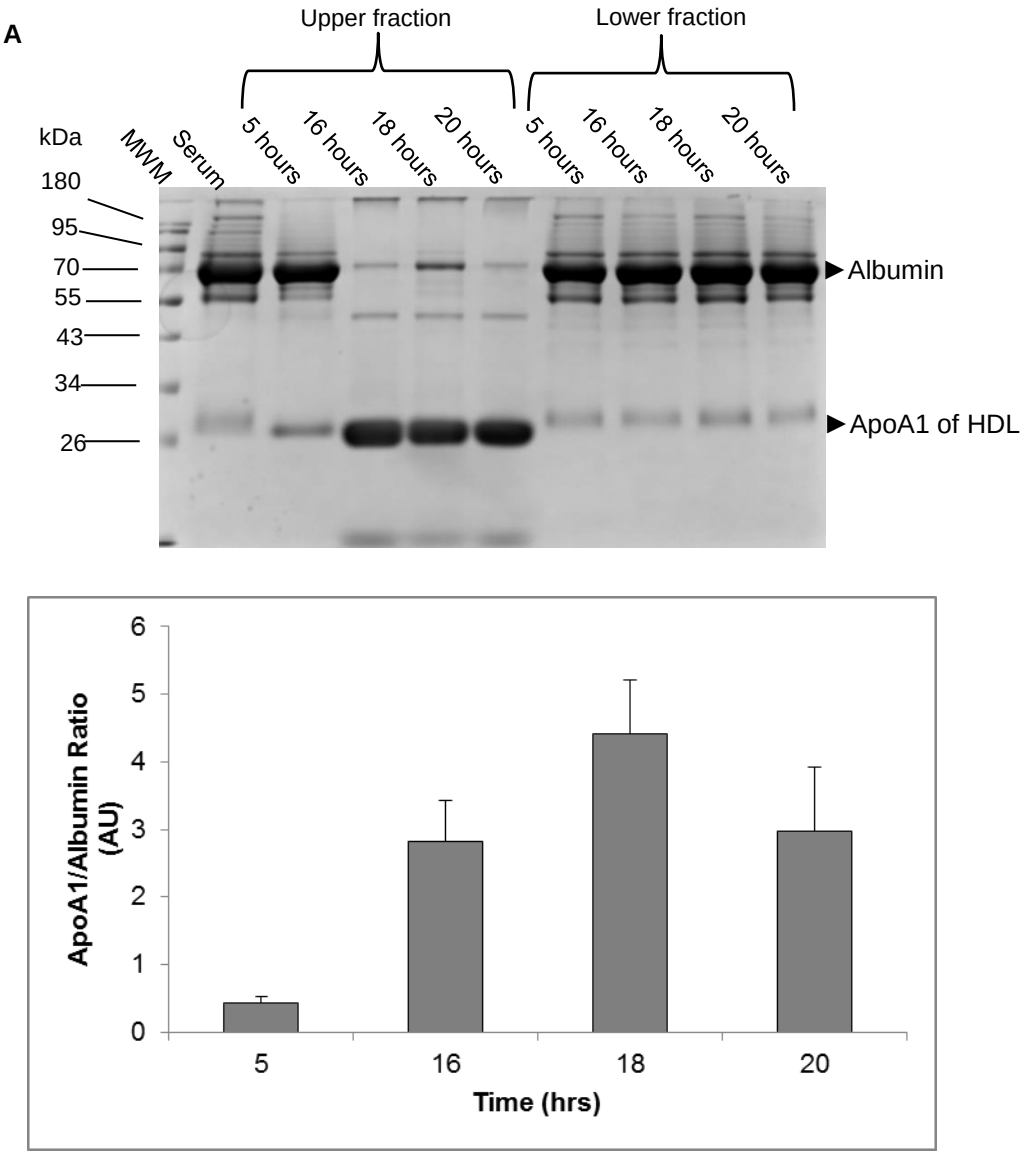



**Figure 20. Optimization of centrifugation duration for HDL isolation using new mini-prep isolation employing Gidez method. ApoB containing lipoproteins were precipitated using heparin and manganese chloride. Supernatants were desalted and density adjusted with sodium bromide prior to ultracentrifugation at 223 000g for 5, 16, 18 and 20 hours. The upper fraction in each ultracentrifuge tube, along with the remaining lower fraction were analysed on 12.5% reducing SDS-PAG gels (A) and densitometry of albumin (70 kDa) and ApoA1 (26 kDa) band intensity in the upper layer was calculated and presented as ApoA1/Albumin ratio as a measure of purity (B). AU: Arbitrary units. (n=3). Data represented as means ± SEM * $p < 0.05$ compared with all other centrifugation times.**

Figure 20 represents the electrophoresis gel of upper and lower fractions isolated after 5, 16, 18 and 20 hours, compared to whole serum. Albumin was localized predominantly in the lower fraction with upper fractions having reduced contamination following centrifugation for at least 16 hours (Figure 20A). Centrifugation for 16, 18 and 20 hours resulted in a significantly higher ApoA1/albumin ratio of 2.82 ± 0.61, 4.41 ± 0.80 and 2.98 ± 0.94 AU, respectively, compared to 5 hours (0.43 ± 0.09 AU, $p < 0.05$) (Figure 20B).

In order to assess antioxidant function of HDL isolated in each of the upper fractions following centrifugation for 5, 16, 18 and 20 hours, the ORAC assay was used (Figure 21).

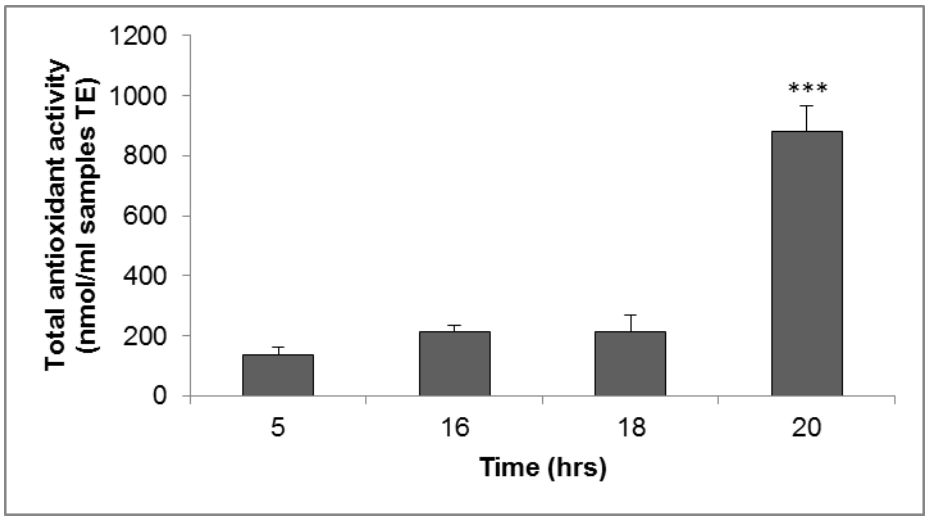



**Figure 21. Antioxidant function of HDL isolated using mini-prep isolation employing Gidez method. Following HDL isolation in upper fractions, HDL samples for each centrifugation duration time were diluted 1:100 in phosphate buffer prior to analysis of total antioxidant capacity in the ORAC assay. Total antioxidant capacity is expressed when corrected for protein concentration. TE: Trolox equivalents. Data represented as means ± SEM. *** $p < 0.001$ (n=9) compared to all other time points**

The 20hr centrifugation produced fractions with a significantly greater total antioxidant capacity of 880.4 ± 84.4 nmol/ml TE compared to all other centrifugation times.

In summary, the initial removal of ApoB containing lipoproteins using this technique isolated pure and functional HDL following centrifugation for 20 hours. This technique was therefore chosen for all further HDL isolation.

# 4. DISCUSSION

The aim of this section was to optimize a method for isolation of HDL from low volume serum samples. Requirements for the isolation were a pure HDL sample, devoid of albumin and LDL contamination, with sufficient yields for further functionality assays. Reducing albumin contamination following traditional ultracentrifugation produced an inefficient yield in pure HDL. Initial removal of VLDL and LDL using the method of Gidez et al, followed by 20 hours of ultracentrifugation produced pure HDL of sufficient yield and antioxidant function (Gidez *et al.*, 1982). This method was therefore adapted to allow for the extraction of pure HDL from an initial small volume (200 µl of serum). This represents the ideal technique for studying HDL function in human and potentially, in animal studies.

*Traditional HDL isolation using ethanol precipitation*

When employing traditional methods for HDL isolation the antioxidant activity of isolated HDL was directly dependent on the presence of albumin in the sample. These results illustrate the importance of removing albumin from the HDL sample as it will skew functionality assays owing to an inherent antioxidant function (Soriani *et al.*, 1994; Cha and Kim, 1996; Bourdon *et al.*, 1999, 2005; Musante *et al.*, 2006). To remove albumin, we employed ethanol precipitation. This did not impair HDL antioxidant function, as evidenced by similar antioxidant capacity between commercial nascent HDL and precipitated commercial HDL. However, ethanol precipitation was not seen as a viable inclusion into the method. Although HDL samples were purer, the protein concentration was below 1 µg/µl and this low yield of HDL was not viable for use in functionality assays, which often require HDL at physiological concentrations exceeding 25 µg/ml.

In order to better optimize the yield and purification using traditional HDL isolation methods, extending centrifugation time to improve HDL purity was explored. Results indicated a time dependent relationship with HDL isolation. Short centrifugation periods of 2.5 and 5 hours, yielded upper fractions with greater albumin levels compared to the longer centrifugation time periods. An optimal time period of 7 hours produced the greatest quantity of ApoA1 (and therefore HDL), in the middle fraction. This quantity of HDL, determined by densitometry, was greater than the 2.5, 5 and 16 hour time points but was not significantly different from the 9 hour time point. LDL was enriched in the upper fraction as expected (Bronzert and Brewer, 1977). The sustained presence of ApoB containing lipoproteins in the middle fraction was of concern as this fraction also contained greater HDL. For functionality assays, the removal of ApoB lipoproteins (and therefore LDL) is important as their susceptibility to oxidation and pro-inflammatory and cytotoxic effects will interfere with quantification of HDL function.

Other than LDL contamination, another concern was that density-shift was not effectively separating HDL at a defined density. HDL was present in each layer and was expected to have been enriched in the lower fraction (Bronzert and Brewer, 1977). This, together with the consistent albumin contamination throughout the middle and lower fractions, brought the validity of this isolation protocol into question. This observation, along with that of the low yield of ethanol precipitated samples, warranted the need for a new novel assay which would remove ApoB lipoproteins, remove albumin and produce HDL at a concentration greater than 1 µg/ µl. It would be important that HDL be separated in an upper fraction, leaving the denser albumin in the lower fraction.

*HDL isolation via the Gidez method*

Using the Gidez method of HDL isolation, ApoB containing lipoproteins were removed from the initial serums sample (Gidez *et al.*, 1982). Density was adjusted using sodium bromide and following density shift, HDL was selectively separated into the upper layer, in a contrast to classical methods (Bronzert and Brewer, 1977; Parker Jr *et al.*, 1983). This fulfils one of the requirements for the isolation technique which was to ensure pure HDL is isolated from LDL and VLDL which could negatively influence HDL functionality assays.

In addition, this isolation technique allowed for isolation of HDL without albumin contamination, which was evident in fractions isolated by previous methods based on classical protocols. Similarly to the traditional HDL isolation method, whether extending ultracentrifugation time improved yield and purity was explored. In this regard, ultracentrifugation for at least 16 hours affected isolation of pure HDL as shown by a prominent ApoA1 band in each of the 16, 18 and 20 hour lanes in the SDS-PAGE gel. Since there was no significant difference in the HDL purity between each time point, HDL antioxidant function was used as a means to distinguish which centrifugation duration would be employed for HDL isolation throughout the remainder of the study.

The total antioxidant capacity was significantly higher for the 20 hour centrifugation time vs 5, 16 and 18 hour time points, when corrected for protein concentration. Therefore HDL, isolated using the Gidez method and centrifuged for 20 hours was both devoid of albumin contamination and indeed functional to a greater extent than HDL fractions centrifuged for shorter periods. This combined with a favourable protein concentration exceeding 1 µg/µl supported the use of the 20 hour centrifugation time for use in all further HDL isolations.

## 5. CONCLUSION

A method to isolate pure HDL from low-volume serum samples (as little as 200 µl) was successfully optimized. This method employed initial removal of VLDL and LDL using the Gidez method followed by density shift ultracentrifugation for 20 hours. Accordingly, this method was used to isolate HDL from participant serum samples for the determination of HDL anti-inflammatory and cholesterol efflux capacity.

# CHAPTER FOUR: ASSOCIATION BETWEEN ETHNICITY AND OBESITY WITH HIGH-DENSITY LIPOPROTEIN (HDL) FUNCTION AND SUBCLASS DISTRIBUTION

## CHAPTER FOUR: ASSOCIATION BETWEEN ETHNICITY AND OBESITY WITH HIGH-DENSITY LIPOPROTEIN (HDL) FUNCTION AND SUBCLASS DISTRIBUTION

The following chapter contains data from the following publication:

Woudberg NJ, Goedecke JH, Blackhurst D, Frias M, James R, Opie LH, Lecour S. Association between ethnicity and obesity with high-density lipoprotein (HDL) function and subclass distribution. *Lipids Health Dis*. 2016;15:1

### INTRODUCTION

Although the leading cause of death in Sub-Saharan Africa remains communicable diseases, the prevalence of ischemic heart disease is increasing and it is predicted to be the leading cause of death in low-income countries by 2030 (Mathers and Loncar, 2006; Moran *et al.*, 2013; Collaborators, 2015). The changes in standardized health care, progressive changes in socio-economic status and greater Westernization have raised the burden of preventable CVD (Akinboboye *et al.*, 2003; Mayosi *et al.*, 2009; Sliwa *et al.*, 2012). In addition, nearly 23% of the worldwide burden of ischaemic heart disease can be attributed to obesity, the prevalence of which has doubled since 1980 (World Health Organization, 2014). Obesity is associated with insulin resistance, which may increase the risk of type II diabetes and dyslipidaemia, as evidenced by an increase in triglycerides and LDL-C and a decrease in HDL-C in these patients (Terry *et al.*, 1989; Williams *et al.*, 1995; Nieves *et al.*, 2003; Goff Jr, 2005).

Ethnic differences in lipid profiles and CVD risk have been documented, attributed to, in part, genetic, socioeconomic and lifestyle differences (Mayosi *et al.*, 2009; Ellman *et al.*, 2015). Black South African women and African Americans exhibit protective lipid profiles, characterised by low LDL-C, low triglyceride and low total cholesterol concentrations (Després *et al.*, 2000; Punyadeera *et al.*, 2001). In addition, cholesterol-attributable mortality is higher in South African white compared to black populations, with only 1.8% mortality attributable to 'sub-optimal' cholesterol levels in black populations (Norman *et al.*, 2007). It was previously thought that a favourable lipid profile in black populations would also be characterised by higher HDL-C concentrations (Seedat *et al.*, 1992; Steyn *et al.*, 1996). However, recent studies conducted in black South African women highlighted a lower or equivalent level of HDL-C than their white counterparts (Punyadeera *et al.*, 2001; Goedecke *et al.*, 2010; Sliwa *et al.*, 2012; Ellman *et al.*, 2015).

The Framingham Heart study suggests an inverse relationship between HDL-C levels and CVD risk (Gordon *et al.*, 1977). However, recent clinical trials aiming to reduce cardiovascular complications by raising HDL-C levels have shown disappointing results (Boden *et al.*, 2011; Schwartz *et al.*, 2012). Elucidation of the complexity of the HDL molecule, has led to a shift

from measuring the quantity of HDL-C to assessing the composition, the distribution of individual HDL subclasses and HDL functionality to try to explain the relationship between HDL and cardiovascular risk (Rizzo *et al.*, 2014; Santos-Gallego, 2015). The risk of ischemic heart disease in African populations has largely been defined by classical risk factors, including the cholesterol component of HDL.

**Given the prevailing doubts about the cardiovascular value of HDL-C, the present study used blood collected from a sample population of black and white, obese and normal-weight South African women, and aimed to explore whether ethnicity and obesity were associated with differences in HDL functionality and subclass distribution.**

2. MATERIALS AND METHODS

## 2.1 Subjects

The sample population consisted of 40 normal-weight (BMI 18-24.9 kg/m$^2$) and obese (BMI > 30 kg/m$^2$) self-reported black and white South African women, who had been enrolled for previous studies and described in details (Goedecke *et al.*, 2009, 2010). Inclusion criteria included (1) age from 18 to 45 years; (2) no known diseases or taking medication for dyslipidemia, diabetes, hypertension, HIV/AIDS, or any other metabolic disorders; and (3) not currently pregnant, lactating, or postmenopausal. The study was approved by the Research Ethics Committee of the Faculty of Health Sciences of the University of Cape Town HREC REF 053/2003.

Basic anthropometric measurements, including height and weight, were taken. Body fat percentage was measured using dual-energy x-ray absorptiometry, while visceral and abdominal subcutaneous adipose tissue were measured using computed tomography as previously described (Goedecke *et al.*, 2010). Fasting serum samples were taken and stored at -80$^0$C prior to use.

## 2.2 HDL isolation (as optimized in Chapter 3)

HDL was isolated from 200 µl aliquots of serum as follows: Serum samples were added to a mixture containing 1 part 500iu/ml heparin (Mucosal, Fresenius) and 2 parts 1.12 (mol/L) manganese chloride solution. Samples were centrifuged at 10 000g for 1 hour at 4°C. The supernatant was dialysed against phosphate buffered saline (PBS, pH 7.4) in Spectra/Por 2 RC membrane (12 000-14 000 kDa) (GIC Scientific, 132676) and 200 µl aliquots were then dissolved in sodium bromide (275.5 mg/ml of supernatant), transferred to thick-wall polycarbonate ultracentrifuge tubes (Beckman, 343775) and centrifuged at 223 000g for 20 hours at 4°C and the upper 70 µl layer was extracted. Purity was confirmed using 12.5% reducing SDS-PAGE stained with Coomassie Blue. The protein concentration of HDL was determined by the modified Lowry method (Markwell *et al.*, 1978). All samples were analysed in duplicate.

## 2.3 PON1 activity assay

Serum samples were diluted 1:10 in phosphate buffer containing 2 mmol/L $CaCl_2$ (pH 8). Diluted serum was added to 96-well plates in triplicate and paraoxon-ethyl substrate (Sigma, D9286) was added. Absorbance at $A_{405}$ was measured at 30 second intervals over 20 minutes. One Unit of activity is defined as 1 nmol of substrate hydrolysed per minute.

### 2.4 PAF-AH activity assay

PAF-AH activity was measured in participant sera using the PAF Acetylhydrolase Assay Kit (Cayman Chemical, 760901). Briefly, serum was added to an equal volume of 5, 5'-dithio-*bis*-(2-nitrobenzoic acid) (DTNB; Ellman's Reagent) and assay buffer in triplicate into clear 96-well plates. All wells were incubated with 2-thio PAF substrate and absorbance at $A_{412}$ measured at 1 minute time intervals for 20 minutes. One Unit of activity is defined as 1 µmol of substrate hydrolysed per minute.

### 2.5 Western blotting

Isolated HDL and serum samples from each of the participants were electrophoresed on reducing 12.5% SDS-PAGE gels with 1.5 µg of HDL protein or 8 µg of serum loaded per well. Samples were run over three separate gels with control samples repeated in each gel. Blots were transferred onto nitrocellulose membranes (Bio-Rad, 162-0113). Ponceau S staining was scanned and used to validate equal loading of wells. Blots were blocked in 5% low fat milk powder in 0.05% Tween in Tris-buffered Saline (TTBS, pH 7.5) and incubated overnight in primary mouse anti-PON1 antibody (1:200) (James *et al.*, 2010) and rabbit anti-PAF-AH (1:400) (Cayman Chemical, 160603). Blots were then washed in TTBS and incubated in goat anti-mouse-HRP conjugated secondary antibody (1:5000) (Bio-Rad, 170 6516) and goat anti-rabbit-HRP conjugated secondary antibody (1:2500) (Santa Cruz Biotechnology, sc-2313), respectively for 1 hour at room temperature. Blots were thoroughly washed in TTBS prior to incubation in Amersham TM ECL™ Western blotting detection reagent (GE Healthcare, RPN2106). Blots were captured in the GeneGnome gel imager. Densitometry of PON1 and PAF-AH blots was quantified using Quantity one software. PON1 and PAF-AH relative expression data were corrected for control samples, repeated in each gel.

### 2.6 ORAC Assay

Isolated HDL samples were diluted (1:50) in phosphate buffer (pH 7.4) prior to analysis for the ORAC assay described in Chapter three (Section 2.4).

### 2.7 Quantification of HDL anti-inflammatory function

HUVEC were purchased from Lonza and were cultured in T75 culture flasks according to supplier specifications. For experimental tests, 30 000 cells were seeded into 12-well culture plates and cultured in RPMI-1640 media supplemented with 20% foetal calf serum (Biochrom BC/S0615), 1 ng/mL vascular endothelial growth factor (VEGF) (Sigma, V7259) and penicillin/streptomycin (Biowest, L0018). Five hours after seeding, the medium was changed

and supplemented with HDL isolated according to optimised protocol at 10 µg/mL. Cells were treated with HDL overnight prior to stimulation with 20 ng/mL murine TNF-α (PeproTech, 315-01A) for 8 hours. Cell pellets were harvested and stored in RNA Protect Cell Reagent (Qiagen, 76526) at -20°C. RNA was isolated using the RNeasy Micro kit (Qiagen, 74004) and cDNA was synthesised using the High Capacity cDNA Reverse Transcriptase Kit (Life Technologies, 4368814). cDNA was quantified using the Qubit High Sensitivity RNA kit (Qiagen, Q32852) and Qubit Fluorometer (LifeTechnologies). cDNA was amplified for 25 cycles using the RT2 SYBR Green qPCR kit (Qiagen, 330500) in the RotorGene6000 (Corbit Lifesciences) with the following primers: VCAM-1 (sense), 5'-GAAGATGGTCGTGATCCTTG-3', and (antisense), 5'-ACTTGACTGTGATCGGCTTC-3'. GAPDH (sense), 5'-CCACCCATGGCAAATTCCATGGCA-3', and (antisense), 5'-TCTAGACGGCAGGTCAGGTCCACC-3'. Results indicate the mean of at least 3 independent experiments ± SEM.

### 2.8 Quantification of HDL subclass distribution

Serum HDL subclass was determined using the Lipoprint® HDL system (Quantimetrix, Redondo Beach, CA) (Hoefner *et al.*, 2001). Briefly, serum (25 µl) was mixed with Lipoprint loading gel (300 µl), containing Sudan black dye which binds proportionally to the cholesterol present in the sample. The mix was placed onto the upper part of the high resolution 3% polyacrylamide gel. Photopolymerisation was carried out for 30 minutes at room temperature and electrophoresis was performed for 50 minutes at 3mA per gel tube. After a rest period of 30 minutes, gel tubes were scanned and analysed using the Lipoware software. The VLDL and LDL remained at the origin [Retention Factor (Rf) = 0.0] while albumin migrated as the leading front (Rf = 1.0). Between these, 10 HDL bands could be detected. HDL-1, HDL-2 and HDL-3 were defined as large HDL; HDL-4, HDL-5, HDL-6 and HDL-7 were defined as intermediate HDL and HDL-8, HDL-9 and HDL-10 were defined as small HDL. Each subclass was quantified and expressed as a percentage of total HDL.

### 2.9 Statistical analysis

Results are presented as mean ± SEM. All variables were normally distributed, therefore two-way analysis of covariance, adjusting for age, was used to compare PON1 activity, PAF-AH activity, antioxidant capacity, relative VCAM expression and HDL subclass distribution between normal-weight and obese black and white women. Pearson correlation coefficients were used to explore the relationships between measures of HDL function and subclass, serum lipids and body composition.

## 3. RESULTS

The body composition, lipid profiles and additional physiological data of the participants have been previously published and are summarised in Table 3 (Goedecke *et al.*, 2010). In brief, ethnic differences included lower visceral adipose tissue (VAT) and higher subcutaneous adipose tissue (SAT) in obese black women compared to obese white women. Critically, black women had lower HDL and total cholesterol concentrations than their white counterparts.

**Table 3. Characteristics and serum lipids of participants included in the study**

| | **White normal-weight (n=12)** | **White obese (n=9)** | **Black normal-weight (n=8)** | **Black obese (n=11)** |
|---|---|---|---|---|
| Age (yr) | 26 ± 2 | 34 ± 2$^{B,C}$ | 23 ± 2 | 27 ± 2 |
| BMI (kg/m$^2$) | 23 ± 1 | 33 ± 1$^{B,C}$ | 22.8 ± 0.9 | 38.5 ± 0.7$^{D}$ |
| Body fat (kg) | 19.2 ± 1.4 | 40.4 ± 1.6$^{C}$ | 17.1 ± 1.7 | 44.9 ± 1.5$^{D}$ |
| Body fat (%) | 29.5 ± 1.4 | 43.7 ± 1.6$^{C}$ | 30.0 ± 1.7 | 46.7 ± 1.5$^{D}$ |
| VAT area (cm$^2$) | 62.2 ± 11.3 | 144.9 ± 13.1$^{B,C}$ | 56.9 ± 13.9 | 95.6 ± 11.8$^{D}$ |
| SAT area (cm$^2$) | 187.0 ± 19.1 | 471.6 ± 22.0$^{B,C}$ | 175.2 ± 23.4 | 594.2 ± 19.9$^{d}$ |
| Serum lipids | | | | |
| HDL-C (mmol/L) | 1.7 ± 0.1$^{a}$ | 1.5 ± 0.1$^{B}$ | 1.3 ± 0.1 | 1.0 ± 0.1 |
| LDL-C (mmol/L) | 2.0 ± 0.2 | 2.5 ± 0.2 | 2.1 ± 0.2 | 2.1 ± 0.2 |
| Total-C (mmol/L) | 4.1 ± 0.2 | 4.6 ± 0.3$^{B}$ | 3.6 ± 0.3 | 3.5 ± 0.2 |
| Triglycerides (mmol/L) | 0.9 ± 0.1 | 1.0 ± 0.1 | 0.5 ± 0.1 | 0.8 ± 0.1 |

**Values are unadjusted means ± SEM. All *p* values are adjusted for age. $^{a}$ *p* < 0.05 and $^{A}$ *p* < 0.01 white normal-weight vs black normal-weight, $^{b}$ *p* <0.05 and $^{B}$ *p* < 0.01 white obese vs black obese, $^{c}$ *p* <0.05 and $^{C}$ *p* < 0.01 normal-weight vs obese white, $^{d}$ *p* < 0.05 and $^{D}$ *p* < 0.01 normal-weight vs obese black. Adapted from (Goedecke *et al.*, 2010). BMI: Body mass index, SAT: Subcutaneous adipose tissue and VAT: Visceral adipose tissue.**

### 3.1 PON1 activity in normal-weight and obese black and white women

In normal-weight white women, PON1 activity was 0.53 ± 0.12 U/L, which is in a similar range to data previously reported in the literature (Kunutsor *et al.*, 2016). PON1 activity of normal-weight and obese, black and white women is presented in Figure 22A. Black women had significantly higher PON1 activity levels than white women (0.78 ± 0.10 U/L vs 0.49 ± 0.09 U/L, $p < 0.05$), with the effect being more pronounced in obese black women compared to obese white women (0.84 ± 0.13 U/L vs 0.45 ± 0.14 U/L, $p < 0.05$) (Figure 22A). Irrespective of ethnicity, PON1 activity did not differ between normal-weight and obese women.

In order to explore whether differences in PON1 activity levels were simply due to differences in PON1 protein expression in HDL, Western blotting was performed on isolated HDL and serum. There were no significant differences in HDL-associated PON1 protein levels between black and white women, nor between normal-weight and obese women (Figure 22B and C). Similarly, there were no differences in PON1 serum expression between black and white women, nor between normal-weight and obese women (Figure 22D and E).

Table 4 shows that PON1 activity correlated positively with LDL levels in both black and white women ($p < 0.05$), positively with total cholesterol ($p < 0.005$) in black women and negatively with total HDL ($p < 0.005$) in white women.

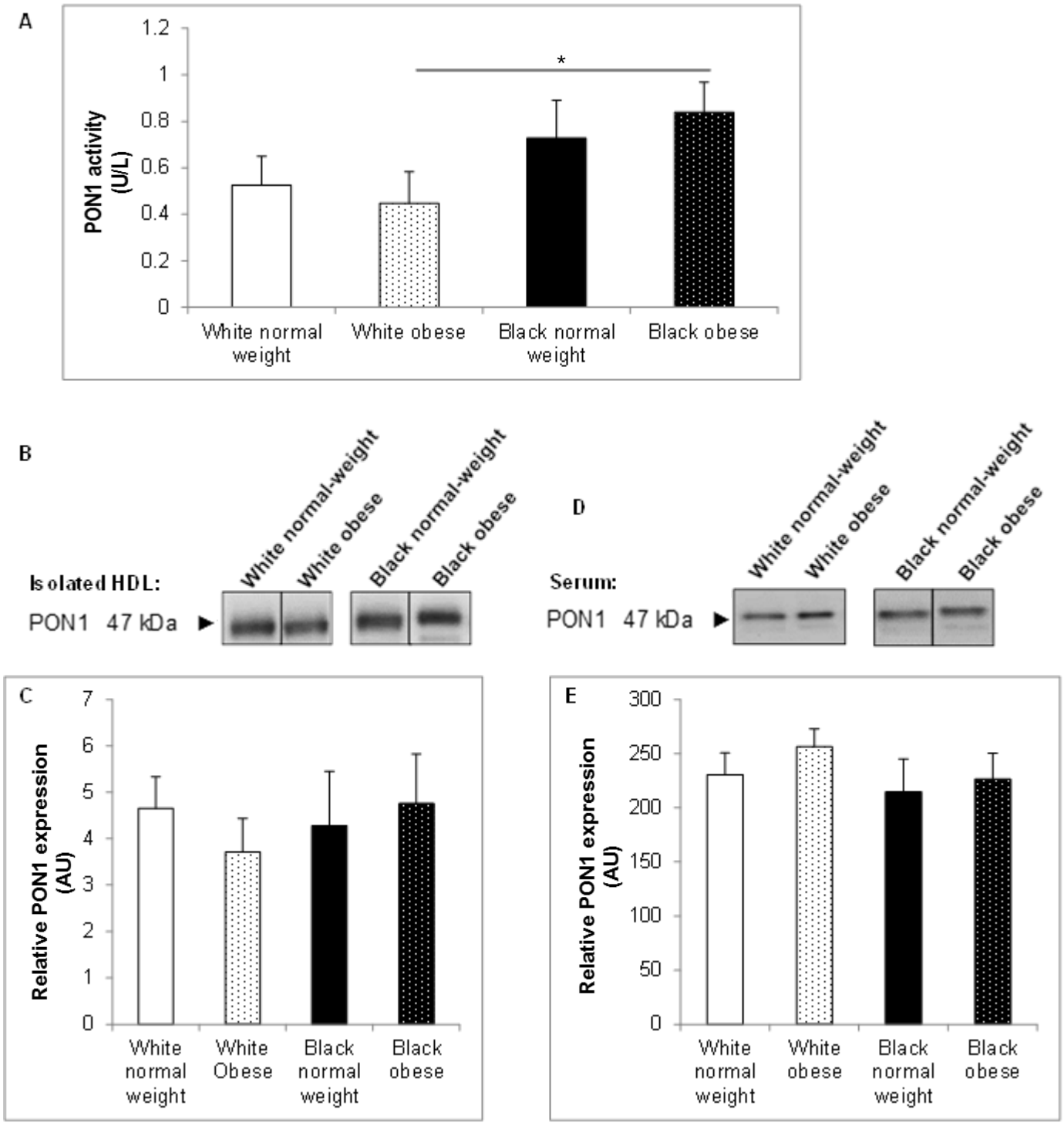



**Figure 22. PON1 activity and protein expression in white and black women. PON1 activity of diluted sera was measured at $A_{405}$ over a 20 minute time interval using the paraoxon-ethyl substrate. One unit of activity is defined as 1 nmol of substrate disintegrated per minute (A). Isolated HDL (B-C) and participant sera (D-E) were run on reducing 12.5% SDS-PAGE gels and transferred to nitrocellulose membrane. Ponceau S staining was used to confirm equal loading. Blots were probed with mouse anti-PON1 antibody. Results are representative of randomized experiments (B) and (D). Densitometry of PON1 expression in HDL (C) and sera (E). AU: Arbitrary units. Results represent means ± SEM * $p < 0.05$.**

### 3.2 PAF-AH activity in normal-weight and obese black and white women

In normal-weight white women, PAF-AH activity was 12.0 ± 1.1 U/L, which is in a similar range to data previously reported in the literature (Gomes *et al.*, 2008). Obese black women had significantly lower PAF-AH activity levels than obese white women (9.34 ± 1.15 U/L vs 13.89 ± 1.21 U/L, $p < 0.05$) (Figure 23A). PAF-AH activity did not differ between normal-weight and obese women.

When examining PAF-AH protein expression in isolated HDL, we found that obese black women had significantly lower levels of HDL-associated PAF-AH than obese white women (5.5 ± 1.7 vs 10.9 ± 1.8 Arbitrary units, AU, $p < 0.05$) (Figure 23B and C), which corresponded to their lower PAF-AH activity ($r = 0.54$, $p < 0.005$). However, there were no differences between black and white obese and normal-weight women in PAF-AH serum expression (Figure 23D and E).

PAF-AH was positively correlated with LDL ($p < 0.005$) and total cholesterol ($p < 0.005$) concentrations in both black and white women (Table 4). In black women only, higher PAF-AH activity was associated with lower fat mass, SAT and triglyceride concentrations ($p < 0.05$).

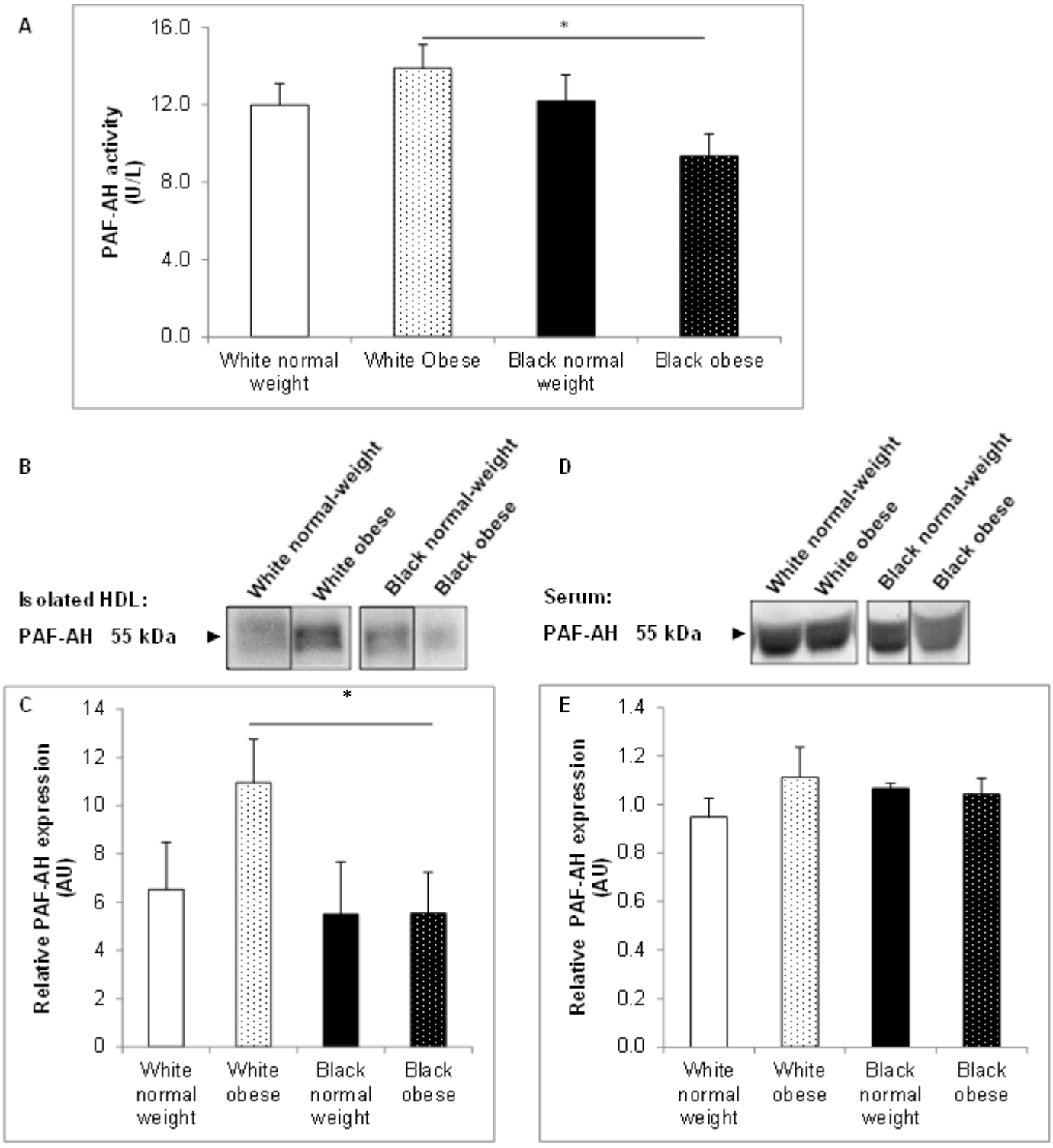



**Figure 23. PAF-AH activity and protein expression in white and black women. PAF-AH activity of diluted sera was measured at A412 over a 20 minute time interval using the PAF Acetylhydrolase Assay Kit. One unit of activity is defined as 1 µmol of substrate disintegrated per minute (A). Isolated HDL (B-C) and participant sera (D-E) were run on reducing 12.5% SDS-PAGE gels and transferred to nitrocellulose membrane. Ponceau S staining was used to confirm equal loading. Blots were probed with rabbit anti-PAF-AH antibody. Results are representative of randomized experiments (B) and (D). Densitometry of PAF-AH expression in HDL (C) and sera (E). AU: Arbitrary Units. Results represent means ± SEM * $p < 0.05$.**

### 3.3 Anti-inflammatory function of isolated HDL

In isolated HDL from white normal-weight women VCAM expression measured in HUVEC cells was 0.98 ± 0.15 AU. No obesity-related or ethnic differences were observed for anti-inflammatory function of isolated HDL (Figure 24).

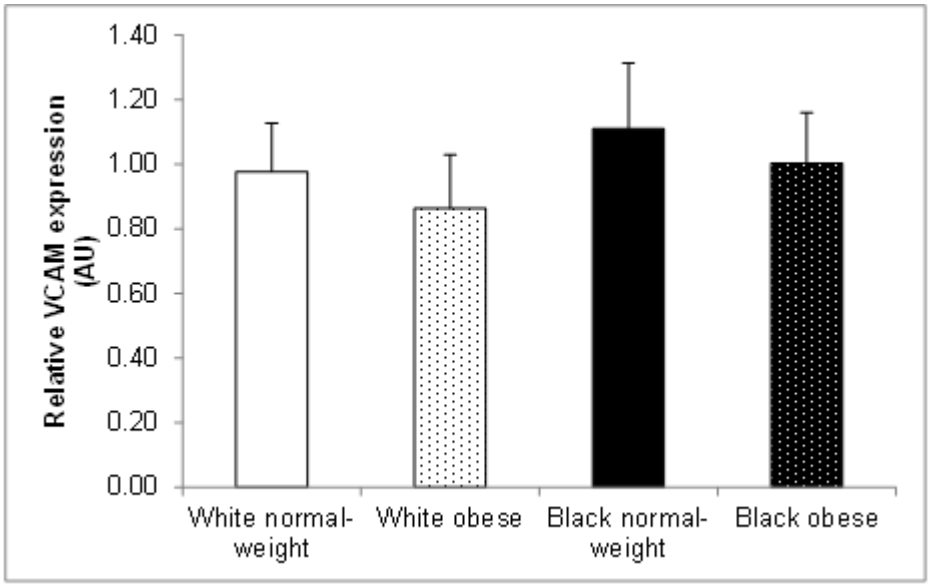



**Figure 24. Anti-inflammatory function of black and white women. HUVEC cells were treated overnight with 10 µg/ml subject HDL. Cells were exposed to 20 ng/ml tumour necrosis factor (TNF) for 8 hours. Cell lysates were harvested and stored in RNAprotect reagent prior to RNA extraction, followed by cDNA synthesis and quantitative real time PCR. AU: Arbitrary units and VCAM: Vascular cell adhesion molecule. Results are presented relative to a no-HDL treatment control. Results are represented as means ± SEM.**

### 3.4 Antioxidant capacity of isolated HDL

In isolated HDL from white normal-weight women total antioxidant capacity, assessed by the ORAC assay, was 653 ± 132 nmol/ml TE. No obesity-related or ethnic differences were observed for antioxidant capacity of isolated HDL (Figure 25).

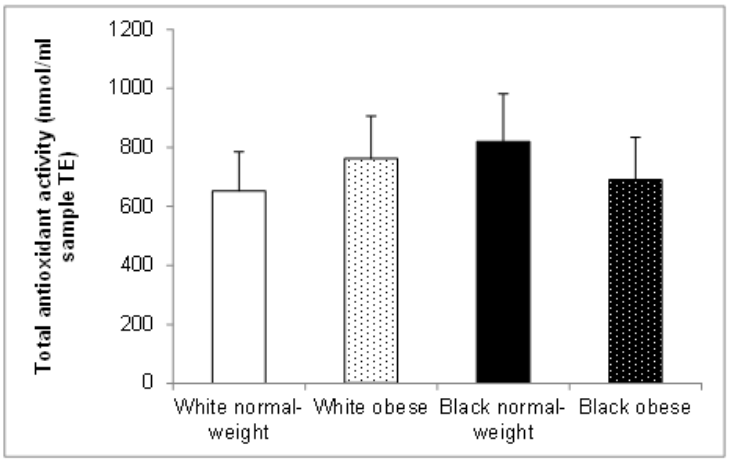



**Figure 25. Antioxidant capacity of black and white women. Isolated subject HDL was diluted in phosphate buffer and measured using the Oxygen Radical Absorbance Capacity (ORAC) assay. TE: Trolox equivalents. Results represent means ± SEM.**

### 3.5 HDL subclass distribution in normal-weight and obese black and white women

Figure 4 shows the distribution of large, intermediate and small HDL subclasses, quantified using the Lipoprint® system. Scans (Figure 26A) were quantified, producing unique HDL subclass profiles (Figure 26B-F). HDL subclass distribution was different between obese and normal-weight women with less large HDL (-10%, $p < 0.05$) and significantly more intermediate HDL (+6%, $p < 0.05$) in the obese women compared to the normal-weight women (Figure 26G). This effect was largely driven by differences between the normal-weight and obese white women (43.1 ± 3.4% vs 32.8 ± 3.8 % for large HDL, $p < 0.05$) (Figure 26G). There were no differences in the percentages of small HDL subclasses between groups.

In the white women, the greater proportion of large HDL subclasses was associated with higher BMI, fat mass, VAT and SAT, as well as higher LDL and triglyceride concentrations ($p < 0.05$, Table 4). In black women, the greater proportion of large HDL was associated with a younger age ($p < 0.05$) and higher HDL-C ($p < 0.005$). Conversely, a greater proportion of intermediate HDL was associated with lower BMI, fat mass, VAT and SAT in white women. A greater proportion of small HDL was associated with increased BMI and fat mass in white women.

There were no associations between measures of HDL functionality and HDL subclass distribution in the combined population sample. However, a greater proportion of large HDL subclasses were associated with lower PAF-H activity was observed in white women only ($r = -0.47$, $p < 0.05$).

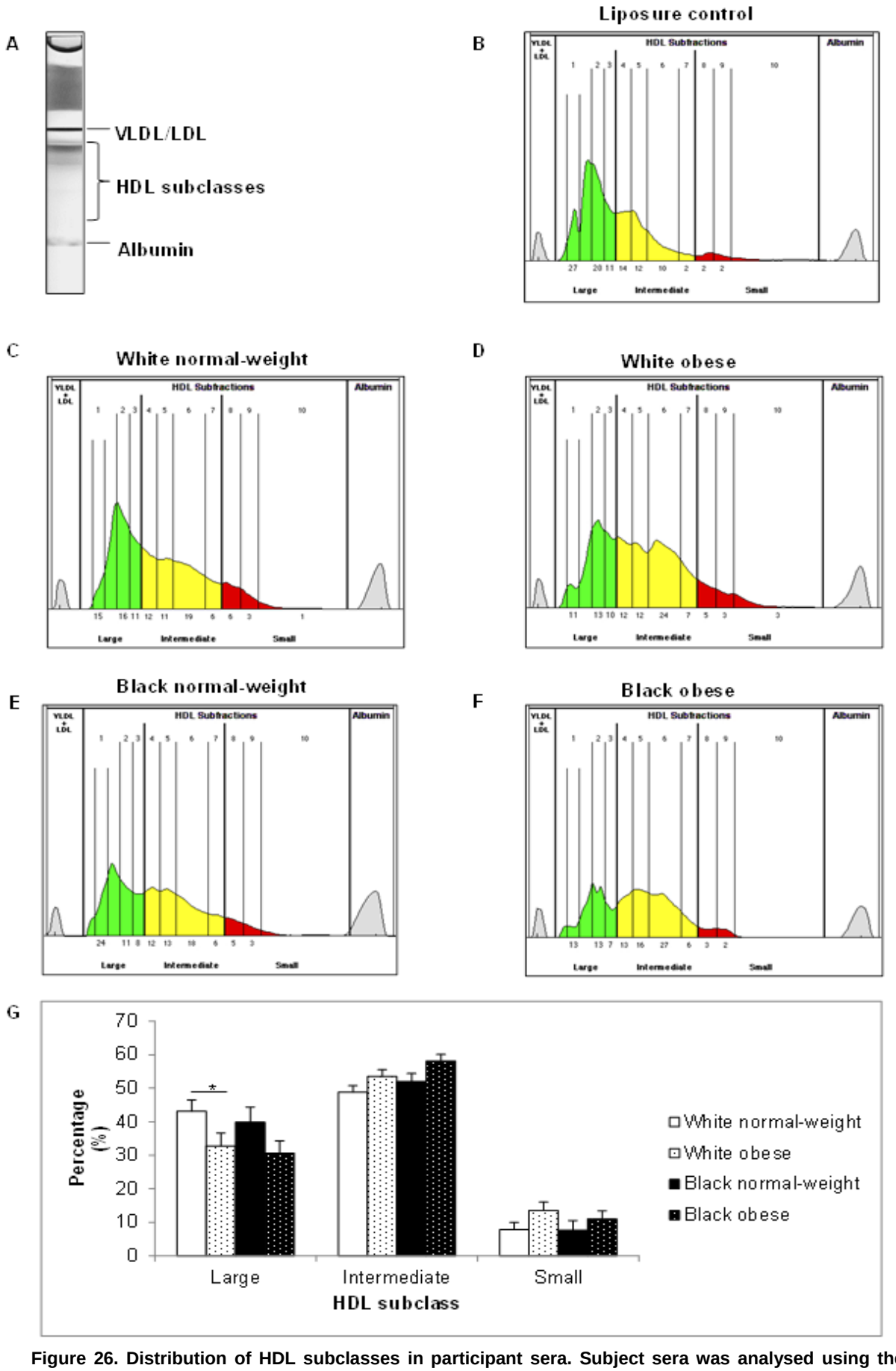



**Figure 26. Distribution of HDL subclasses in participant sera. Subject sera was analysed using the Lipoprint® system and analysed using Lipoware software. Representative scan (A) and scan result (B) of Liposure control indicating HDL subclass bands. Representative scan results from white and black obese and normal-weight women (C-F). Percentages of large, intermediate and small HDL subclasses (G). VLDL: Very low-density lipoprotein. Results represent means ± SEM. * $p < 0.05$**

**Table 4. Associations between HDL functionality measures, HDL subclass, body composition and serum lipids in black and white South African women**

| | Ethnicity | Age (year) | BMI ($kg/m^2$) | Fat (kg) | VAT ($cm^2$) | Total SAT ($cm^2$) | HDL (mmol/L) | LDL (mmol/L) | Triglycerides (mmol/L) | Total cholesterol (mmol/L) |
|---|---|---|---|---|---|---|---|---|---|---|
| PON1 Activity | Black | -0.07 | 0.30 | 0.26 | 0.19 | 0.19 | 0.29 | 0.59* | 0.13 | 0.66** |
| | White | -0.40 | 0.14 | 0.16 | -0.25 | -0.09 | -0.62*** | 0.45* | 0.21 | 0.28 |
| PAF-AH activity | Black | -0.18 | -0.44 | -0.47* | -0.25 | -0.48* | 0.05 | 0.65*** | -0.51* | 0.53* |
| | White | 0.02 | 0.42 | 0.39 | -0.02 | -0.10 | -0.37 | 0.85*** | 0.22 | 0.80*** |
| Large HDL subclass | Black | -0.52* | -0.32 | -0.30 | -0.18 | -0.31 | 0.69** | 0.067 | -0.19 | 0.25 |
| | White | -0.31 | -0.52* | -0.48* | -0.48* | -0.48* | 0.32 | -0.49* | -0.48* | -0.48* |
| Intermediate HDL subclass | Black | 0.24 | 0.43 | 0.41 | 0.17 | 0.45 | -0.64*** | 0.17 | -0.15 | -0.11 |
| | White | 0.40 | 0.46* | 0.44* | 0.58* | 0.51* | -0.33 | 0.36 | 0.52* | 0.34 |
| Small HDL subclass | Black | 0.52* | 0.08 | 0.08 | 0.11 | 0.06 | -0.29 | -0.24 | 0.42 | -0.25 |
| | White | 0.20 | 0.52* | 0.47* | 0.34 | 0.41 | -0.27 | 0.55** | 0.40 | 0.55** |

**Values are Pearson correlation coefficients. BMI: Body Mass Index; VAT: Visceral Adipose Tissue and SAT: Subcutaneous Adipose Tissue**

*** $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$**

# 4. DISCUSSION

In this study, the aim was to examine whether ethnicity and obesity may be associated with differences in HDL functionality and subclass distribution. Data showed that, despite lower levels of HDL-C, black women had higher PON1 activity levels compared to white women. In contrast, the activity and protein expression of PAF-AH was lower in obese black compared to obese white women. Obesity was not associated with a difference in the activity of these enzymes, but was associated with a shift in HDL subclass from large to intermediate and small HDL, an effect which was largely driven by differences between normal-weight and obese white women.

The relatively low incidence of myocardial infarction in the South African black population has long been thought to be related to higher HDL-C levels in comparison to white populations. However, recent studies provide evidence for lower HDL-C in South African black populations compared South African white populations, despite clear reduced risk of acute myocardial infarction (Goedecke *et al.*, 2010; Sliwa *et al.*, 2012). Recent research suggests that the quality of HDL, rather than its quantity, may be a more important factor to consider as a CVD risk factor (see review, (Rizzo *et al.*, 2014)).

Low serum PON1 activity levels have been associated with an increased risk for major adverse cardiovascular events (MACE), including MI and stroke in an American study (Tang *et al.*, 2012). The novel finding of higher PON1 activity levels in black women compared to white women, independent of protein expression levels is in contrast to other studies in the USA that found that African Americans had lower or similar PON1 activity levels than their white counterparts, despite similar HDL-C concentrations (Gaillard *et al.*, 2011; S. J. Healy *et al.*, 2015).

Accordingly, it is hypothesised that the low rate of cholesterol-attributable mortality (Norman et al. 2007), and particularly the low incidence of myocardial infarction in black populations (Sliwa *et al.*, 2012) may be explained by higher PON1 activity levels. However, this hypothesis would need to be tested as a study in a mixed race South African population found that PON1 activity was not associated with CVD risk (Macharia *et al.*, 2014).

Data also indicated that increased PON1 activity in black women was not related to a greater amount of HDL-associated PON1 protein, which may relate to genetic factors. PON1 polymorphisms, such as the promotor region small nucleotide polymorphism (SNP) L-55M, causes a reduction in serum PON1 activity, and is much less frequent in oriental and black

populations (Phuntuwate *et al.*, 2005; Mackness and Mackness, 2015). Additionally, PON1 polymorphisms can result in lower activity with PON1 serum protein levels unchanged (Phuntuwate *et al.*, 2005).

Associations between lower PON1 activity and typical markers of risk, including higher LDL and triglyceride concentrations, which have been reported previously in an American white young adult population were shown (Breton *et al.*, 2014). Of interest was a negative correlation between PON1 activity and HDL-C in white but not black women. Failure to show this association in black women is unexpected because HDL is physiologically associated with PON1 and has been shown in other studies to be negatively correlated with HDL-C (Mackness *et al.*, 1993b; Razav *et al.*, 2012). As PON1 activity remains consistently high in black women, it is possible that PON1 activity may be a marker of HDL function in black women, independent of HDL-C levels.

PAF-AH is another HDL-associated enzyme whose primary physiological role is maintenance of PAF metabolism and anti-thrombotic functions (Stafforini *et al.*, 1987; McIntyre *et al.*, 2009). Data has shown significantly lower PAF-AH activity in obese black women compared to obese white women. Overall, PAF-AH activity was correlated with PAF-AH expression in HDL while PAF-AH serum expression remained unchanged amongst the groups. Obese black women therefore expressed significantly less HDL-associated PAF-AH than obese white women. Furthermore, reduced PAF-AH activity was associated with increased fat mass and SAT in black women only. PAF-AH is mainly associated with LDL and a smaller proportion with HDL, which translate into different physiological functions of PAF-AH activity (Tselepis and Chapman, 2002). Reduced HDL-associated PAF-AH activity has been shown to be associated with increased risk of CVD (Kakafika *et al.*, 2003). Accordingly, the data suggests that reduced HDL-associated PAF-AH activity in obese black women may be associated with a reduction in anti-atherogenic HDL function. However, previous studies have shown that PON1 activity modulates HDL-associated PAF-AH activity (Kakafika *et al.*, 2003). It is therefore, proposed that higher PON1 activity in obese black women may circumvent reductions in PAF-AH activity. However, further studies are needed to test this hypothesis.

Surprisingly, despite the findings regarding PON1 activity, there were no significant group differences in antioxidant capacity of isolated HDL. This was similarly found in a study comparing diet-induced weight loss in overweight American participants (Aicher *et al.*, 2012). It is proposed that, in the ORAC assay, measurement of total antioxidant capacity may produce different results to a specific antioxidant assay such as PON1 activity, owing to contributions of additional HDL

components. Similarly, no differences in the expression of VCAM in HUVEC cells treated with isolated HDL were found. Since HDL has been shown to reduce expression of a number of endothelial adhesion molecules, additional markers would need to be used to further confirm the findings in this study.

This is the first report of the distribution of HDL subclasses in an African population. In this study, a similar profile of HDL subclass distribution was observed between black and white women. However, obesity was associated with reduced large HDL subclasses and concomitant higher intermediate and small subclasses; largely driven by differences between normal-weight and obese white women. In support of this finding, markers of increased adiposity, BMI, fat mass, percentage fat, VAT and SAT correlated with differences in large and intermediate subclasses in white, but not black women. These findings were confirmed by Spearman non-parametric testing. This shift has been previously shown in non-African male and female obese populations, where a decrease in average HDL particle size and increased concentrations of smaller HDL subclasses were reported (James *et al.*, 1997; Tian *et al.*, 2006; Magkos *et al.*, 2008). In this case, HDL subclass distribution does not explain changes in functionality related to ethnicity. HDL subclasses were not significantly different between normal-weight and obese black women, while HDL subclasses differed by obesity in white women. A similar trend was observed in LDL subclasses in the same population of women, where differences in LDL subclass in normal-weight and obese women were observed in white, but not black women (Goedecke *et al.*, 2010).

Longitudinal data on a black South African cohort also indicate that despite increases in weight, HDL-C concentrations remained consistently low in black women (Chantler *et al.*, 2015). In spite of this, it was shown that black women display greater HDL antioxidant functionality in comparison to white women, indicating the importance in measurement of HDL quality instead of total HDL cholesterol levels.

In addition to the limited number of patients recruited in this study, another important limitation to acknowledge is the different background, social status, education, lifestyle that has not been taken into account and that may contribute to differences observed between ethnicities

## 5. CONCLUSIONS

Despite lower HDL-C, black women had higher PON1 activity levels compared to white women. In contrast, the activity and protein expression of PAF-AH was lower in obese black compared to

obese white women. Obesity was associated with a shift in HDL subclass from large to intermediate and small HDL. It is acknowledged that the small sample size limits the conclusions which can be drawn, however data suggest that obesity and ethnicity may affect HDL functionality and HDL subclass. It is therefore suggested that future studies examining the association between HDL and cardiovascular risk should focus on examining the role of HDL subclass and functionality. A number of functionality assays were considered in this study, however, additional measures such as reverse cholesterol efflux can be considered in similar studies in the future. Longitudinal studies are required to determine if HDL subclass and function are indeed important risk factors for CVD. In addition, future studies will be required to establish baseline values for normal HDL subclass distribution for specific populations to better assess CVD risk.

# CHAPTER FIVE: EFFECTS OF EXERCISE TRAINING (12-WEEKS) ON HDL FUNCTIONALITY AND SUBCLASS IN OBESE BLACK SOUTH AFRICAN WOMEN

## CHAPTER FIVE: EFFECTS OF EXERCISE TRAINING (12-WEEKS) ON HDL FUNCTIONALITY AND SUBCLASS, IN OBESE BLACK SOUTH AFRICAN WOMEN

### 1. INTRODUCTION

Data presented in Chapter 4 highlighted that despite having lower HDL-C than their white counterparts, black South African women displayed greater PON1 activity. The functionality of HDL in African women therefore differs from their white counterparts, suggesting that, although African women have lower HDL-C levels, the quality of their HDL may be superior to the white African women (Woudberg et al. 2016). In addition, obesity was associated with shifts in HDL subclass distribution, with lower large HDL and greater intermediate and small HDL subclasses associated with obese compared to normal-weight women. Common interventions to limit obesity include diet and aerobic and/or resistance exercise training. Exercise training interventions result in a reduction in weight, body fat mass and cardiovascular risk factors such as blood pressure, total cholesterol, LDL-C and increases in HDL-C (Wood *et al.*, 1988; Lavie and Milani, 1997; Hawley, 2004; Slentz *et al.*, 2004; Curioni and Lourenco, 2005; Ohta *et al.*, 2005). Indeed, as little as 30 minutes of exercise per day can also increase the concentration of HDL-C in diabetic patients (Argani *et al.*, 2014).

Recent studies have suggested that exercise training may influence HDL function but the association between exercise, changes in HDL function and subclass in obese women is still unclear (Blazek *et al.*, 2013).

**The aim of the study was therefore to examine the effects of exercise training (12-weeks) on HDL functionality and subclass in obese black South African women.**

## 2. MATERIALS AND METHODS

### 2.1 Participants

Forty-five women were recruited during 2015 and 2016 from the Western Cape, South Africa. Inclusion criteria included: 20-35 years in age, obese (BMI 30-40 kg/m$^2$), weight stable for 6 months, black South African (both biological parents isiXhosa), sedentary (not participating in exercise training (>1 session of >20 min per week) within the last 12 months), on injectable contraceptive (depot medroxyprogesterone acetate, 400 mg) for a minimum of 2 months, no known illness or chronic disease, not taking any medications, and had no surgical procedures within the last 6 months. This study was approved by the Human Research Ethics Committee at the University of Cape Town (HREC REF:054/2015) and participants provided written consent prior to testing.

### 2.2 Study design

Women were block randomized into either control (n=22) or exercise (n=23) conditions. The exercise intervention consisted of 12 weeks of supervised aerobic and resistance exercise training, whilst the control participants were instructed to continue their normal physical activity and dietary patterns. Participants attended two pre- and post-testing testing sessions. During the first session, anthropometrical measurements were taken and participants completed a maximal graded exercise test for the assessment of maximal oxygen consumption ($VO_2$max). After a minimum of 48 hour recovery from the previous testing session, participants returned for a second session that included fasting (10-12 hours) venous blood collection. Venous blood samples were later analysed for HDL-C concentration, HDL functionality and subclass distribution.

### 2.3 Exercise intervention

The exercise intervention consisted of 12-weeks of supervised aerobic and resistance training at a moderate-vigorous intensity for 40-60 minutes, 4 days per week by a trained facilitator. Exercises included cardiovascular exercises in the form of aerobic dance, boxing, running, skipping, and stepping that were performed at a moderate-vigorous intensity (75-80% maximal heart rate, $HR_{max}$). Resistance exercises included the participants using their own body weight and progressed to the use of equipment (e.g. bands and free weights). These exercises included, squats, lunges, bicep curls, push up and shoulder press with a prescribed intensity of 60-70% $HR_{max}$. A heart rate monitor (Polar A300, Kempele, Finland) was worn by participants at all training

sessions to ensure the prescribed exercise intensity was maintained throughout the 12-week period.

### 2.4 Nutritional and physical activity standardization

Prior to participation in the study, all participants were informed (verbal and written) of the importance of maintaining their normal dietary patterns throughout the 12-week training period. Prior to the start of all testing sessions participants refrained from any physical activity for 72 hours, and the consumption of alcohol and caffeine for 24 hours.

### 2.5 Graded exercise test

$VO_{2max}$ was measured using a treadmill-based (C, Quasar LE 500 CE, HP Cosmos, Nussdorf-Traunstein, Germany) graded exercise test. Participants were familiarized to the equipment prior to the test. The test commenced at $3km.h^{-1}$ and a 6% gradient and increased every minute to a gradient of 16%, thereafter the pace and gradient were increased steadily until volitional exhaustion. Heart rate was recorded each minute throughout the protocol and the highest heart rate for this test was used as $HR_{max}$. Pulmonary gas exchange was measured by determining $O_2$ and $CO_2$ concentrations and ventilation to calculate $VO_2$ using a metabolic gas analysis system (CPET, Cosmed, Rome Italy). The system was calibrated according to the manufacturer's instructions.

### 2.6 HDL isolation

HDL was isolated from aliquots of serum as previously described in Chapter four (Section 2.2).

### 2.7 Quantification of HDL subclass distribution

Serum HDL subclass was determined using the Lipoprint® HDL system (Quantimetrix, Redondo Beach, CA) as described in Chapter four (Section 2.8).

### 2.8 Quantification of HDL reverse cholesterol efflux capacity

HDL induced reverse cholesterol efflux was quantified using a modified method (Sankaranarayanan *et al.*, 2011). Briefly, RAW264.7 cells, generously donated by Prof Gil Dealtry (Nelson Mandela Metropolitan University), were proliferated in RPMI-1640 media supplemented with 10% foetal calf serum and penicillin/streptomycin prior to seeding (100 000 cells/well) in 24-well culture plates for 16 hours. Labelling media was prepared by adding 4 µCi/ml of [$^3$H] cholesterol (Perkin Elmer, NET139001MC) to RPMI-1640 medium containing 2 µg/ml of acyl-CoA

cholesterol acyltransferase (ACAT) inhibitor (Sandoz, Sigma, S9318) and supplemented with 5% foetal calf serum. Cells were then incubated in labelling media for 24 hours. Cells were washed with minimum essential eagle (MEM) in HEPES buffer prior to addition of 25 µg/ml of isolated HDL in MEM-HEPES for 4 hours. Cell culture media was extracted and added to Ultima Gold scintillant (Perkin Elmer, 6013327). Counts per minute (CPM) were enumerated using TriCarb® Liquid Scintillation Analyzer and QuantaSmart™ software with 2 Sigma terminator 0.5 and 30 minute count time. Reverse cholesterol efflux capacity was calculated as label present in the cell media relative to the untreated control.

### 2.9 Quantification of HDL anti-inflammatory function

HDL anti-inflammatory function was measured as previously described in Chapter four (Section 2.7).

### 2.10 PON1 activity assay

Serum PON1 activity was measured as described in Chapter four (Section 2.3).

### 2.11 PAF-AH activity assay

Serum PAF-AH activity was measured as described in Chapter four (Section 2.4).

### 2.12 Western blotting

Isolated HDL and serum samples from each of the participants were electrophoresed on reducing 12.5% SDS-PAGE gels with 1.2 µg of HDL protein or 8 ug of serum loaded per well. Samples were run over multiple gels with control samples repeated in each gel. Blots were transferred onto nitrocellulose membranes (Bio-Rad, 162-0113). Ponceau S staining was used to validate equal loading of wells. Blots were blocked in 5% low fat milk powder in 0.05% TTBS, pH 7.5 and incubated overnight in primary mouse anti-PON1 antibody (1:200) (James *et al.*, 2010), and rabbit anti-PAF-AH (1:400) (Cayman Chemical, 160603). Blots were then washed in TTBS and incubated in goat anti-mouse-HRP conjugated secondary antibody (1:5000) (Bio-Rad, 170 6516) and goat anti-rabbit-HRP conjugated secondary antibody (1:2500) (Santa Cruz Biotechnology, sc-2313), respectively for 1 hour at room temperature. Blots were thoroughly washed in TTBS prior to incubation in Amersham TM ECLTM Western blotting detection reagent (GE Healthcare, RPN2106). Blots were captured in the GeneGnome gel imager. Densitometry of PON1 and PAF-AH blots was quantified using Quantity one software. PON1 and PAF-AH relative expression data were corrected for control samples, repeated in each gel.

### 2.13 Statistical analysis

Results are presented as mean or as percentage changes relative to baseline ± SEM. All variables were tested for normality prior to analysis. Non-normally distributed data were log transformed prior to statistical analysis and included serum PON1, serum and HDL PAF-AH expression. Two-way repeated measures analysis of variance was used to compare changes in anthropometry, $VO_{2max}$, HDL-C, cholesterol efflux capacity, anti-inflammatory function, PON1 activity, PAF-AH activity and HDL subclass distribution over the 12-week period between the exercise and control groups, using Fischer posthoc analysis. Unpaired t-tests were used to test differences in percentage changes in cholesterol efflux capacity, anti-inflammatory function, PON1 activity, PAF-AH activity and HDL subclass distribution over the 12-week period between control and exercise conditions. Pearson's correlation coefficients for the associations between anthropometry, fitness, HDL-C, HDL function and subclass were determined at baseline in the combined sample, and for percentage changes in the combined sample. $p < 0.05$ was deemed statistically significant and statistical tests were performed using Statistica (version 13.2, Dell Inc, 2016).

3. RESULTS

### 3.1 Changes in anthropometry, fitness and HDL-C

Exercise training adherence, expressed as the percentage attendance of total number of sessions, was 80.3±3.0% (range of 60.4-100%.). Exercise training resulted in a significant increase in fitness ($VO_{2max}$) compared to control ($p$ <0.05 for interaction, Table 5), BMI, waist and hip circumference and waist/hip ratio (WHR) decreased in response to the 12-week intervention in the exercise group only ($p$ <0.05 for interaction). HDL-C values were within the physiological range (Piepoli *et al.*, 2016), whilst $VO_{2max}$ was lower than values associated with healthy, exercising South Africans (Keytel *et al.*, 2005). HDL-C did not vary between groups or over time ($p$ = 0.238 for interaction).

**Table 5. Changes in anthropometry, fitness and HDL-C as a result of the exercise intervention**

| | Control (n=22) | | Exercise (n=23) | | *p* value | | |
|---|---|---|---|---|---|---|---|
| | Pre-testing | Post-testing | Pre-testing | Post-testing | Group | Time | Interaction |
| Age (yrs) | 24 ± 1 | | 23 ± 1 | | 0.157 | - | - |
| BMI (kg/m$^2$) | 33 ± 1 | 34 ± 1 | 34 ± 1 | 34 ± 1 | 0.493 | 0.678 | **0.010** |
| Waist (cm) | 103 ± 2 | 106 ± 2* | 104 ± 2 | 100 ± 2** | 0.406 | 0.548 | **0.001** |
| Hip (cm) | 118 ± 2 | 119 ± 2 | 114 ± 1 | 113 ± 1* | **0.045** | 0.359 | **0.022** |
| WHR | 0.88 ± 0.01 | 0.89 ± 0.02 | 0.91 ± 0.01 | 0.89 ± 0.01 | 0.436 | 0.892 | **0.044** |
| HDL-C (mmol/L) | 1.03 ± 0.06 | 1.06 ± 0.06 | 1.02 ± 0.05 | 0.98 ± 0.05 | 0.571 | 0.966 | 0.238 |
| $VO_2$ Max (ml/Kg/min) | 23.7 ± 0.8 | 22.7 ± 0.9 | 25.2 ± 0.7 | 27.7 ± 0.7* | **0.002** | 0.202 | **0.003** |

**Results represent means ± SEM. Unadjusted p values testing for significance of the grouping variable (Control vs Exercise), time (intervention duration) and the interaction (Group*Time). For Fischer post-hoc testing following interaction effect: * $p$ < 0.05 ** $p$ < 0.005 pre vs post-testing. BMI: Body mass index and WHR: Waist/hip ratio.**

### 3.2 Shift in HDL subclass distribution following exercise intervention

The distribution of HDL subclasses in response to the exercise intervention are presented in representative scan sections of large (Figure 27A and B), intermediate (D and E) and small (G and H) subclass distributions, and as percentages of large, intermediate and small HDL-C (Figure 27C, F and I). At baseline, the distribution of large, intermediate and small HDL subclasses were not different between groups ($p = 0.803$, $p = 0.701$ and $p = 0.485$, respectively). The distribution of intermediate HDL subclasses was similar between groups in response to the intervention ($p = 0.523$ for interaction) whilst there was a decrease in the distribution of small HDL subclasses in the exercise group ($p < 0.005$ for interaction). When examining differences in percentage changes in HDL subclass distributions, the change in the distribution of large and small HDL subclasses differed between groups in response to the intervention ($p < 0.05$). Small HDL subclasses increased in the control group and decreased in the exercise group ($p < 0.005$).

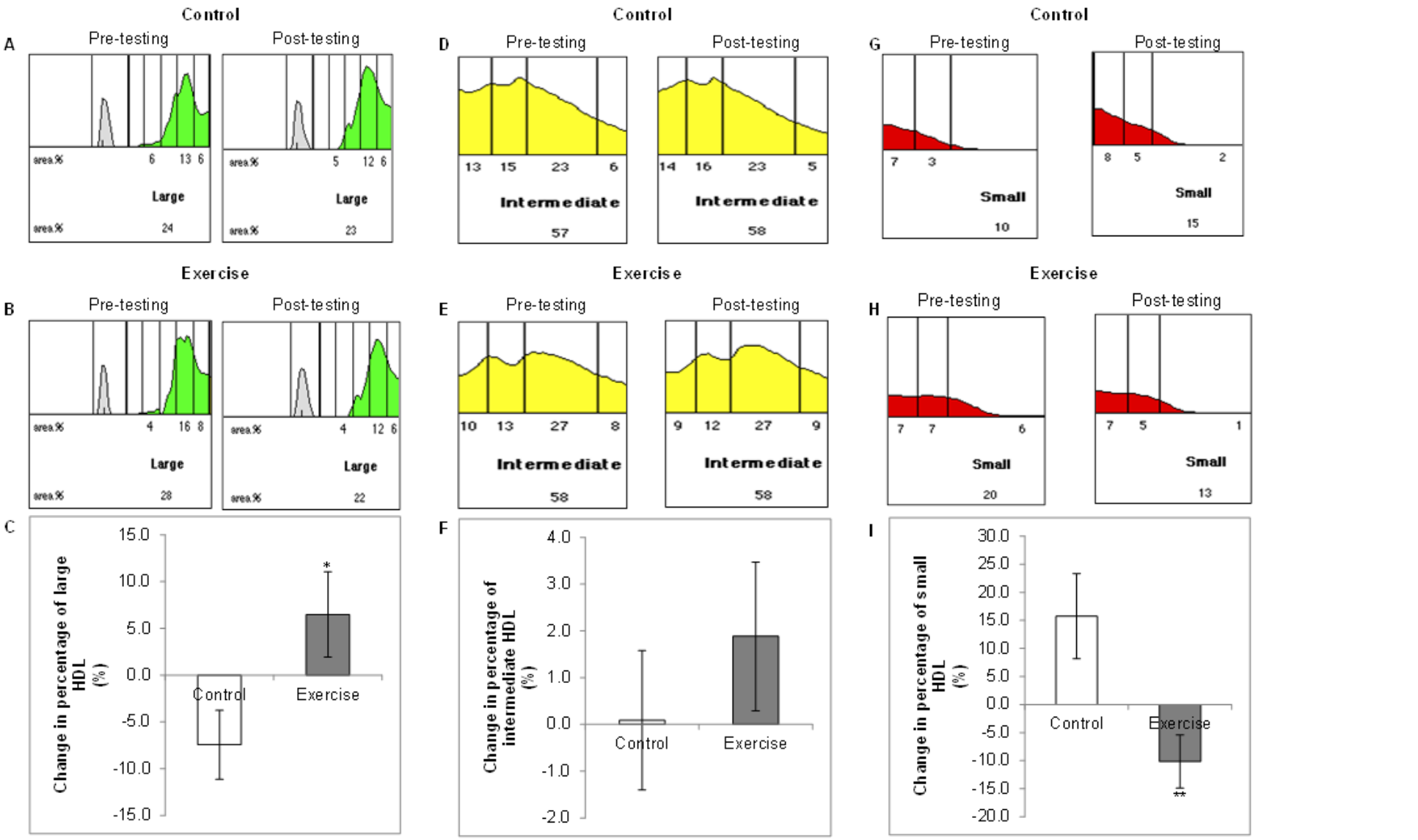



**Figure 27. Distribution of HDL subclasses in participant sera in response to the intervention. Participant sera was analysed using the Lipoprint® system and analysed using Lipoware software. Representative scan results of control (A,D and G) and exercise (B, E and H) sera pre and post testing. Changes in the percentages of large (C), intermediate (F) and small (I) HDL subclasses. Results as percentage changes relative to a baseline. Results are means ± SEM.* $p < 0.05$ and ** $p < 0.005$ significance for t-test.**

### 3.3 Changes in HDL function in response to the 12-week exercise intervention

*Cholesterol efflux capacity*

At baseline cholesterol efflux capacity did not differ between control and exercise groups (3.77 ± 0.22 vs 3.63 ± 0.20 AU, *p* =0.808 respectively). HDL-mediated cholesterol efflux capacity did not change in response to a 12-week exercise intervention and the percentage changes did not differ between groups (*p* = 0.476), (Figure 28).

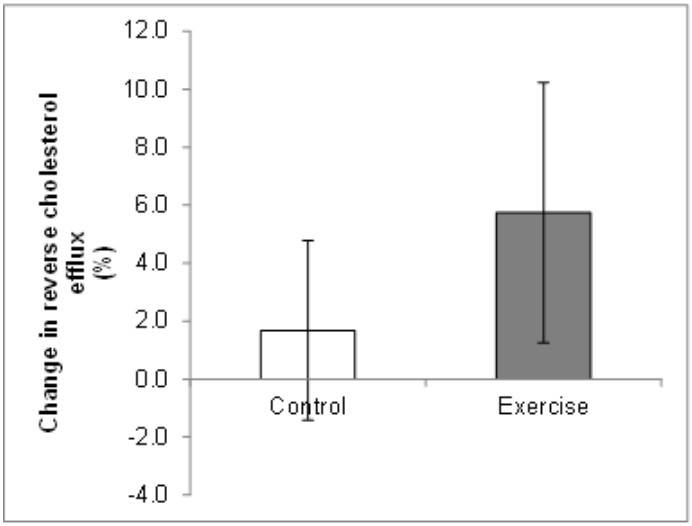



**Figure 28. Changes in reverse cholesterol efflux capacity of control and exercise participants in response to the intervention. [$^3$H-Cholesterol] was effluxed from RAW264.7 cells for 4 hours prior to scintillation counting. Cholesterol efflux capacity represents the mean radiolabel present in culture media relative to that of an untreated control. Results are represented as percentage changes relative to a baseline. Results are means ± SEM.**

*Anti-inflammatory function*

At baseline HDL anti-inflammatory function (expressed as relative reduction in VCAM expression in HUVEC cells) did not differ between control and exercise groups (0.47 ± 0.07 vs 0.50 ± 0.09 AU, *p* =0.504 respectively) (Figure 29). HDL anti-inflammatory function did not change in response to a 12-week exercise intervention and the percentage changes did not differ between groups (*p* = 0.476).

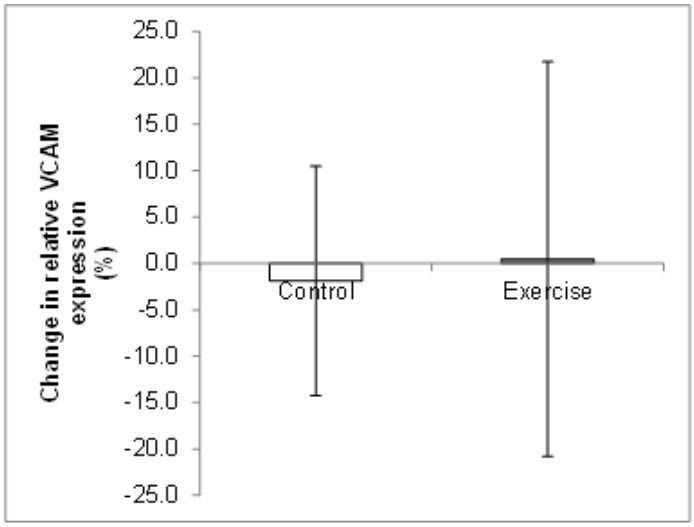



**Figure 29. Changes in anti-inflammatory function of control and exercise participants in response to the intervention. HUVEC cells were treated with 10 µg/ml participant HDL prior to 20 ng/ml tumour necrosis factor (TNF) treatment for 8 hours. Results are calculated relative to a no-HDL treatment control. Cell lysates were harvested and stored in RNA protect reagent prior to RNA extraction, followed by cDNA synthesis and quantitative real time PCR. Results as percentage changes relative to a baseline. VCAM: Vascular Cell Adhesion Molecule. Results are means ± SEM.**

*PON1 activity*

At baseline, serum PON1 activity did not differ between the control and the exercise groups (0.90 ± 0.07 vs 0.83 ± 0.05 U/L, $p$ = 0.173, respectively). After 12 weeks, serum PON1 activity decreased in response to the exercise intervention only ($p < 0.05$ for interaction). The percentage change was therefore significantly different between exercise and control groups ($p < 0.05$, Figure 30A). In contrast, changes in serum and HDL PON1 expression levels did not differ between groups ($p$ = 0.751 and $p$ = 0.464 respectively, Figure 30B-E) or in response to the intervention ($p$ = 0.888 and $p$ = 0.697 for interaction, respectively). The association between PON1 activity and expression was explored at baseline in all participants and serum PON1 activity was positively correlated with serum and HDL PON1 expression (r = 0.48, $p < 0.05$, and r = 0.57, $p < 0.005$ respectively). However, percentage changes in PON1 activity were not associated with changes in serum and HDL PON1 expression (r = - 0.05, $p$ = 0.817, and r = 0.09, $p$ = 0.633 respectively).

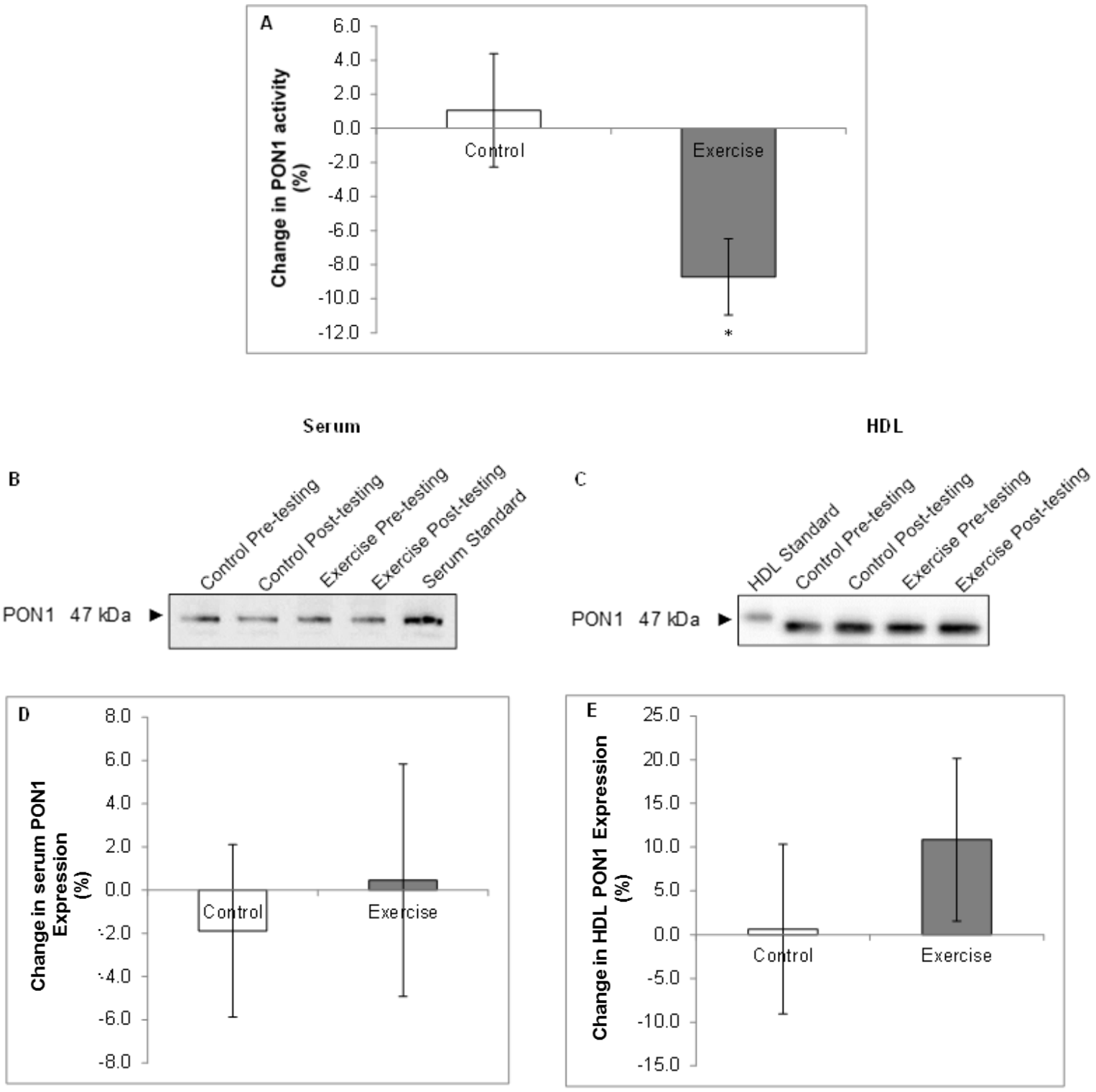



**Figure 30. Changes in paraoxonase activity and protein expression in control and exercise participants in response to the intervention. Paraxonase activity of diluted sera was measured at $A_{405}$ over a 20 minute time interval using the paraoxon-ethyl substrate. One unit of activity is defined as 1 nmol of substrate disintegrated per minute (A). Participant sera (B and D) and isolated HDL (C and E) were run on reducing 12.5% SDS-PAGE gels and transferred to nitrocellulose membrane. Ponceau S staining was used to confirm equal loading. Blots were probed with mouse anti-PON1 antibody. Results are representative of randomized experiments (B) and (C). Results are percentage changes in densitometry relative to a baseline in sera (D) and HDL (E). Results are means ± SEM.* $p < 0.05$ significance for t-test.**

*PAF-AH activity*

At baseline, serum PAF-AH activity did not differ between the control and the exercise groups (12.7 ± 1.4 vs 15.2 ± 1.2 U/L, $p = 0.311$, respectively). There was no difference in PAF-AH activity between groups in response to the intervention ($p = 0.112$ for interaction) and no difference in the percentage change in PAF-AH activity between groups (Figure 31A $p = 0.093$). In contrast, the percentage change in serum PAF-AH expression differed between groups, with a decrease in the exercise group only (Figure 31D $p < 0.005$). Changes in HDL PAF-AH expression were not different between groups over time ($p = 0.493$). When exploring the association between PAF-AH activity and expression no associations were found between PAF-AH activity and serum and HDL PAF-AH expression at baseline (r = 0.02, $p = 0.921$, and r = 0.09, $p = 681$ respectively) or between changes in activity and expression over the 12-week intervention (r = 0.38, $p = 0.055$, and r = 0.17, $p = 0.441$ respectively).

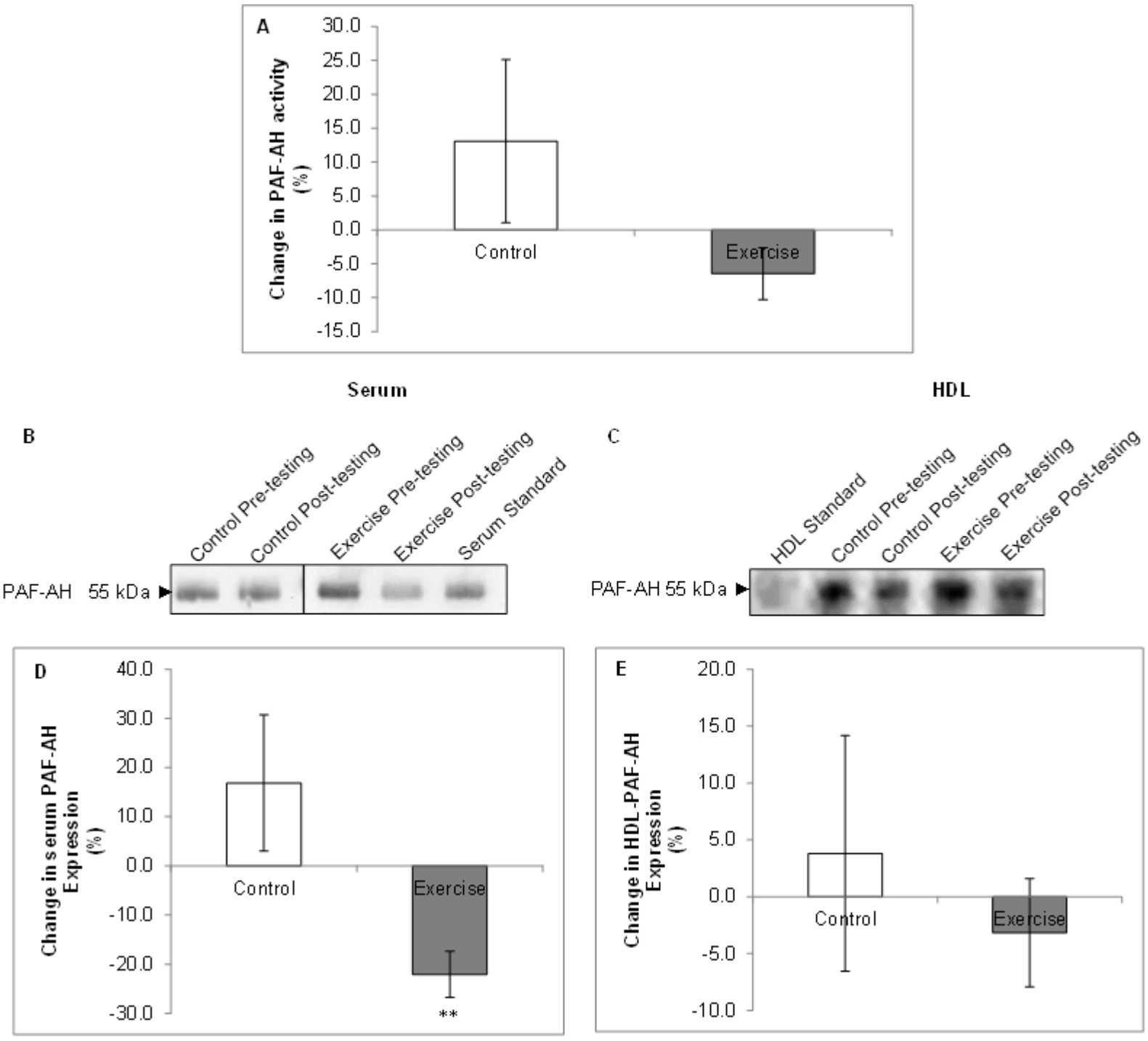



**Figure 31. Changes in PAF-AH activity and protein expression in control and exercise participants in response to the intervention. PAF-AH activity of diluted sera was measured at $A_{412}$ over a 20 minute time interval using the PAF Acetylhydrolase Assay Kit. One unit of activity is defined as 1 µmol of substrate disintegrated per minute (A). Participant sera (B and D) and isolated HDL (C and E) were run on reducing 12.5% SDS-PAGE gels and transferred to nitrocellulose membrane. Ponceau S staining was used to confirm equal loading. Blots were probed with rabbit anti-PAF-AH antibody. Results are representative of randomized experiments (B) and (C). Results are percentage changes in densitometry relative to a baseline in sera (D) and HDL (E).**

****p*<0.005 significance for t-test.**

### 3.4 Relationships between anthropomorphic measures, fitness and HDL function and subclass

At baseline, higher BMI was associated with lower cholesterol efflux capacity ($p < 0.05$), less large HDL subclasses and lower HDL-C ($p < 0.05$) (Table 6). There were no significant associations between the changes in fitness, body composition and HDL-C and changes in HDL function (Table 6), however, a decrease in BMI and increase in HDL-C in the combined sample was associated with an increase in the percentage of large HDL subclasses.

### 3.5 Relationships between measures of HDL functionality and HDL subclass

At baseline, RCT was positively associated with anti-inflammatory function. Similarly, higher HDL anti-inflammatory function was associated with a greater percentage of large HDL and lower percentage of intermediate HDL subclasses ($p < 0.05$, Table 6). In addition, an increase in cholesterol efflux capacity and PON1 activity were associated with increases in the percentage of intermediate and small HDL subclasses, respectively (Table 7).

**Table 6. Associations between HDL functionality and subclass measures with body composition and HDL-C in all participants at baseline**

| | BMI | WHR | $VO_{2max}$ | HDL-C | Cholesterol efflux capacity | Anti-inflammatory function | PON1 activity | PAF-AH activity | Large HDL | Intermediate HDL | Small HDL |
|---|---|---|---|---|---|---|---|---|---|---|---|
| BMI | | 0.202 | -0.199 | -0.265 | -0.423* | -0.260 | -0.148 | -0.053 | -0.375* | 0.311 | 0.298 |
| WHR | | | -0.108 | -0.377* | -0.253 | -0.517 | -0.174 | -0.130 | -0.222 | 0.037 | 0.304 |
| $VO_{2max}$ | | | | -0.053 | 0.148 | -0.287 | -0.209 | 0.048 | -0.138 | 0.109 | 0.090 |
| HDL-C | | | | | 0.301 | 0.036 | 0.108 | 0.263 | 0.421* | -0.356 | -0.322 |
| Cholesterol efflux capacity | | | | | | 0.677* | -0.208 | 0.291 | 0.194 | -0.252 | 0.071 |
| Anti-inflammatory function | | | | | | | 0.484 | -0.093 | 0.535* | -0.545* | -0.377 |
| PON1 activity | | | | | | | | 0.183 | -0.044 | -0.018 | 0.073 |
| PAF-AH activity | | | | | | | | | 0.277 | -0.280 | -0.191 |
| Large HDL | | | | | | | | | | -0.775** | -0.845** |
| Intermediate HDL | | | | | | | | | | | 0.318 |
| Small HDL | | | | | | | | | | | |

**Values are Pearson correlation coefficients. BMI: Body Mass Index and WHR: Waist/hip ratio. * $p < 0.05$, ** $p < 0.005$**

**Table 7. Associations between changes in HDL functionality and subclass measures with body composition and HDL-C in all participants**

| | ΔBMI | ΔWHR | $\Delta VO_{2max}$ | ΔHDL-C | ΔCholesterol efflux capacity | ΔAnti-inflammatory function | ΔPON1 activity | ΔPAF-AH activity | ΔLarge HDL | ΔIntermediate HDL | ΔSmall HDL |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ΔBMI | | 0.636** | 0.044 | 0.165 | 0.094 | -0.175 | -0.075 | 0.225 | -0.401* | 0.147 | 0.295 |
| ΔWHR | | | -0.091 | -0.096 | -0.006 | -0.258 | -0.120 | 0.171 | -0.366* | 0.227 | 0.095 |
| $\Delta VO_{2max}$ | | | | -0.007 | 0.087 | -0.306 | -0.317 | -0.284 | 0.191 | 0.061 | -0.274 |
| ΔHDL-C | | | | | 0.299 | 0.227 | 0.425* | -0.006 | -0.096 | -0.022 | 0.310 |
| ΔCholesterol efflux capacity | | | | | | 0.102 | 0.312 | -0.211 | -0.212 | 0.395* | -0.043 |
| ΔAnti-inflammatory function | | | | | | | -0.156 | -0.236 | -0.112 | 0.193 | 0.096 |
| ΔPON1 activity | | | | | | | | 0.051 | -0.275 | -0.153 | 0.509* |
| ΔPAF-AH activity | | | | | | | | | 0.252 | -0.077 | -0.239 |
| ΔLarge HDL | | | | | | | | | | -0.561** | -0.652** |
| ΔIntermediate HDL | | | | | | | | | | | -0.112 |
| ΔSmall HDL | | | | | | | | | | | |

**Values are Pearson correlation coefficients. BMI: Body Mass Index and WHR: Waist/hip ratio * p < 0.05, ** p < 0.005**

## 4. DISCUSSION

The aim of this study was to explore whether exercise training would result in modification of the quality of HDL, the functionality and subclass distribution. Although HDL-C was unchanged, a 12-week exercise training intervention in obese black South African women resulted in reductions in PON1 activity and PAF-AH expression and percentages of small HDL subclasses. Notably, changes in PON1 activity and cholesterol efflux capacity were associated with changes in HDL subclass distribution.

Exercise interventions are routinely prescribed for obese individuals with the aim of reducing the risk of cardio-metabolic complications, and have been shown to promote an increase in HDL-C (Kelley and Kelley, 2006; Kodama *et al.*, 2007; Argani *et al.*, 2014). The current study, however, did not find any change in HDL-C following the intervention. No previous studies have examined the benefits of exercise on HDL-C levels in an African population, who often have lower basal HDL-C levels, compared to other populations (Sliwa *et al.*, 2008; Goedecke *et al.*, 2010).

*Exercise intervention associated with changes in HDL subclass distribution*

In the present study, it was explored whether exercise training may alter HDL subclass distribution in obese black South African women. Here, evidence suggests that exercise training may revert HDL subclass distribution back to a “non-obese” state in a black South African sample population. These results are supported by a decrease in small HDL and an increase in large HDL in the exercise training group compared to the non-training control group. Additionally, negative associations between the percentage of large HDL subclasses and markers of greater BMI and WHR at baseline and associations between an increase in large HDL subclasses and decrease in BMI in all participants support these findings. In a similar sample population (Chapter four), it was previously reported that an association between obesity and lower levels of large and high levels of smaller HDL subclasses exists (Woudberg et al. 2016). It is proposed that BMI and weight loss may mediate changes in HDL subclass distribution, however, in this study, changes in body weight (-1.0 ± 0.6%) and WHR (-1.7 ± 0.8%) were minor. Results are supported by a study in elderly non-obese participants where a 6-week endurance training resulted in an increase in large HDL subclasses (Riedl *et al.*, 2010). Other studies including exercise interventions showed that exercise training resulted in changes in HDL subclasses, favouring increases in larger HDL subclasses (Ribeiro *et al.*, 2008; Casella-Filho *et al.*, 2011).

*Exercise intervention associated with changes in HDL function*

Cholesterol efflux capacity, the primary function of HDL *in vivo*, did not change following the intervention. Data are consistent with previous studies conducted in African American populations (Aicher *et al.*, 2012). In this study, a 6 month diet programme of reduced fat and energy, combined with low-intensity exercise, showed improvements in fitness and weight loss, however, these changes were not associated with changes in cholesterol efflux capacity (Aicher *et al.*, 2012). In contrast, cholesterol efflux capacity was associated with baseline BMI. Similarly, in a study examining the relationship between body composition and HDL RCT, found that an increase in waist circumference was an accurate predictor of impairment in cholesterol efflux capacity (Attia *et al.*, 2010). Comparing cholesterol efflux between obese and lean subjects, it was found that there was an association between increased BMI and lower cholesterol efflux capacity in obese subjects (Sasahara *et al.*, 1998). Commensurate with these findings, improvement in cholesterol efflux capacity in individuals undergoing exercise interventions were only significant in those individuals with significant weight loss, and associations between an increase in body weight and a decrease in cholesterol efflux were observed (Lesna *et al.*, 2009). Accordingly, the lack of clinically significant changes in anthropometry in our study may explain the lack of change in HDL cholesterol efflux capacity in response to the intervention.

In this study, the exercise intervention did not improve HDL anti-inflammatory function. In contrast, a 21-day dietary and exercise intervention, which resulted in a 3.2% decrease in BMI, found that HDL anti-inflammatory function was improved in response to the intervention in obese men (Roberts *et al.*, 2006). These data suggest that substantial weight loss, not observed in this study, rather than exercise may influence changes in HDL anti-inflammatory function.

The unexpected findings of this study were the decrease in both the antioxidative and anti-thrombotic activities of PON1 and PAF-AH, respectively, in response to the exercise intervention. This is in contrast to other studies that found improvements in PON1 activity and overall HDL antioxidant function after moderate aerobic exercise interventions carried out over 3 or 4 months in metabolic syndrome and type 2 diabetic patients respectively (Ribeiro *et al.*, 2008; Casella-Filho *et al.*, 2011). The reasons for the different response in PON1 activity to the exercise intervention in our study are not entirely clear, but may be due to the fact that the participants in our study were normolipidemic, and/or relate to differences in the type and intensity of the intervention. Black South African women exhibited higher PON1 activity when compared to obese white South African women, independent of serum PON1 expression levels (Chapter four) (Woudberg et al. 2016). PON1 activity is largely modulated by genetic and environmental factors such as smoking and intake of antioxidants, and these factors may contribute to differences between studies (reviewed by (da Costa Vieira *et al.*, 2001). In the

current study, changes in PON1 activity were not associated with changes in anthropometry, nor with changes in fitness, suggesting that the beneficial aspects of exercise training may result in a compensatory reduction in PON1 activity owing to reduction in oxidative stress. Indeed, studies in overweight adolescents and type 2 diabetic patients have both reported how exercise interventions may improve cardiovascular health by reducing oxidative stress (Vinetti *et al.*, 2015; Li *et al.*, 2017).

In the present study, exercise was also associated with a decrease in the serum expression of PAF-AH. Again, this is in apparent contrast to the literature, which indicated that a short term (3 weeks) diet and exercise intervention in obese subjects increased PAF-AH activity (Roberts *et al.*, 2006). PAF-AH is associated with HDL and LDL, with much of the beneficial aspects specific to the HDL fraction (Tselepis and Chapman, 2002). A positive association between PAF-AH activity in LDL-C has previously been shown in a similar population (Woudberg et al. 2016) and indicate that activity levels may not relate specifically to the HDL-associated fraction of PAF-AH. Unlike previous research in obese black South African women (Chapter four) (Woudberg et al. 2016), the current study showed no association between HDL-PAF-AH expression and PAF-AH activity. These results make it difficult to ascribe whether the exercise training-induced change in serum expression and activity were related to the beneficial HDL-associated PAF-AH activity.

Considering the function and subclass of HDL-C together is relevant as both can be modified concurrently and may be associated with each other (Hernáez *et al.*, 2017). The study has demonstrated an association between a decrease in small and intermediate HDL subclasses and a decrease in PON1 activity and cholesterol efflux capacity, respectively. PON1 is preferentially associated with smaller HDL subclasses, suggesting that decreases in PON1 activity are associated with decreases in the distribution of small HDL subclasses (Davidson *et al.*, 2009). Different HDL subclasses have inherent functional differences however associations between measures of HDL size and function have seldom been undertaken, and have not done using the Lipoprint® System (Kontush *et al.*, 2003; Zerrad-Saadi *et al.*, 2009; Kontush and Chapman, 2010; Camont *et al.*, 2013; Gugliucci *et al.*, 2013; Hernáez *et al.*, 2017). Therefore, novel evidence shows that changes in traditional measures of HDL function can be linked to new measures of HDL size.

Unfortunately, this study was limited with a relatively small sample size, which certainly limits conclusions regarding HDL anti-inflammatory function in particular. Unfortunately, the findings regarding the associations between changes in body composition, HDL function and subclass were not maintained when analysed using Spearman non-parametric testing. Although, participants were instructed to maintain their normal dietary patterns, the participant dietary

details will be published in a subsequent publication and not in this study. There was, however, good adherence to the exercise training, allowing for adequate interpretation of its effects on HDL function and subclass distribution.

## 5. CONCLUSION

Despite no change in HDL-C concentration, our study provides the first evidence that exercise training may revert the HDL subclass distribution to a “non-obese” state as well as alter HDL antioxidative and anti-thrombotic function. HDL cholesterol efflux capacity was associated with BMI at baseline, however, was not associated with the minor changes in body composition as a result of exercise training. This suggests that improvements in HDL function and subclass are related to obesity and weight loss and not necessarily only to exercise per se. This study provides evidence how exercise training may alter HDL function and subclass distribution in an African population. Measures of HDL function and subclass were shown to be associated in this study. This suggests that studying HDL subclass and function may therefore be a more sensitive approach to assess CVD risk in this population.

# CHAPTER SIX: DIFFERENCES IN HDL COMPOSITION, SUBCLASS AND FUNCTION ASSOCIATED WITH HYPERTENSION AND HEART FAILURE

## CHAPTER SIX: DIFFERENCES IN HDL COMPOSITION, SUBCLASS AND FUNCTION ASSOCIATED WITH HYPERTENSION AND HEART FAILURE

### 1. INTRODUCTION

Studies conducted in Chapter 4 and 5 have highlighted the association of HDL subclass and function with major CVD risk factors such as ethnicity and obesity.

Hypertension is another major risk factor for CVD with hypertension related complications accounting for at least 9.4 million annual deaths worldwide (WHO, 2013). An increase in hypertension prevalence in developing countries is associated with the adoption of Westernized diets and a decrease in physical activity (Bello, 2013). In 2015, the highest contributor to disability adjusted life years (DALY's) was high systolic blood pressure, and deaths due to raised blood pressure have increased in the last 25 years (GBD 2015 Disease and Injury Incidence and Prevalence Collaborators, 2016; Forouzanfar *et al.*, 2017; Noubiap *et al.*, 2017). Critically, central and sub-Saharan Africa remain as regions with the highest age-adjusted DALY's with 20-25% of adult Nigerians estimated as hypertensive (Ogah, 2006; Forouzanfar *et al.*, 2017).

Chronic elevated systolic blood pressure is the principal cause of left ventricular hypertrophy (LVH) and evidence suggests that LVH progresses to hypertensive HF (Verdecchia *et al.*, 1990; Izzo, 2004). Indeed, hypertension remains a major cause of HF in developing countries (Mendez and Cowie, 2001; Onwuchekwa and Asekomeh, 2009). In a cohort of 1 515 patients, hypertensive HF was the most common form of HF in Abuja, Nigeria (Ojji *et al.*, 2013).

In hypertensive patients, HDL-C is lower (Paynter *et al.*, 2011) and HDL subclass size is lower (Zhang *et al.*, 2015), however, little is known how hypertension and hypertensive HF may affect HDL function. The pathogenesis of hypertension proposes a number of causal factors relating to diet, renal function, stress and endothelial dysfunction due to impaired nitric oxide release and oxidative stress (Oparil *et al.*, 2003). It is therefore proposed that HDL function may be impaired in hypertensive patients since HDL is important in controlling oxidative stress in the endothelium and maintaining normal endothelial function. HDL stimulates eNOS activity through the binding of S1P to its specific receptors (Nofer *et al.*, 2004; Igarashi *et al.*, 2007; Besler *et al.*, 2011).

**The aim of the study was therefore to explore whether HDL composition, function and subclass distribution in Nigerian patients diagnosed with hypertension and hypertensive HF may differ from healthy controls.**

## 2. MATERIALS AND METHODS

### 2.1 Subjects

The sample population consisted of 80 outpatients from the University of Abuja Teaching Hospital and were recruited as previously described (Ojji *et al.*, 2013). Briefly, patients were grouped into three primary groups: i) control patients (n=30) who complained of non-specific chest pain but were non-hypertensive with no cardiac risk factors; ii) hypertensive patients (n=28) displaying no hypertrophy and iii) hypertensive patients with HF (n=22) diagnosed using echocardiography. Hypertension was defined according the Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC 7) guidelines (US Department of Health and Human Sevices, 2004). HF was diagnosed according to the European Society of Cardiology Guidelines (Nieminen *et al.*, 2005), and functional status of the HF subjects was according to the guidelines of the New York Heart Association Functional classification (NHYA 1964). Exclusion criteria included patients with dyslipidaemia and diabetes mellitus and any patients who were active smokers. The study was approved by the Research Ethics Committees of the Faculty of Health Sciences of the University of Cape Town (HREC REF: 745/2016) and the University of Abuja teaching Hospital (REF: HREC/PR/530). Fasting serum samples were taken and stored at -80°C prior to use.

### 2.2 HDL isolation

HDL was isolated from aliquots of serum as previously described in Chapter four (Section 2.2).

### 2.3 Measurement of apolipoproteins A1 and M content in HDL

Isolated HDL and serum samples from each of the participants were electrophoresed on reducing 12.5% SDS-polyacrylamide (SDS-PAGE) gels with 1.5-2 µg of HDL protein loaded per well. Samples were run over multiple gels with control samples repeated in each gel. Blots were transferred onto nitrocellulose membranes (Bio-Rad, 162-0113). Ponceau S staining was used to validate equal loading of wells. Blots were blocked in 5% low fat milk powder in 0.05% TTBS, pH 7.5, and incubated overnight in separate primary antibody solutions: rabbit anti-ApoM, (Abcam, 47711) (1:1000) and mouse anti-ApoA1, (Calbiochem, 178422) (1:1000). Blots were then washed in TTBS and incubated in goat anti-rabbit, (Li-Cor, 92632221) and goat anti-mouse, (Li-Cor®, 92632210) cocktail for 1 hour at room temperature. Blots were thoroughly washed in TTBS prior to visualization using the Li-Cor® digital scanner. Blots were captured in the GeneGnome gel imager and Li-Cor digital scanner. Densitometry of blots was

quantified using Quantity one and Li-Cor® software. ApoA1 and ApoM relative expression data were corrected for control samples, repeated in each gel.

### 2.4 Measurement of S1P content in isolated HDL

The concentration of S1P in isolated HDL was measured using mass spectroscopy as described previously (Brulhart-Meynet *et al.*, 2015). Briefly, analyses were performed on liquid chromatography mass spectroscopy (LC-MS/MS) system consisting of a 5500 QTrap® triple quadrupole linear ion trap (QqQLIT) mass spectrometer equipped with a TurboIon Spray™ interface (AB Sciex, Framingham, MA, USA) and an Ultimate 3000 Series (Thermo Fisher Scientific Inc., Whaltham, MA, USA). Data acquisition and analysis were performed using AnalystTM software (version 1.6.2; AB Sciex, Framingham, MA, USA).

Substances were separated using a Chromolith RP-18 analytical column (100 mm x 3 mm Merck, Darmstadt, Germany). Mobile phase was constituted of a mixture of water (A) and methanol (B). A gradient was used starting from 50 to 100% in 1.5 minutes, maintained during 8 minutes, and re-equilibrated at 50% in 0.1 minutes during 1.4 minutes for an overall analysis time of 11 minutes. The autosampler was kept at 6°C.

The TurboIon Spray™ interface was operated in the negative ionization mode. The parameters of the source were used with the following settings using nitrogen as curtain and nebulizer gas: capillary voltage -4.0 kV, temperature 625°C, curtain gas 20 psi, collision gas -8, ion source gas-1 45 psi, and ion source gas-2 45 psi. For the multiple reaction monitoring parameters, a dwell time of 50 ms, a declustering potential of -100 eV, and a collision energy of -40 eV were used. The precursor ion was at 378.2 m/z. The product ion of 78.8 m/z was selected. The MS/MS experiments were based on collision-induced dissociation occurring in the collision cell (quadrupole 2), with nitrogen as collision gas set at 10.

### 2.5 Quantification of HDL subclass distribution

Serum HDL subclass was determined using the Lipoprint® HDL system (Quantimetrix, Redondo Beach, CA) as described in Chapter four (Section 2.8).

### 2.6 PON1 activity assay

Serum PON1 activity was measured as described in Chapter four (Section 2.3).

### 2.7 Quantification of HDL anti-inflammatory function

HDL anti-inflammatory function was measured as previously described in Chapter four (Section 2.7).

### 2.8 Quantification of HDL-induced endothelial stimulation

In order to quantify HDL function relating to maintaining normal endothelial function, induction of eNOS in endothelial cells stimulated with isolated HDL was assessed. Mouse endothelial cell line bEnd.3 cells, generously provided from Prof Brenda Kwak (University of Geneva), were cultured on plates coated with 1.5% gelatin (Sigma, G1890) in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, 41966-029) containing foetal bovine serum (FBS) (Biochrom,S0415) and 1% penicillin/streptomycin (Gibco, 15140122) at 37ºC, 5% $CO_2$. Once cells reached 100% confluence, cells were incubated in DMEM containing 1% FBS overnight. Media was changed to DMEM with no FBS for 1 hour prior to treatment with subject HDL (100 µg/ml) for 10 minutes. Cells monolayers were treated with lysis buffer containing Tris-HCl (50mM, pH 7.4), NaCl (150mM), glycerol (10%), EDTA (2mM), ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) (2mM), Triton X-100 (1%v/v), β-glycerophosphate (40mM), sodium fluoride (50mM), a mixture of protease inhibitors (Roche, Mannheim, Germany) and phenyl-methyl-sulfonyl fluoride (1mM). Cell lysates were incubated at 4ºC for three 10 minute intervals interspersed with vortexing. Lysates were centrifuged at 10 000g for 10 minutes at 4ºC and supernantants retained. Protein concentration was determined using the method described by Bradford (Bradford, 1976).

Cell lysates (20 µg/lane) were loaded onto reducing 10% SDS-PAGE gels and transferred onto nitrocellulose membranes. Ponceau S staining was used to validate equal loading of wells. Blots were blocked in 5% low fat milk powder in TTBS for 1 hour at room temperature. Blots were washed in TTBS and incubated in rabbit anti-phosphoeNOS (Cell signalling, 9571, Ser1177). Membranes were stripped and reprobed with rabbit anti-total eNOS (Cell signalling, 32027). Relative bands were visualised and densities quantified with the Chemidoc™ system (Biorad, 1708265). Pathway activation was quantified using the ratio between phospho-eNOS with total eNOS.

### 2.9 Statistical analysis

Non-normally distributed data including: patient age, BMI, systolic and diastolic blood pressures, lipid profiles, ApoA1, ApoM expression, S1P content, HDL subclass distribution, PON1 activity and anti-inflammatory function were presented as medians ± interquartile range (IQR). K-Wallis and Mann-Whitney nonparametric testing was used to compare differences between groups. For comparisons between groups regarding echocardiographic

measurements, samples were log transformed prior to one-way analysis of covariance with Scheffe posthoc testing. For endothelial stimulation, comparisons to non-treated controls were tested using students t-tests and results are presented as means ± SEM. All Statistical analysis was performed using Stata/SE version 14.2 (StataCorp®, College Station, Texas).

3. RESULTS

### 3.1 Patient clinical characteristics

Patient groups did not differ by BMI and gender. Hypertensive patients with and without HF were older and presented with a higher systolic and diastolic blood pressure ($p < 0.001$) as well as a higher total cholesterol level ($p < 0.05$) than control patients, (Table 8). In contrast, triglycerides, LDL-C and HDL-C did not differ between the groups

**Table 8: Clinical characteristics of patients**

| Parameter | Control (n=30) | Hypertensive (n=28) | Hypertensive and HF (n=22) | *p* value |
|---|---|---|---|---|
| Age (years) | 25 ± 3 | 46 ± 10 | 53 ± 9 | < 0.001 |
| Gender (male) | 47% | 43% | 59% | 0.499 |
| BMI (kg/m$^2$) | 29 ± 6 | 27 ± 5 | 27 ± 6 | 0.705 |
| Systolic blood pressure (mmHg) | 109 ± 15 | 140 ± 20$^A$ | 140 ± 20$^A$ | < 0.001 |
| Diastolic blood pressure (mmHg) | 71 ± 5 | 90 ± 6$^A$ | 90 ± 10$^A$ | < 0.001 |
| HDL-C (mmol/L) | 1.1 ± 0.5 | 1.3 ± 0.7 | 1.3 ± 0.5 | 0.296 |
| LDL-C (mmol/L) | 2.9 ± 0.9 | 2.9 ± 1.0 | 3.5 ± 1.7 | 0.093 |
| Total-C (mmol/L) | 4.1 ± 1.3 | 4.9 ± 1.4$^a$ | 5.2 ± 2.1$^a$ | 0.025 |
| Triglycerides (mmol/L) | 1.1 ± 0.7 | 1.3 ± 0.8 | 1.3 ± 0.3 | 0.990 |

**BMI: Body mass index. Results presented as medians ± IQR. $^A$ $p < 0.005$ compared to Control, $^a$ $p < 0.05$ compared to Control.**

### 3.2 Patient echocardiographic characteristics

Echocardiographic measurements indicate that hypertensive and HF patients had higher end diastolic diameter (EDD), end systolic diameter (ESD), left atrial area (LAA), right atrial area (RAA) and lower tricuspid annular pulmonary excursion (TAPSE) than both control and hypertensive patients, Table 9. Additionally, hypertensive and HF patients had higher early mitral filling (ME) than control patients ($p < 0.05$). There were no differences in atrial filling (MA) between hypertensive and hypertensive and HF patients while hypertensive patients had greater inter-ventricular septal diameter in diastole (IVSD) compared to control patients ($p < 0.05$).

**Table 9: Patient echocardiographic characteristics**

| Parameter | Control | Hypertensive | Hypertensive and HF | *p value* |
|---|---|---|---|---|
| IVSD | 0.80 ± 0.11 | 1.07 ± 0.59[a] | 0.82 ± 0.32 | 0.011 |
| EDD | 4.52 ± 0.89 | 4.44 ± 1.02 | 6.65 ± 0.39[A,B] | <0.001 |
| ESD | 2.38 ± 0.66 | 2.50 ± 0.47 | 6.01 ± 1.25[A,B] | <0.001 |
| LAA | 15.1 ± 4.0 | 15.7 ± 1.8 | 26.3 ± 7.2[A,b] | <0.001 |
| RAA | 13.6 ± 2.4 | 11.1 ± 0.8 | 27.2 ± 8.2[A,B] | <0.001 |
| ME | 0.64 ± 0.21 | 0.64 ± 0.06 | 0.91 ± 0.34[a] | 0.007 |
| MA | 0.48 ± 0.23 | 0.60 ± 0.31 | 0.32 ± 0.27 | 0.120 |
| DT | 168 ± 24 | 195 ± 69 | 91 ± 27[A,B] | <0.001 |
| TAPSE | 23.5 ± 3.4 | 22.7 ± 5.6 | 15.6 ± 4.9[A,b] | 0.001 |

**IVSD: Inter-ventricular septal diameter in diastole. EDD: End Diastolic Diameter. ESD: End Systolic Diameter. LAA: Left Atrial Area. RAA: Right Atrial Area. ME: Early Mitral Filling. MA: Atrial Filling. DT: Deceleration Time. TAPSE: Tricuspid Annular Pulmonary Excursion. Results presented as medians ± IQR. [A] $p < 0.005$ compared to Control, [a] $p < 0.05$ compared to Control. [B] $p < 0.005$ compared to hypertensive, [b] $p < 0.05$ compared to hypertensive**

### 3.3 Hypertension and HDL composition

Control and hypertensive with/without HF patients had equivalent expression levels of ApoA1 (0.95 ± 1.45, 1.02 ± 2.56 and 1.25 ± 1.35, respectively). ApoM expression was lower in hypertension and HF patients compared to control (3.4 ± 1.6 vs 4.5 ± 1.7 AU, $p = 0.07$, Figure 32) but did not differ from the hypertensive group.

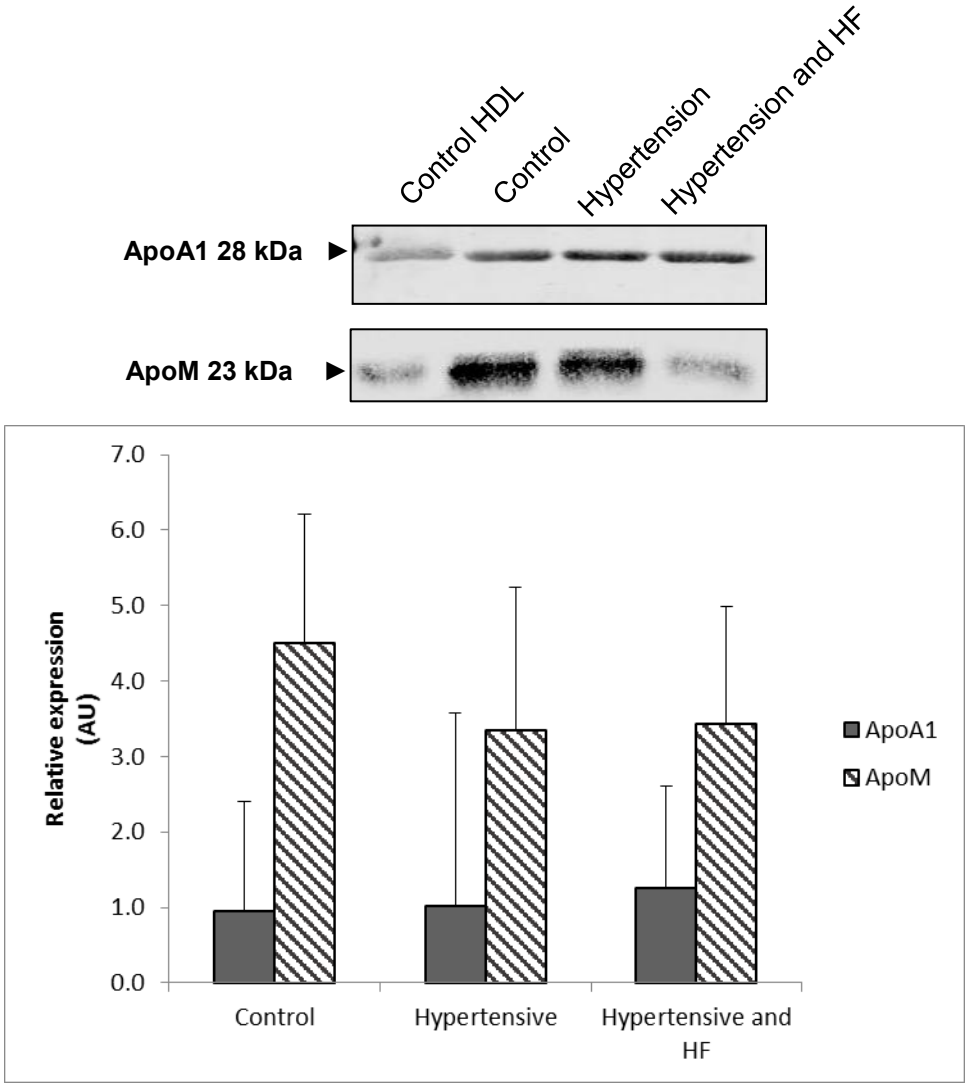



**Figure 32. Apolipoprotein A1 and M expression patient HDL. HDL from control, hypertension and hypertension and HF patients was isolated and were run on reducing 12.5% SDS-PAGE gels and transferred onto nitrocellulose membranes. Blots were probed with mouse-ApoA1 and rabbit-ApoM. Representative blots of ApoA1 and ApoM expression. Densitometry was quantified using Li-Cor® software. AU: Arbitrary units and HF: Heart failure. Results represent medians ± IQR.**

The S1P level in HDL isolated from control patients was 201 ± 73 pmol per mg of HDL, which is in a similar range to data previously reported in the literature (Brinck *et al.*, 2016). S1P concentration was lower in hypertension and HF patients compared to control (165 ± 55 vs 201 ± 73 pmol/mg, $p < 0.05$ vs control, Figure 33), but it did not differ from the hypertensive group.

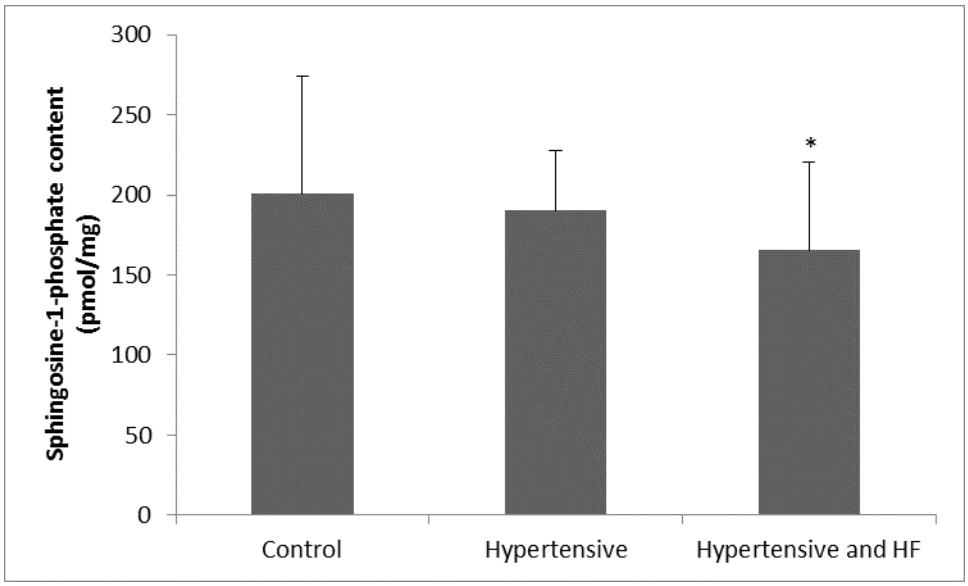



**Figure 33. S1P content of isolated patient HDL. HDL from control, hypertension and hypertension and HF patients was isolated and S1P content in isolated HDL was quantified using liquid chromatography mass spectroscopy. AU: Arbitrary units and HF: Heart failure. Results represent medians ± IQR. S1P: pmol/mg of total protein. * $p < 0.05$ compared to control.**

### 3.4 Hypertension and HDL subclass distribution

The distribution of large, intermediate and small HDL subclasses, quantified by the Lipoprint® System is presented in Figure 34. Representative profiles are shown in Figure 34A-C for the subclass distributions of control, hypertension and hypertension and HF patients. The subclass distribution profile was significantly different in hypertension and HF patients compared to control patients with lower large (48 ± 15 vs 63 ± 7%, $p < 0.005$), higher intermediate (45 ± 7 vs 34 ± 5%, $p < 0.005$) and small HDL (7 ± 9 vs 2 ± 4%, $p < 0.05$).

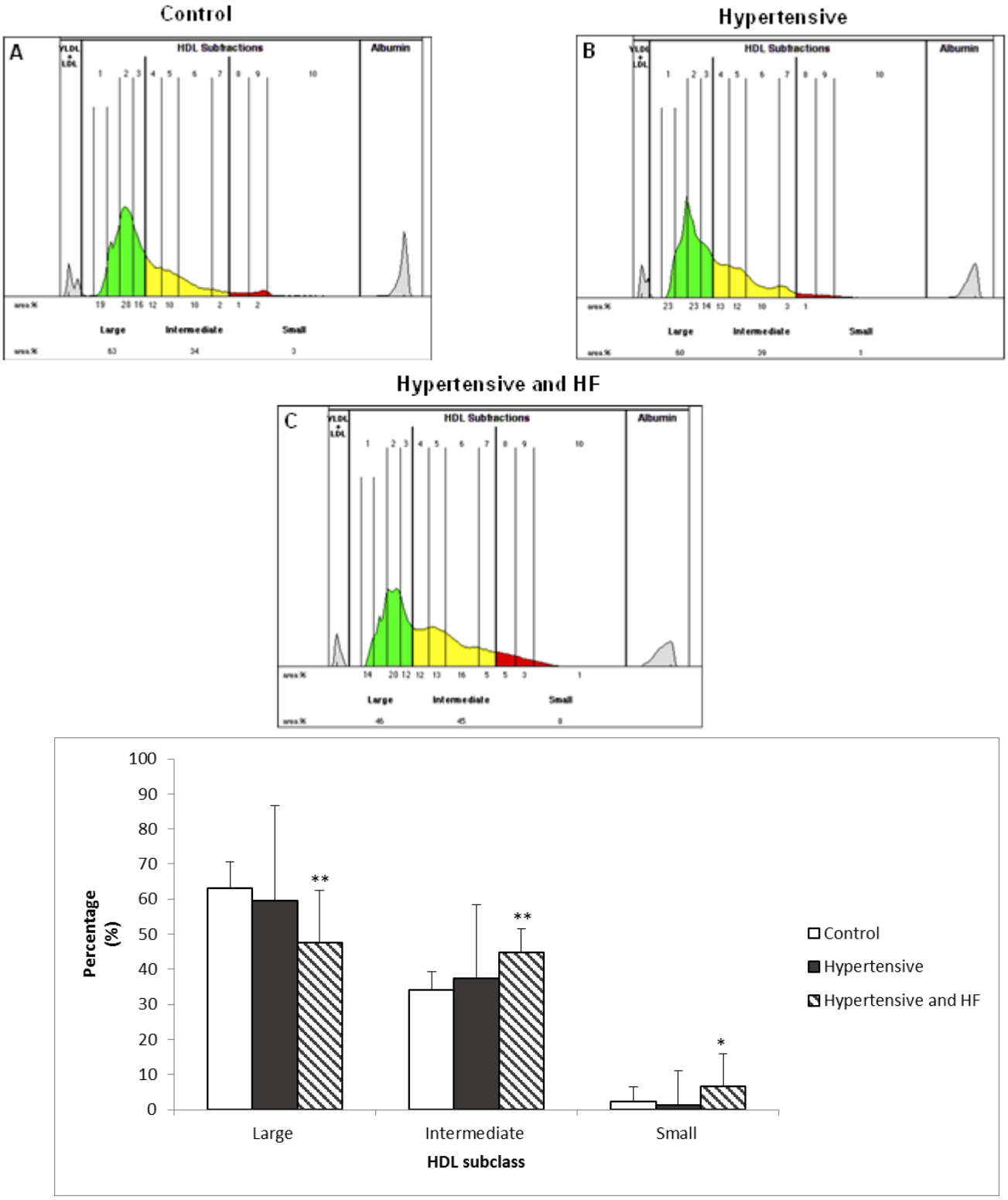



**Figure 34. Patient HDL subclass distribution. Patient sera were analysed using the Lipoprint® system and Lipoware software. Representative scan results from control, hypertensive and hypertensive and HF patients (A-C, respectively). Percentages of large, intermediate and small HDL subclasses (D). VLDL: Very low-density lipoprotein. HF: Heart failure Results represent medians ± IQR. ** $p < 0.005$ * $p < 0.05$ compared to control.**

### 3.5 Hypertension and HDL functionality

*Hypertension and HDL antioxidative function*

As a measure of the antioxidative function of HDL, the serum activity of PON, an HDL-associated antioxidative enzyme was quantified (Figure 35). PON activity in control patients was 0.38 ± 0.20 U/L, which was in a similar range to data previously reported in the literature and in Chapters four and five of this thesis (Kunutsor et al., 2016). PON activity did not differ amongst the groups (0.38 ± 0.20 vs 0.46 ± 0.33 vs 0.49 ± 0.29 U/L respectively).

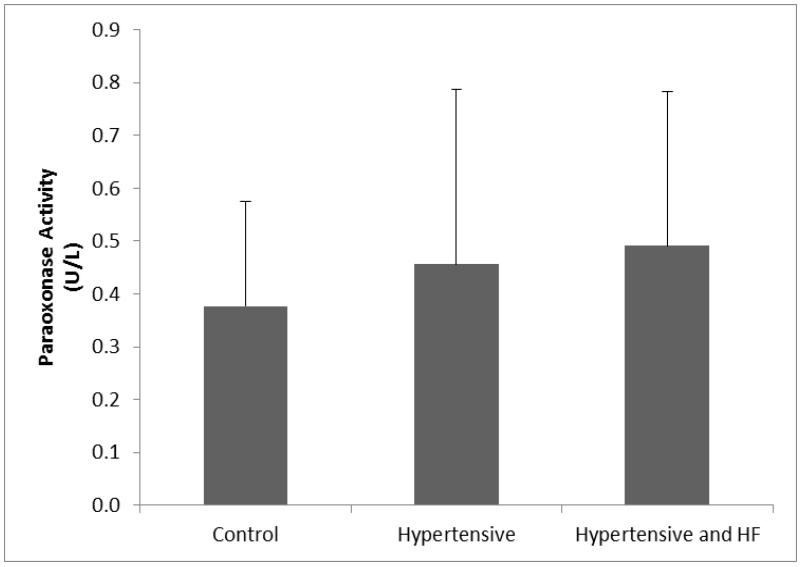



**Figure 35. Patient PON1 activity. PON1 activity was measured in patient serum. One Unit of activity is defined as 1 nmol of substrate disintegrated per minute. HF: Heart failure. Results represent medians ± IQR.**

*Hypertension and HDL anti-inflammatory function*

The anti-inflammatory function of patient HDL was assessed by quantifying relative reduction in TNF-α mediated activation of VCAM expression in HUVEC cells (Figure 36). There were, no differences between patient groups (1.2 ± 0.2 vs 1.0 ± 0.6 vs 1.0 ± 0.9 AU, respectively).

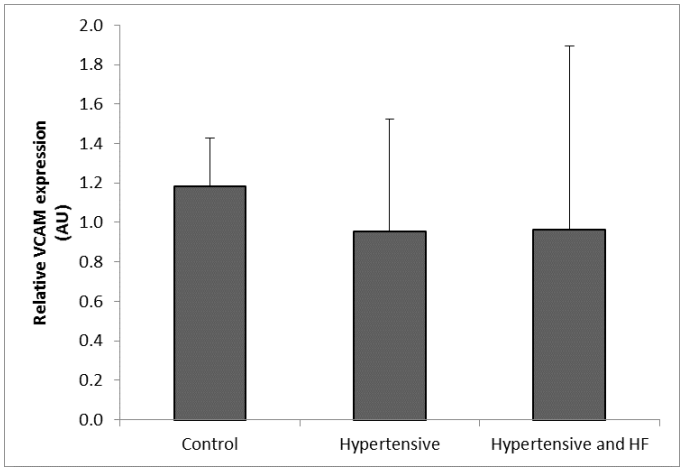



**Figure 36. Patient HDL anti-inflammatory function. Determined by Vascular Cell Adhesion Molecule (VCAM) expression relative to tumour necrosis factor (TNF) treated cells, pre-treated with isolated HDL. Human Umbillical Vein Endothelial Cells (HUVEC) were treated with isolated HDL followed by TNF-α. HDL anti-inflammatory function was calculated as VCAM expression in HUVEC cells relative to a control untreated with HDL while a non-TNF treated control was also run. HF: Heart failure AU: Arbitrary units. Results represent medians ± IQR.**

*Hypertension and activation of eNOS by HDL*

In order to assess how known impairment of normal endothelium function under hypertensive conditions may have associations with HDL, a cell culture model was used to quantify eNOS activation by HDL (Figure 37). The relative intensity of phosphorylated eNOS, relative to total eNOS was used to calculate eNOS activation. The effects of isolated patient HDL on eNOS activation was compared to activation in cells not treated with HDL. Only control patient HDL significantly increased eNOS activation compared to untreated cells (1.27 ± 0.12 vs 0.94 ± 0.08, $p < 0.05$). HDL isolated from hypertension and hypertension and HF patients failed to significantly activate eNOS under these conditions (1.16 ± 0.13 and 1.15 ± 0.14, non-significant, respectively).

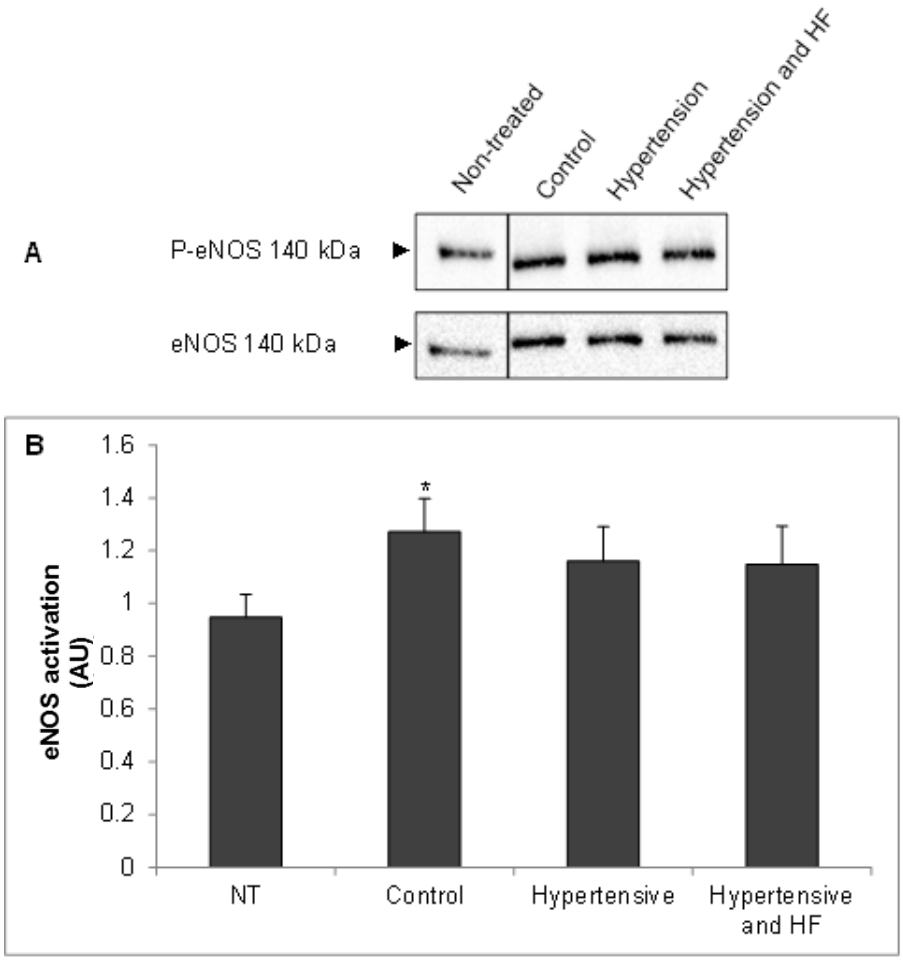



**Figure 37. Patient HDL induced activation of endothelial nitric oxide synthase (eNOS). Isolated HDL was used to treat bEND.3 cells for 10 minutes. Cell lysates were harvested and run on reducing 10% SDS-PAGE gels. Gels were transferred onto nitrocellulose membranes and were probed with rabbit anti-phosphoeNOS and reprobed with rabbit anti-total eNOS. Representative blot of eNOS activation following treatment of HDL from control, hypertensive and hypertensive and HF patients as well as a non-treated control (A). Activation of eNOS was calculated as intensity of phosphorylated bands relative to total eNOS (B). HF: Heart failure, NT: No treatment and AU: Arbitrary units. Results represent means ± SEM. * $p < 0.05$ compared to non-treated control.**

### 3.6 Relationships between measures of HDL composition, function and subclass and patient characteristics

In order to explore whether HDL composition, function and subclass may be associated with differences in patient characteristics, all patients were combined and Spearman correlation coefficients were calculated and presented in the matrix in Table 10. Greater age was associated with higher systolic and diastolic blood pressure and percentage of intermediate HDL subclasses and with lower percentages of large HDL subclasses ($p < 0.005$). Higher ApoA1 content was associated with lower relative VCAM expression (greater anti-inflammatory function) ($p < 0.05$). There was no association between measures of HDL function and subclass distribution.

**Table 10. Associations between HDL composition, functionality and subclass measures with patient characteristics**

| | Age | BMI | Systolic BP | Diastolic BP | ApoA1 expression | ApoM expression | S1P content | PON1 activity | Anti-inflammatory function | Large HDL | Intermediate HDL | Small HDL |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Age | | 0.045 | 0.810** | 0.710** | 0.058 | -0.436 | -0.427 | 0.333 | -0.018 | -0.700* | 0.727* | 0.284 |
| BMI | | | 0.284 | 0.140 | -0.196 | -0.182 | 0.145 | -0.500 | -0.364 | Null | -0.064 | 0.147 |
| Systolic BP | | | | 0.880** | -0.067 | -0.481 | -0.535 | 0.050 | -0.261 | -0.485 | 0.407 | 0.139 |
| Diastolic BP | | | | | -0.098 | -0.382 | -0.452 | -0.093 | -0.280 | -0.485 | 0.451 | 0.212 |
| ApoA1 expression | | | | | | -0.176 | 0.041 | -0.571 | -0.636* | 0.042 | -0.067 | 0.337 |
| ApoM expression | | | | | | | -0.064 | 0.027 | 0.182 | 0.354 | -0.282 | 0.037 |
| S1P content | | | | | | | | -0.023 | -0.091 | 0.263 | -0.191 | 0.064 |
| PON1 activity | | | | | | | | | 0.379 | -0.424 | 0.492 | 0.138 |
| Anti-inflammatory function | | | | | | | | | | -0.054 | 0.054 | -0.156 |
| Large HDL | | | | | | | | | | | -0.973** | -0.642* |
| Intermediate HDL | | | | | | | | | | | | 0.615* |
| Small HDL | | | | | | | | | | | | |

**Values are Spearman correlation coefficients. BMI: Body Mass Index, BP: Blood pressure, S1P: Sphingosine-1-phosphate and PON: Paraoxonase; * $p < 0.05$, ** $p < 0.005$**

# 4. DISCUSSION

The aim of the current preliminary study was to examine how hypertension and HF may be associated with a change in HDL composition, subclass and function. It was found that hypertension and HF patients had a reduced S1P content, lower distributions of large HDL subclasses and possibly displayed impaired activation of endothelial eNOS. This preliminary study therefore provides novel evidence how hypertension and HF may be associated with differences in HDL composition and function.

Hypertension risk is high in African populations (Mendez and Cowie, 2001; Onwuchekwa and Asekomeh, 2009). It is estimated that up to 42% of adult Nigerians may be hypertensive (Ulasi *et al.*, 2011; Mozaffarian *et al.*, 2015). In developing countries, the cause of HF is mainly due to hypertension, in contrast to higher income countries, whereby HF has predominantly ischemic causes (Celermajer *et al.*, 2012). Casual factors in hypertension include endothelial dysfunction and oxidative stress (Oparil *et al.*, 2003). It is therefore, surprising how relatively few studies have considered how HDL functional impairment and structural changes may be linked with hypertension.

In the current study, hypertensive and HF patients had lower S1P levels than controls. It is hypothesized that this may be attributed to a lower ApoM expression, although there were no significant associations between S1P content and ApoM expression. ApoM is an HDL protein which has a conformation that allows for specific binding to S1P and therefore HDL serves as the primary carrier of S1P. The ApoM-S1P complex mediates the vasoprotective functions of HDL *in vivo* (Christoffersen *et al.*, 2011). The S1P component of HDL has been shown to induce nitric oxide release (Nofer *et al.*, 2004); prevent ischemic injury and trigger cardioprotective signalling pathways (Theilmeier *et al.*, 2006; Frias *et al.*, 2012); reduce OxLDL related cytotoxicity in HUVEC cells (Kimura *et al.*, 2001); induce prostacyclin release via activation of cyclooxygenase-2 (Liu *et al.*, 2012) and prevent LDL oxidation (Kontush *et al.*, 2007). Reduced HDL-associated S1P in HF patients would therefore have deleterious consequences for patients. In addition, we found an association ($r = -0.535$, $p < 0.05$) between higher S1P and lower systolic blood pressure. This further suggests that S1P content may be linked with the pathophysiology of hypertension.

S1P signalling pathways have been implicated in the pathogenesis of hypertension (Meissner *et al.*, 2017). Activation of eNOS by S1P is important in maintaining endothelial structural integrity, reducing vasoconstriction and promoting pro-survival pathways (reviewed by Xing et al. 2015). In the present study, isolated HDL from both hypertensive and hypertensive and HF

patients, failed to significantly activate endothelial eNOS in a cell culture model, an effect which may be associated with a reduction in HDL-associated S1P in these patients.

The importance of eNOS in hypertension was first demonstrated in knockout mice who developed hypertension (Huang *et al.*, 1995). Literature suggests that there are associations between polymorphisms in the eNOS gene in patients with chronic heart disease and these were linked to endothelial dysfunction (Yakovleva *et al.*, 2008; Teplyakov *et al.*, 2010). Polymorphisms in the eNOS gene have since been proposed to potentially explain the high genetic risk for hypertension and HF in African Americans (Yancy, 2000; Li *et al.*, 2004). Similarly, the high risk of hypertension in African populations may be potentially explained by impaired eNOS activation, as a result of diminished S1P content as shown in this study (Mendez and Cowie, 2001; Sliwa *et al.*, 2008; Onwuchekwa and Asekomeh, 2009).

The present study found no differences in HDL anti-inflammatory function between groups. The present study was limited by low sample numbers, however, it was shown that 43% of all diseased patients displayed pro-inflammatory HDL. Increasing the population numbers may improve and validate this finding further. Additionally, it was found that greater expression of ApoA1 was associated with improved anti-inflammatory function. This suggests that changes in HDL anti-inflammatory function may be mediated by changes in ApoA1 expression. This was similarly shown in pro-inflammatory HDL, which was characterised by a decrease in ApoA1 content (Van Lenten *et al.*, 1995; Cabana *et al.*, 1996; Lewis *et al.*, 2004). Failure to show differences in ApoA1 content between groups may further explain why no between-group differences in HDL anti-inflammatory function were observed.

Interestingly, the present study found no difference in PON1 activity between groups. In contrast, patients with enhanced morning surge of high blood pressure had lower PON1 activity (Kaypaklı *et al.*, 2016). Similarly, increased oxidative stress was associated with decreased PON1 activity in white coat hypertensive patients (Kishimoto *et al.*, 2004). Patients with essential hypertension displayed polymorphism in the PON1 gene (Turgut Cosan *et al.*, 2016). However, PON1 polymorphisms, such as the promotor region SNP L-55M, causes a reduction in serum PON1 activity and are much less frequent in oriental and black populations (Phuntuwate *et al.*, 2005; Mackness and Mackness, 2015). Additionally, in a population of black and white African women, black women displayed higher PON1 activity when compared to white women, (Chapter four) (Woudberg et al. 2016). It is therefore suggested that these findings may explain why diseased patients maintained healthy levels of PON1 activity. Future studies examining genetic aberrations in the PON1 gene would, however, need to be conducted.

Pertinent to understanding HDL in relation to disease, is the consideration of HDL subclasses. HDL subclass distribution shifts are associated with obesity (Chapter four) (James *et al.*, 1997; Magkos *et al.*, 2012) and most recently, with hypertension (Paynter *et al.*, 2011; Zhang *et al.*, 2015). In this latter study, hypertensive status was associated, in an aged population, with lower percentages of large and higher percentages of small HDL subclasses (Zhang *et al.*, 2015). A similar shift in HDL subclass distribution was similarly shown in patients with coronary heart disease (Oravec *et al.*, 2011). The current study is the first to examine how HDL subclass distribution may differ in patients with hypertensive HF compared to healthy patients. Similar to the studies mentioned above, our data show the same shift in HDL subclasses in hypertensive and HF patients compared to controls.

A major limitation of the study design was that the control patients were significantly younger than both disease patient groups. Indeed, age was associated with increased systolic and diastolic blood pressure and with decreased large HDL subclasses. This latter observation makes it difficult to determine whether the differences in HDL subclass distribution between HF patients and control are related to disease status or age. Indeed, HDL subclass size reduction and changes in subclass distribution are linked to aging (Dobiasova *et al.*, 1992; Tian *et al.*, 2010; Rajalahti *et al.*, 2016). Another major limitation to our study was the low sample numbers which limits the conclusions that may be drawn. However, as a preliminary study, it is the first study to examine differences in HDL composition, function and subclass in an African hypertensive sample population.

## 5. CONCLUSION

There is a high prevalence and risk of hypertension and hypertensive HF in Africa. Pathophysiological consequences of hypertension may be associated with impaired HDL function. In spite of no differences in HDL-C, hypertensive and HF patients displayed lower S1P content in HDL compared to control patients. In addition, all hypertensive patients HDL displayed impaired activation of endothelial eNOS. Hypertensive and HF patients displayed a shift in HDL subclass distribution, with lower large and greater intermediate and small HDL subclasses compared to controls. This study therefore provides novel evidence that hypertension and HF may have consequences for HDL structure, function and subclass distribution. Additional studies will be required to better understand the exact role of the modification of HDL in the pathophysiology of hypertension and HF.

# CHAPTER SEVEN: CONCLUSIONS AND PERSPECTIVES

## CHAPTER SEVEN: CONCLUSIONS AND PERSPECTIVES

### 1. SUMMARY OF FINDINGS

The primary aim of the PhD thesis was to examine how different CVD risk factors may be associated with changes in HDL quality instead of HDL-C quantity. Modern research into lipidology is beginning to suggest a more pronounced focus on HDL functionality, composition and subclasses (HDL quality) (Egom *et al.*, 2013; Santos-Gallego, 2015). To better address this, the overall study was subdivided to focus on three sample populations, each with defined CVD risk factors.

1. A sample population of normal-weight and obese black and white South African women to explore whether ethnicity and obesity were associated with changes in HDL function and subclass distribution (Chapter four).

2. A sample population of black obese women, half of which will underwent an exercise intervention to explore whether exercise alters HDL function and subclass distribution (Chapter five).

3. A sample population of hypertensive population of Nigerian patients with or without HF along with healthy controls to explore how hypertension and HF were associated with changes in HDL composition, subclass distribution and function (Chapter six).

Each sample population revealed different aspects of the association between CVD risk factors and HDL. The common finding among all three was that CVD risk was not associated with HDL-C concentrations. In the first sample population, black South African women had lower HDL-C levels than their white counterparts. This however, did not translate into any impairment in HDL function. To our knowledge, we demonstrated for the first time in an African sample population, that any potential reduction in risk for ischemic heart disease in black Africans may be attributed to greater HDL function (Rossouw, 1983; Akinboboye *et al.*, 2003; Mayosi, 2007; Sliwa *et al.*, 2008; Agyemang *et al.*, 2009; Woudberg *et al.*, 2016). This was evident in higher PON1 activity (Figure 38). The second major finding from this sample population was an inverse association between, large HDL subclasses and a higher BMI. This was commensurate with findings in European obese populations (James *et al.*, 1997; Magkos *et al.*, 2008).

Following on from this first sample population, it was explored how an exercise training intervention, in obese black South African women, may revert HDL function and subclass to a “non-obese” state. HDL-C was not altered by the 12-week exercise training intervention, however, there were associations between exercise training and HDL function and subclass.

These associations included inverse associations between RCT and large HDL with lower BMI while exercise training was linked to a decrease in small HDL subclasses. This aptly follows on from the previous study indicating that exercise training alters HDL subclass distribution, via weight loss, however this weight loss was minimal following the exercise intervention (Figure 38). Critically, we showed for the first time, a significant association between HDL functionality and HDL subclass distribution, measured by the Lipoprint® System. The Lipoprint® System was established 16 years ago and has never been applied to an African sample population. HDL subclasses have their own defined functions, therefore links between traditional measures of HDL function and a relatively new method quantifying HDL subclass, are critical to the future of HDL research (Camont *et al.*, 2013).

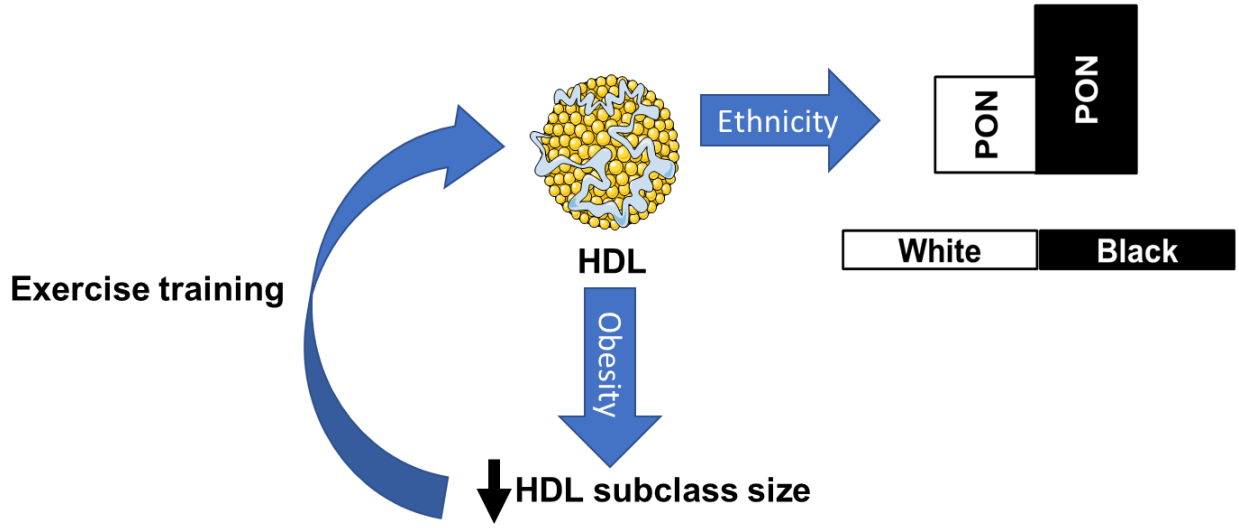



**Figure 38. Summary of major findings from the first (Chapter four) and second (Chapter five) sample populations. Obesity was associated with lower HDL subclass size, whilst an exercise training intervention reverts HDL subclass distribution to a non-obese state. Ethnicity was associated with a difference in HDL function wherein black South African women had greater PON1 activity than their white counterparts.**

In the final sample population, we expanded our focus to include measures of HDL composition, quantifying the expression of ApoA1, ApoM and S1P. The sample population comprised healthy controls, hypertensive patients and hypertensive and HF patients. Similarly to previous sample populations, there were no differences in HDL-C between groups and no associations between HDL-C and the risk of CVD. Hypertensive HF patients, in particular, demonstrated diminished HDL function and modifications in HDL composition. Patients had a reduction in ApoM and S1P content compared to controls, which we hypothesize, could contribute to functional impairment in endothelial eNOS activation. Similar to our findings in obesity, hypertension as a CVD risk factor was again associated with a lower percentage of large HDL subclasses (Figure 39) (Zhang *et al.*, 2015; Woudberg *et al.*, 2016).

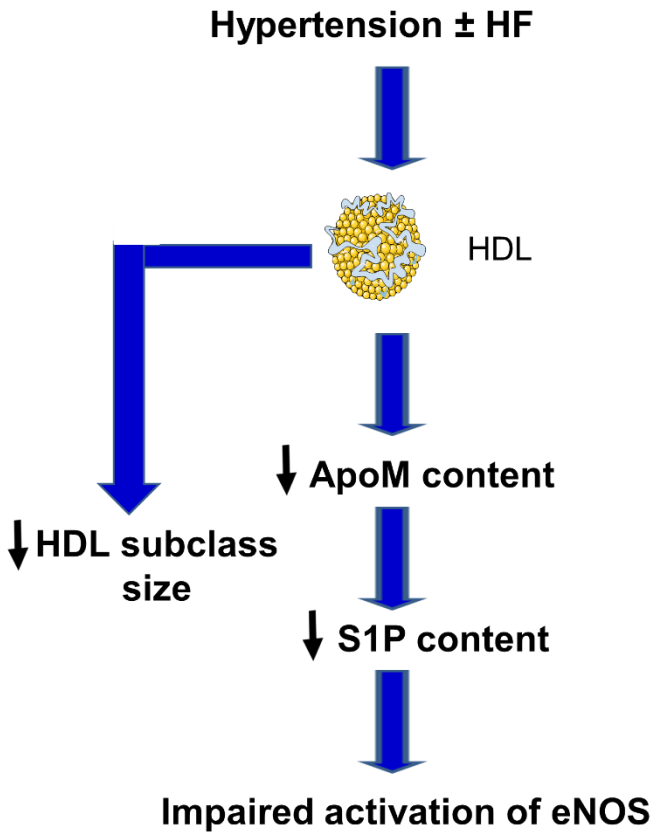



**Figure 39. Summary of major findings from the third sample population (Chapter six). Hypertension and hypertension with HF patients have lower ApoM expression, compared to healthy controls. This possibly leads to reduced S1P content, which impairs HDL-induced activation of eNOS. In addition, hypertension and HF is associated with lower HDL subclass size compared to healthy controls.**

All three of the studies therefore underline the relevance of focussing on HDL quality over quantity. In all sample populations, patients at differing levels of CVD risk showed similar HDL-C levels, however, the functionality of patient HDL and the distribution of subclasses were different.

## 2.LIMITATIONS

### *Sample numbers*

The main limitation linked to all three studies were the numbers of patients and participants included in each sample population. These opportunistic studies were not specifically powered for the outcomes described in the thesis. We acknowledge that this limits the conclusions and interpretation of the findings. Particularly in the Nigerian sample population, missing echocardiographic data greatly limited the depth and significance of our findings. In this case, echocardiographic data validated the model of hypertensive HF but could not be associated with HDL subclass and function as had been originally planned.

Whilst sample numbers were low, making the studies largely "preliminary" in nature, the relevance becomes important when considering the novelty of the studies. In all sample populations, HDL subclass and function were considered concurrently as potential biomarkers of CVD risk which contrasts to recent literature, in which markers of HDL function and HDL subclass were considered separately (Khera *et al.*, 2011; Tang *et al.*, 2012; Rohatgi, *et al.*, 2014; Tian *et al.*, 2014; Martin *et al.*, 2015). Considering both markers together may be particularly relevant in an African setting, due to the increasing prevalence of myocardial infarction and ischemic heart disease, likely linked to the epidemiological transition (Kakou-Guikahue *et al.*, 2016; Traina *et al.*, 2017). Finding novel biomarkers of CVD risk in such a population is critical.

In addition, data from this thesis provides a foundation for future studies. In the first part of the thesis, a novel method of HDL isolation of pure HDL samples from low serum volumes was established (Chapter three). In the same way, HDL subclass analysis and new measures of HDL functionality measurement have been established in robust and repeatable experimental models. These techniques can therefore be applied to a number of different sample populations considering HDL quality in relation to CVD. Across the thesis the number of analytical tests progressively increased. This allowed for a deeper analysis of HDL functionality.

### *Study design*

As stated previously, the studies included in the thesis were opportunistic studies, therefore, whilst there was input into the HDL analysis strategy, overall design of the larger studies was not possible. For the first two population studies, young black and white South African women were selected for inclusion. Women were only selected in the original study to focus specifically on insulin resistance in women, reducing gender-related variability. It is

acknowledged that background, social status, education, lifestyle that had not been taken into account and that may contribute to differences observed between ethnicities. In the first sample population, associations between HDL subclass and obesity were described, however, this trend was largely driven by findings in white women only. Further studies should be undertaken to understand why these effects were not reflected in the black women. Therefore, ideally, as a follow-up regarding an exercise training intervention in obese women, both black and white obese women should have been included. The project whose samples were used in the thesis had already been established and focused more on black South African women and their risk of type 2 diabetes due to increased insulin resistance (Jennings *et al.*, 2008; Knight *et al.*, 2011). Despite this flaw in the cohesiveness of the design, we still demonstrated changes in HDL subclass in black African women in response to an exercise training intervention. These responses were indeed, mediated by small, but significant, changes in BMI.

In the Nigerian sample population, control samples were not age matched to their diseased counterparts. Whilst the prevalence of HF is increased in aged populations, it is important to ensure populations are age matched for statistically relevant comparisons (Bleumink *et al.*, 2004). To test age as a confounder, we performed Spearman correlation matrices. Percentages of large HDL subclasses correlated negatively with age suggesting that the difference between groups was driven by an older age in the hypertensive HF patients.

The results of this thesis were based on the findings from cross-sectional and intervention studies. It is therefore difficult to state whether HDL function or HDL subclass distribution may be a useful marker to assess possible risk of heart disease. The studies did not examine the Framingham risk score but rather focussed on specific risk factors of heart disease and how these may be causally related to differences in HDL quality. The observations are therefore descriptive and not predictive. As such, we cannot accurately state which measure of HDL function or indeed whether specific HDL subclasses correlate to an increase or a decrease of CVD risk per se.

*Methodology*

The activities of PON1 and PAF-AH enzymes were quantified in serum only. Both are associated *in vivo* with HDL, with PON1 in particular, almost completely HDL bound (James and Deakin, 2004). However, attributing activities of specific enzymes as strict HDL “functions” is challenging. To address this, we performed western blotting in both isolated HDL and in serum. This allowed us to attribute activities of PON1 and PAF-AH as HDL-specific or not HDL-specific.

The prevalence of polymorphisms in the PON1 gene, particularly those which are ethnicity-specific, make a genetic aspect of the research potentially interesting. Future work regarding PON1 activity and expression would be improved by examining genetic aberrations in the expression of the PON1 gene in a large cohort of patients.

Our studies utilized the Lipoprint® System for the first time in African sample populations. As a caveat, it is important to consider that nomenclature for HDL subclass depends on the methods used for quantification and separation (Asztalos et al. 2011). It is therefore worth considering that this study therefore designates HDL subclasses as large, intermediate and small. Much of the existing literature describes two principal HDL subclasses, HDL2 and HDL3. It is well established that HDL2 is the larger of the HDL subclasses and HDL3 is the smaller (Williams *et al.*, 1992; Rosenson *et al.*, 2011). To better demonstrate these differences in traditional methods of HDL subclass isolation, and the Lipoprint® System, pure HDL2 and HDL3, generously donated by Dr Miguel Frias, were separated on the Lipoprint® System and distributions of large, intermediate and small subclasses quantified (Figure 40).

HDL2 is largely represented by large HDL but HDL3 is not clearly defined. It was expected that HDL3 would be represented by intermediate or small HDL subclasses. For this reason, Lipoprint® data can only be compared with similar data as the system does not strictly represent HDL3 by intermediate or small HDL subclasses. This was a potential limitation of the study, however, the ease of use of the system, approval for the system's use in clinical diagnosis in the United States and increased interest from private clinical laboratories in South Africa will further increase application in the broader scientific community. Therefore, it will become easier to compare findings from the Lipoprint® System with others in the literature.

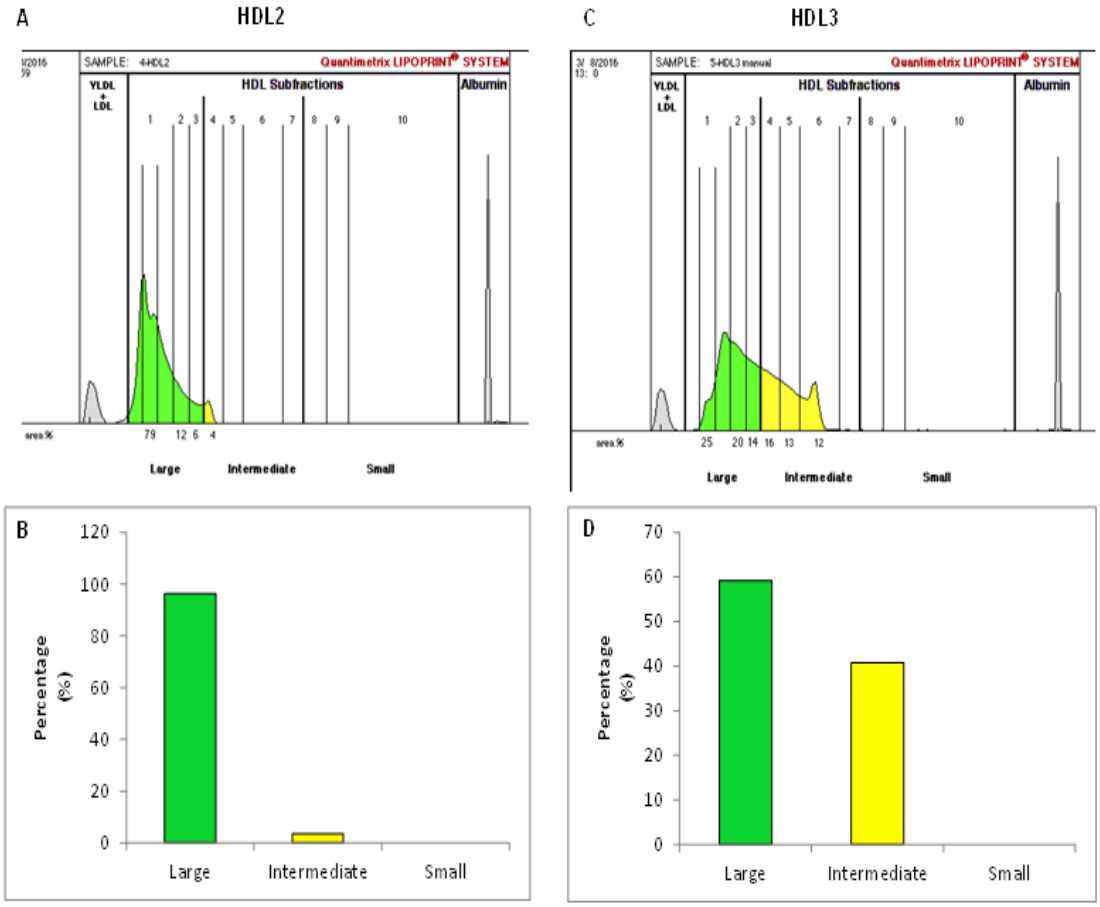



**Figure 40. Distribution of HDL subclasses of pure HDL2 and HDL3 analysed in the Lipoprint® System. Data was analysed using Lipoware software. Representative scan results of HDL2 (A) and HDL3 (C). Percentages of large, intermediate and small HDL subclasses designated by the Lipoprint® System for HDL2 (B) and HDL3 (D). VLDL: Very low-density lipoprotein.**

## 3. HDL QUALITY AS A USEFUL BIOMARKER: FUTURE RESEARCH

Our data in this thesis suggest how HDL quality may more closely relate to CVD risk factors than HDL quantity. Discussed by Santos-Gallego, HDL-C concentration does not consistently reflect the concentration of the HDL molecule, which can be fully or partially loaded with cholesterol (Santos-Gallego, 2015). Only 5% of total HDL-C is derived from macrophage cholesterol efflux, demonstrating how HDL-C is a poor indicator of RCT function (Santos-Gallego, 2015). HDL-C also does not represent other important HDL functions, including vasodilatory or anti-inflammatory functions (Santos-Gallego, 2015). Therefore, it is worth considering how HDL quality may translate into a useful biomarker.

*Reverse cholesterol efflux*

Of the multiple HDL functions, RCT has been the most extensively studied as a potential biomarker (Khera *et al.*, 2011; Santos-Gallego *et al.*, 2011; Rohatgi, *et al.*, 2014). RCT successfully predicted severity of coronary artery disease events while HDL-C failed to do so (Khera *et al.*, 2011). Analysis from the JUPITER trial recently indicated that RCT was inversely associated with incident of cardiovascular events in patients on potent statin therapy (Khera *et al.*, 2017). RCT has therefore shown potential as a viable biomarker in high profile studies,. However, similarly to the assessment of HDL anti-inflammatory function, RCT relies on cell-culture based models. Robustness and standardization of these techniques are very limited. Results will be affected by multiple variables including cell lines, cell passages, culture conditions and reagent use. Cell-culture is also not cost effective and is not suitable for a clinical diagnosis setting, due to time constraints linked to the method. In addition, RCT measurements traditionally rely on the use of radioactive materials. New regulations will restrict the usage of these materials in clinical laboratories. However, new techniques using alternative fluorescent labels of cholesterol have been optimized (Sankaranarayanan *et al.*, 2011).

*HDL S1P content*

A second option is consideration of HDL S1P content, which has been suggested as a useful biomarker, reviewed by (Egom *et al.*, 2013). MI and coronary artery disease patients had a lower S1P-HDL concentration, which could be raised using *in vitro* S1P loading (Sattler *et al.*, 2010, 2015). S1P uptake deficiency of HDL in coronary artery disease patients presented as a potential marker of HDL dysfunction (Sattler *et al.*, 2010). This observation was extended to patients with coronary in stent restenosis (Jing *et al.*, 2015) and in type 2 diabetic patients (Brinck *et al.*, 2016). S1P is inversely correlated with HbA1c in type 2 diabetic patients, and the concentration of S1P is correlated with cardiac specific anti-apoptotic capacity (Brinck *et*

*al.*, 2016). S1P levels in HDL serum fractions successfully discriminated subjects with ischemic heart disease from those without (Argraves *et al.*, 2011).

To measure S1P accurately in a diagnostic setting, clinical laboratories would need to use liquid chromatography mass spectroscopy. Mass spectroscopy as a diagnostic tool has potential, however, use is limited due to a substantial initial investment estimated as much as 400 000 Euros in 2012 (Van den Ouweland and Kema, 2012). Additional costs include routine maintenance, training of specialized staff and a constant supply of liquid nitrogen (Van den Ouweland and Kema, 2012). Method standardization, availability of commercial certified reference materials and establishing concordance among methods should allow for increased application of mass spectroscopy as routine tests in clinical laboratories, notwithstanding the aforementioned cost concerns (Kushnir *et al.*, 2011).

*PON1 activity*

PON1 activity measurement is a third potential option as an HDL-specific biomarker. Low serum PON1 activity was associated with increased risk for Major Adverse Cardiovascular Events (MACE) and coronary artery disease (Granér *et al.*, 2006; Tang *et al.*, 2012). The predictive power of PON1 activity was significant in detecting the development of vascular complications in type 2 diabetic patients (Mogarekar *et al.*, 2016). Results of a systematic review and meta-analysis, showed that polymorphisms in the PON1 gene were significantly associated with heart diseases, including MI and coronary artery disease (Hernández-Díaz *et al.*, 2016). Using patients from the Prevention of Renal and Vascular End-Stage Disease (PREVEND) study, a log-linear relationship between PON1 activity and CVD risk was demonstrated (Kunutsor *et al.*, 2016). Incorporation of PON1 activity as a diagnostic test is simple, reliable, repeatable, fast, readily automated and inexpensive (Ferre *et al.*, 2002).The only perceived draw-back is the toxicity of the paraoxon-ethyl substrate.

*HDL subclass distribution using the Lipoprint® System*

Finally, the Lipoprint® System, utilized for the first time in an African setting, is a relatively new technique for rapid (11 samples/ 2 hours) measurement of HDL and LDL subclass in serum samples (Hoefner *et al.*, 2001). The ease of use of this method and a relatively low initial investment of $25 000 make this technique a particularly attractive tool for clinical purpose. The use of this equipment does not require extensive training and samples will not require any pre-treatment prior to analysis. HDL and LDL subclass analysis has been applied to patient populations with CVD and suggested as a potential biomarker in patients with CVD risk factors (Lofgren *et al.*, 2004; Nakou *et al.*, 2008; Goedecke *et al.*, 2010; Oravec *et al.*, 2011, 2014; Makariou *et al.*, 2012; Filippatos *et al.*, 2013; Woudberg *et al.*, 2016). The LDL subclass test

has been approved by the Food and Drug Administration (FDA) for use as a diagnostic test in the United States. Further studies with large number of patients at risk of CVD may lead to approval of the HDL subclass test as more data becomes available.

Diagnosis of HF is routinely done using echocardiography. Unfortunately, echocardiography is not easily accessible in low-income countries (Ogah *et al.*, 2006). In addition, specialised training as well as the variability in data interpretation subject to the operator with echocardiography analysis highlights the need to establish simple novel biomarkers which can distinguish HF patients from those with hypertension (Ojji *et al.*, 2013). HDL subclass may therefore be attractive biomarkers in this setting. In the third sample population, our preliminary study suggested that it may be possible, with greater patient numbers, that a decrease in large HDL subclasses may distinguish hypertensive patients and hypertensive HF patients.

# 4. FINAL CONCLUSIONS

The thesis has conclusively demonstrated how HDL quality and not HDL-C concentrations differ in individuals at risk of CVD. CVD risk factors such as obesity and hypertension were associated with less large HDL subclasses combined with greater intermediate and small HDL subclasses. Exercise training, as an intervention for obese individuals, reverted HDL subclass distribution to a “non-obese” state. Markers of HDL function were associated with ethnicity, were modified by exercise training and were impaired in patients at high risk of CVD. Future research considering CVD risk factors can therefore consider HDL quality as a potential biomarker of CVD risk. Literature data suggests that PON1 activity and HDL subclass distribution be used as potential biomarkers for CVD risk due to the low cost, reproducibility and ease of use. In order for this to be fully realised, longitudinal studies on large patient cohorts will need to be carried out to confirm the predictive power of these potential biomarkers. Similar to the studies included in this thesis, studying both measures of HDL function and subclass concurrently will be beneficial and important for assessing CVD risk, particular in African populations.

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