Investigating high-density lipoprotein (HDL) subfractions, composition and functionality in people living with HIV

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List of abbreviations

ABC	ATP binding cassette	HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ACAT	Acyl-CoA cholesterol acyltransferase	HIV	Human immunodeficiency virus
Apo	Apolipoprotein	HMG-CoA	3-hydroxy-3-methyl-glutaryl-coenzyme A
ART	Antiretroviral therapy	hsCRP	High-sensitivity C-reactive protein
AZT	Azidothymidine	ICAM	Intercellular adhesion molecule
BMI	Body mass index	IDL	Intermediate-density lipoprotein
BSA	Bovine serum albumin	IQR	Interquartile range
cAMP	Cyclic adenosine monophosphate	LCAT	Lecithin cholesterol acyltransferase
CETP	Cholesterol ester transfer protein	LDL	Low-density lipoprotein
CIMT	Carotid intima-media thickness	LDL-C	Low-density lipoprotein cholesterol
CKD	Chronic kidney disease	LC-MS/MS	Liquid chromatography tandem mass spectrometry system
CPM	Counts per minute	Lp-PLA_2	Lipoprotein-associated phospholipase A2
CRP	C-reactive protein	MEM	Minimum essential eagle
CVD	Cardiovascular disease	MiR	MicroRNA
DAD	Data Collection on Adverse Events of Anti-HIV Drugs	NCD	Non-communicable disease
DM	Diabetes Mellitus	NHLS	National Health Laboratory Service
DTNB	5,5'-dithio-bis-(2-nitrobenzioic acid)	NF-κB	Nuclear factor of kappa-light-chain-enhancer of activated B cells
eNOS	Endothelial nitric oxide synthase	NMR	Nuclear magnetic resonance
FMD	Flow-mediated dilation	NNRTI	Non-nucleoside-reverse transcriptase inhibitor
GGE	Gradient gel electrophoresis	NO	Nitric oxide
GGT	Gamma-glutamyl transferase	NRTI	Dideoxynucleoside reverse transcriptase inhibitor
Hb	Haemoglobin	ORAC	Oxygen radical absorbance capacity
HDL	High-density lipoprotein	oxLDL	Oxidised LDL
HDL-C	High-density lipoprotein cholesterol	PAF	Platelet-activating factor

PAF-AH	Platelet-activating factor-acetylhydrolase
PBS	Phosphate-buffed saline
PI-3	Phosphatidyl-inositol-3
PI	Protease Inhibitor
PLTP	Phospholipid transfer protein
PLWH	People living with HIV
PON	Paraoxonase
PURE	Prospective Urban and Rural Epidemiology
RCT	Reverse cholesterol transport
REPRIEVE	Randomized Trial to Prevent Vascular Events in HIV
Rf	Retention factor
S1P	Sphingosine-1-phosphate
sdLDL	Small-dense LDL
SEM	Standard error of mean
SMART	Strategies for Management of Anti-Retroviral Study
SSA	Sub-Saharan Africa
SR-B1	Scavenger receptor type 1 receptors
TNF-α	Tumour necrosis factor-alpha
TTBS	Tween in tris-buffered saline
VCAM	Vascular cell adhesion molecule
VLDL	Very-low-density lipoprotein

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Abstract

Background: Although antiretroviral therapy (ART) increases survival in individuals living with human immunodeficiency virus (HIV), this population faces an increased risk for cardiovascular disease (CVD). There is mounting evidence that the distribution, composition, and functionality of high-density lipoprotein (HDL) subfractions are altered in the presence of cardiovascular risk factors. We aimed to explore whether HIV and/or ART modulate HDL subfractions and functionality in a population of people living with HIV (PLWH).

Methods: Fifty healthy HIV-negative control patients (HIV free control), 44 HIV-infected patients yet to receive any ART treatment (HIV ART-naïve) and 50 HIV-infected patients receiving ART (ART-treated) were included (South African cohort). HDL functionality was assessed by measuring reverse cholesterol efflux capacity, anti-oxidative activity (paraoxonase-1 (PON-1) activity) and anti-thrombotic activity (platelet-activating factor acetylhydrolase (PAF-AH) activity). HDL subfractions were measured using the Lipoprint® system.

Results: HIV ART-naïve patients had lower HDL cholesterol than HIV-negative or ART-treated patients (1.05 ± 0.46 vs 1.33 ± 0.39 vs 1.31 ± 0.74 mmol/L, respectively, $p < 0.05$). The percentage of the largest subfraction of HDL (HDL-1) was higher in HIV ART-naïve patients compared to HIV-negative patients (12.46 ± 6.33 vs 9.43 ± 4.41%, $p< 0.05$). The HIV ART-naïve patients also displayed a change in HDL composition, with decreased levels of apolipoprotein A-I compared to HIV ART-treated patients and HIV-negative patients (38.5 ± 7.5 vs 43.8 ± 13.4 vs 45.5 ± 8.1 μmol/L, respectively, $p < 0.05$). Large HDL was inversely correlated with CD4+ count (r = -0.279, $p < 0.01$) and small HDL was positively correlated with CD4+ count (r = 0.333, $p < 0.01$). Although HDL functionality was not different between groups, PON-1 activity positively correlated with small HDL (r=0.19, $p<0.05$).

Conclusion: Our study suggests that HIV infection is associated with a change in HDL composition and a shift in HDL subfraction distribution, favouring a higher percentage of large HDL subfractions, which may contribute to the increased risk of CVD in HIV patients. More in-depth studies should be conducted to better understand the exact role of HIV and/or ART on the modification of HDL.

CHAPTER ONE: INTRODUCTION

1.1 Cardiovascular disease (CVD)

Non-communicable diseases (NCDs) are diseases that are not transmissible from one person to another. The four major NCDs are cardiovascular disease (CVD), diabetes, cancer, and lung disease. CVD is a general term for conditions affecting the heart and blood vessels and is the primary cause of death globally, responsible for 17.9 million deaths every year with the majority (>80%) of deaths due to CVD occurring in low- and middle-income countries (World Health Organization, 2019a). In sub-Saharan Africa (SSA), although communicable diseases remain the leading cause of death, the prevalence of CVD continues to increase (Mayosi et al., 2009; Hyle et al., 2017; Hamid, Groot & Pavlova, 2019; Yuyun et al., 2020). In South Africa, CVD accounts for the largest percentage of deaths among the NCDs (figure 1) and these numbers are increasing year by year. (Yuyun et al., 2020).

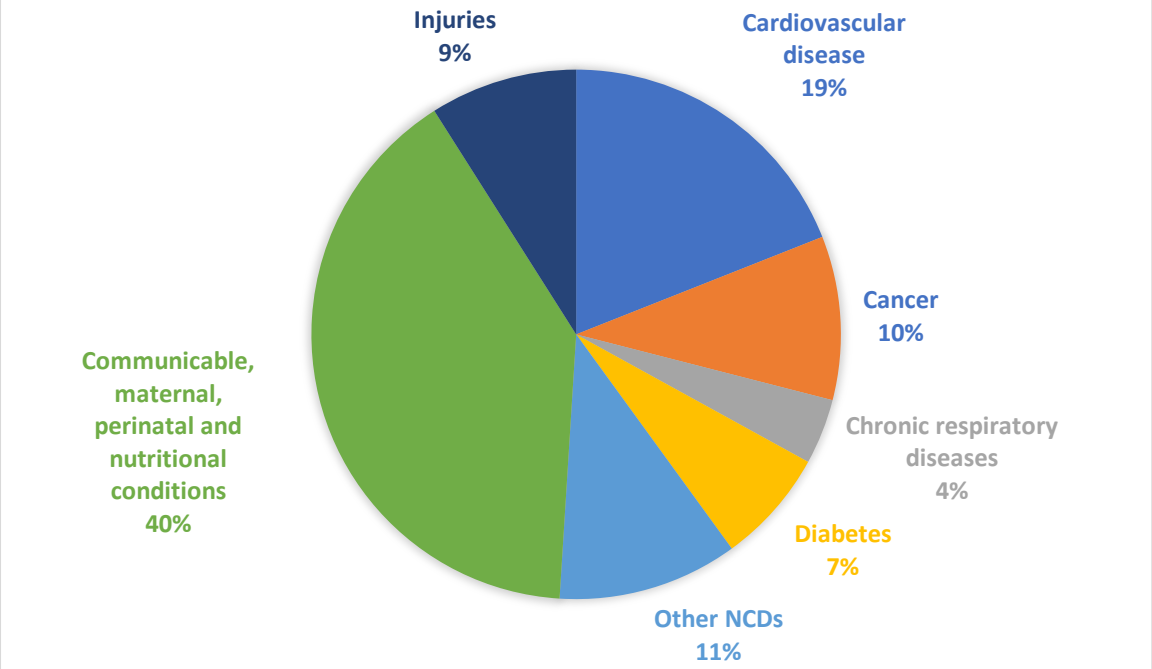


Figure 1. NCDs are estimated to account for 51% of all deaths in South Africa with CVD accounting for the largest percentage among them. Adapted from (WHO, 2019).

The growing burden of preventable CVD in Africa can, at least in part, be attributed to globalization, rapid urbanization and population growth over the last 30 years (Mensah et al., 2015). There has been a well-observed increase in the burden of classical risk factors for CVD such as obesity, hypertension and diabetes mellitus (DM) type 2 (Bentham et al., 2017; Zhou et al., 2017; International Diabetes Foundation, 2019). The prevalence of obesity in Africa has approximately doubled from 1980 to 2015, from 6.2% to 12.7% (Chooi, Ding & Magkos,

2019). It is estimated that the global prevalence of hypertension is highest in Africa compared to the rest of the world, with nearly half of those aged 25 or above being hypertensive (World Health Day, 2013; Ataklte et al., 2015; World Health Organization, 2017). Similarly, the prevalence of DM in adults is approximately 12.7% in Africa (International Diabetes Foundation, 2020).

Throughout the countries in this region, there is a growing body of evidence that the incidence of CVD is also increasing due to the contraction and management of communicable diseases such as the human immunodeficiency virus (HIV) and tuberculosis (Sliwa & Ntusi, 2019).

1.2 Human immunodeficiency virus (HIV)

HIV is a virus that targets the host immune system, specifically attacking and destroying CD4 cells, thus increasing the patient's susceptibility to other diseases (see review, (Moir, Chun & Fauci, 2011)). Over 37.7 million people are living with HIV worldwide, with approximately two-thirds in SSA (UNAIDS, 2021). South Africa, specifically, has at least 7 million people living with HIV (PLWH) (World Health Organization, 2019b). There is still no cure or an effective vaccine against the disease, however, there have been major advances in HIV management. Indeed, antiretroviral therapy (ART) has altered the course of the epidemic, making the once-fatal disease, a chronic and manageable condition (Deeks, Lewin & Havlir, 2013). ART suppresses viral replication within the body, allowing CD4 recovery and the return to normal immune function (Simon, Ho & Abdool Karim, 2006). PLWH with access to ART can now expect an improved, near-normal life expectancy (Remais et al., 2013). However, this longevity can result in clinical challenges for these patients, including an increased risk for CVD (Friis-Møller et al., 2003; Freiberg et al., 2013; Smit et al., 2017; Bekker et al., 2018; Vachiat et al., 2019).

1.3 Demographics of HIV-related CVD

Research in high-income countries has shown that the incidence of CVD is higher in the population of PLWH compared to the HIV-negative population and both HIV and ART are risk factors for the development of CVD (Ballocca et al., 2016; Hyle et al., 2017). An American study reported that infection with HIV is associated with a 50% increased risk of acute myocardial infarction (Freiberg et al., 2013). Unfortunately, data on clinical CVD amongst PLWH in SSA are limited and it is unclear if the results seen in high-income countries can

directly be translated into the African context, especially since there are distinctive demographic, and socio-economic differences between the PLWH of North America, Europe and SSA. Most of the HIV-positive population in high-income countries consists of white men who have sex with men, sex workers and intravenous drug users (figure 2) (UNAIDS, 2017). Furthermore, PLWH in high-income countries typically have high cardiovascular risk profiles reflected by a higher prevalence of smokers, hypertensive and DM type 2 patients as opposed to the general population (Triant et al., 2007; Mdodo et al., 2015). Conversely, HIV is predominantly transmitted within the general population in SSA (figure 2) and PLWH in SSA are mainly black heterosexual women (UNAIDS, 2014). Unlike the rest of the world, people from SSA may have fewer traditional CVD risk factors than the general population (Clark et al., 2015).

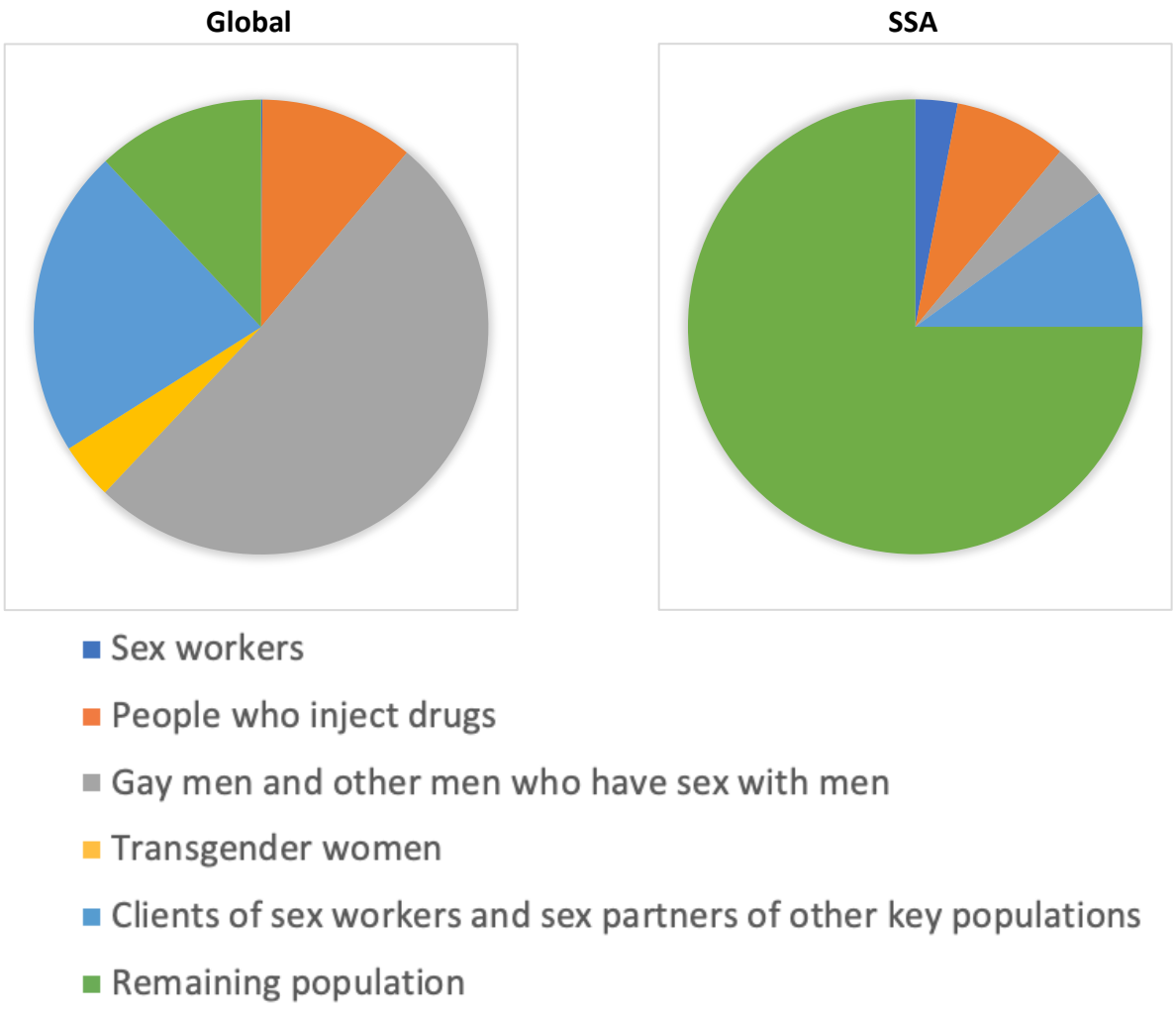


Figure 2. Differences in the distribution of new HIV infections by population group (aged 15-49 years, 2018) between the entire world and SSA. Adapted from (UNAIDS special analysis, 2019).

Furthermore, most of the research from high-income countries has focussed on a subtype of HIV that is not predominant in Africa. HIV-1, Group M, subtype C makes up 55 to 60% of all HIV-1 infections in SSA, however, the majority of research has been performed on Caucasian populations with HIV-1, subtype B (Manga, 2015). The two subtypes differ as much as 30% in their genome (Peeters, 2001). Therefore, we cannot exclude that subtype C HIV infections may have a slightly altered pathophysiology of HIV-related CVD in comparison to subtype B infections. In 2016, South Africa implemented the test-and-treat policy and the country has the world's largest ART program, subsidized by the government (Sliwa & Ntusi, 2019). Although the majority of PLWH in South Africa are receiving ART, the lack of resources in many other developing nations in Africa results in far fewer patients being ART-treated. Indeed, statistics from 2018, show that the global ART coverage rate is 62%, and of the remaining 38%, most of the people reside in SSA (UNAIDS, 2017). It has been predicted that the ART coverage rate is likely to drop and the death rate may increase due to complications related to the COVID-19 pandemic for PLWH (Jewell et al., 2020). When studying HIV-related CVD, one should therefore consider separating the population into treated versus untreated groups and consider the type of ART offered to the patients as the treatment itself may impact the risk for CVD (Manga, 2015).

1.4 Pathogenesis of HIV-related CVD

1.4.1 Pathogenesis of atherosclerosis

Atherosclerosis is a disease that revolves around chronic inflammation. Pro-atherogenic stimuli, such as hypercholesteremia, and pro-inflammatory cytokines, increase the permeability of the endothelium. It is these changes in endothelial homeostasis along with an increase in oxidative stress, that lead to the onset of atherosclerosis (Ross, 1999). The damaged endothelium allows lipids, particularly apolipoprotein B (apoB)-containing low-density lipoprotein cholesterol (LDL-C), to enter the endothelium and they undergo modifications including oxidation (Ross, 1999; Nguyen et al., 2019). Endothelial cells increase the expression of leukocyte adhesion molecules, such as vascular cell adhesion molecule (VCAM) and intercellular adhesion molecule (ICAM), which serve as binding sites for inflammatory monocytes and leucocytes (Hwang et al., 1997; Ridker, Buring & Rifai, 2001). The monocytes then infiltrate the lumen via chemotaxis, and they differentiate into macrophages in the

presence of stimulatory growth factors. The macrophages absorb cholesterol in the form of oxidised low-density lipoprotein (oxLDL) forming foam cells which secrete additional cytokines and growth factors to further accentuate the process (figure 3) (Nguyen et al., 2019). Foam cells form the basis of the atherosclerotic plaque and their aggregation results in a 'fatty streak' in the vessel wall (Yu et al., 2013). Under chronic conditions, smooth muscle cells and platelets are recruited, and the fatty streak becomes a fibrous plaque. Once in the intima, the smooth muscle cells proliferate and produce extracellular matrix proteins that form a fibrous cap that overlies the plaque. Continuous inflammation and smooth muscle cell recruitment cause the proteins to degrade, and the plaque becomes unstable. If the plaque ruptures, thrombogenic material is released into circulation and thrombus can form and possibly cause an arterial occlusion (Ross, 1999; Ginter & Simko, 2013).

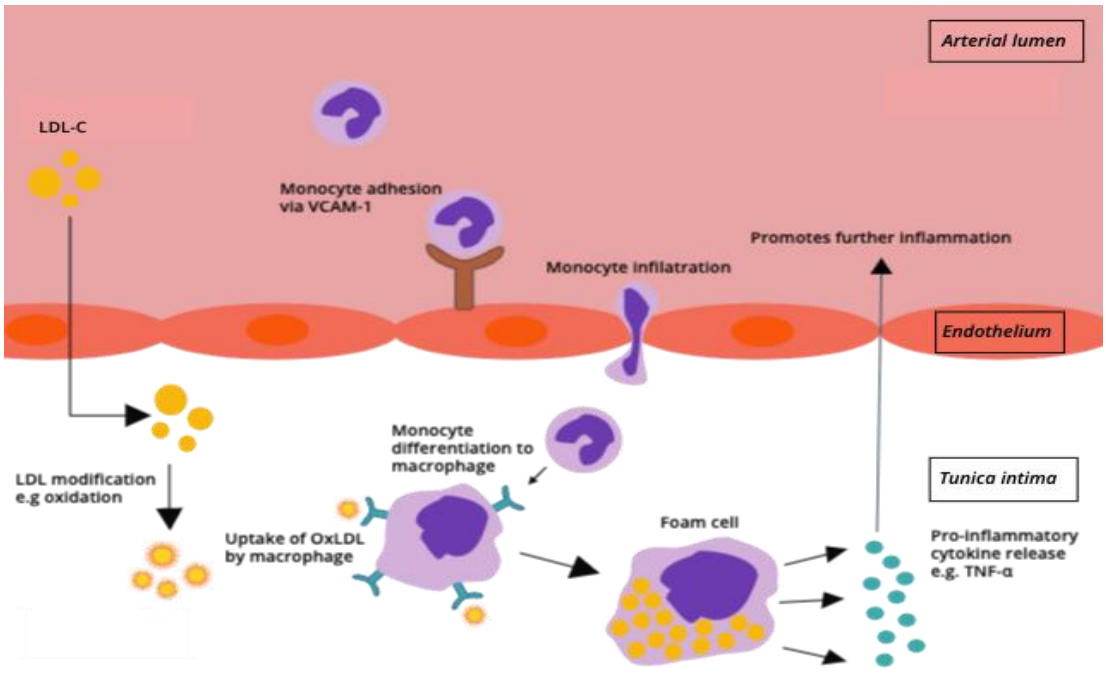


Figure 3. Key events in the formation of the atherosclerotic plaque: Pro-inflammatory stimuli increase the permeability of the endothelium allowing lipids to enter the endothelium and undergo modifications. The endothelium also expresses more adhesion molecules which serve as binding sites for monocytes which then infiltrate the lumen via chemotaxis. The monocytes differentiate into macrophages and take up oxLDL via scavenger receptors. The build of lipids within the macrophage leads to the formation of foam cells which release more pro-inflammatory cytokines thus promoting further inflammation. Adapted from (Nguyen et al., 2019).

1.4.2 The roles of HIV and ART in the pathogenesis of HIV-related CVD

The pathophysiological mechanisms behind the increased risk of CVD in HIV infection remain unclear but potential contributors to the aetiology of HIV related CVD include well-known CVD risk factors, HIV itself and the long term effects of ART (Zanni et al., 2014; Freiberg & So-Armah, 2015; Marincowitz et al., 2019). The general population, irrespective of HIV infection status, has a substantial burden of CVD risk factors such as obesity and hypertension (Triant et al., 2007). However, HIV infection itself determines a state of chronic inflammation, immune activation, metabolic abnormalities and vascular dysfunction (Beltrán et al., 2015). In this regard, persistent inflammation and immune activation may be the most significant contributors to increase CVD risk in HIV-infected individuals with increased concentrations of markers of monocyte activation and relatively higher levels of nonclassical (CD14-CD16+) monocytes (Toribio et al., 2017; Teer et al., 2019). Chronic inflammation and immune activation alter how lipids are processed/transported and they exacerbate structural modifications to these lipids via reactive oxygen species or the activation of enzymes such as lipoprotein-associated phospholipase A2 (Lp-PLA_2) (Funderburg & Mehta, 2016). Before the rollout of effective ART, many patients experienced dyslipidaemia which was potentially related to the inflammation caused by acute HIV infection (Grunfeld et al., 1989). The typical ART-naïve lipid profile displays increased triglycerides and decreased total cholesterol, LDL- and high-density lipoprotein cholesterol (HDL-C) (Grunfeld et al., 1992; Shor-Posner et al., 1993; Anastos et al., 2007). An increase in circulating LDL-C, together with an increase in macrophage activity, increases the likelihood of oxLDL formation, thus promoting foam cell accumulation (Mujawar et al., 2006). Additionally, the presence of chronic co-infections common to PLWH, such as the Hepatitis C virus, herpes family viruses and chronic kidney disease (CKD) may contribute to increased inflammation and CVD risk (Nwagha et al., 2010).

The first ART to be approved for use in patients in 1987 was a direct-acting dideoxynucleoside reverse transcriptase inhibitor (NRTI) known as azidothymidine (AZT). However, it was only used in advanced cases due to its toxicity (Vella et al., 2012). Over the next decade, drugs from different classes were developed, including non-nucleoside-reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs) and doctors started prescribing ART in combination as a three-drug regimen (Vella et al., 2012). Over the years, ART regimens have changed towards having drugs with fewer side effects and greater adherence (Domingo et al., 2018). Today, there are many different types of ART drugs available and most PLWH receive

first-line treatment as a fixed-dose combination drug consisting of NRTIs and NNRTIs. Second-line ART regimens are used when patients develop treatment failure to first-line treatments and they usually contain a boosted PI and two NRTIs (Vella et al., 2012; Alene et al., 2019). South Africa has a high ART coverage rate thanks to the test-and-treat policy implemented in 2016 and PLWH have their ART regimens changed if they present with any side-effects however much fewer PLWH are receiving ART in the rest of SSA and those have a more limited choice in terms of regimen (Sliwa & Ntusi, 2019; Assefa et al., 2020).

There is an ongoing debate regarding the relative contributions of viral factors versus ART side-effects to the development of CVD in PLWH (Behrens, Grinspoon & Carr, 2005; Vachiat et al., 2017). The Strategies for Management of Anti-Retroviral Study (SMART study) reported that patients assigned to discontinued ART exhibited an increased risk for CVD compared to those exposed to ART drugs (El-Sadr et al., 2006). Since then, it has been suggested that viral infection increases the risk of CVD even in patients with complete viral suppression following ART treatment (Beltrán et al., 2015). However, to further complicate the matter, the risk profiles may vary function to the class of ART (Dubé & Cadden, 2011). ART may lead to a series of physiological and biochemical disorders affecting the kidney, the liver and the cardiovascular system (Currier, 2008; Klimas, Koneru & Fletcher, 2008; Marin et al., 2009).

PIs, including stavudine and zidovudine, were the first antiretroviral drugs associated with increased CVD risk: their introduction into a clinical setting coincided with the first reported cases of ischaemic heart disease in HIV patients (Henry et al., 1998). NNRTIs such as efavirenz are also associated with increased CVD risk (Behrens, Grinspoon & Carr, 2005). Efavirenz is still a commonly prescribed first-line drug in South Africa even though it is known for its adverse effects on lipid and glucose metabolism (Sinxadi et al., 2016). The Data Collection on Adverse Events of Anti-HIV Drugs Study (DAD study) confirmed the relationship between ART and CVD, reporting a significantly increased occurrence of acute myocardial infarction, with an increased risk of 26% after 6 years of treatment with both PIs and NNRTIs (Friis-Møller et al., 2003). The harmful side effects of the older ART regimens include dyslipidaemia, altered glucose metabolism and have been associated with increased carotid intima-media thickness (CIMT), a measure of artery wall thickness and a common marker for subclinical atherosclerosis (Gleason et al., 2016). Patients on ART, often present with low-grade chronic inflammation along with raised levels of total cholesterol and LDL-C (Lazzaretti

et al., 2012). ART-associated lipid abnormalities are most evident in PI-based treatment, an effect which may be explained by its direct effect on the liver (Henry et al., 1998). NNRTIs also have deleterious effects, usually over a longer period. Their mechanisms of inducing lipid abnormalities are likely related to impaired adipose tissue function and resulting dyslipidaemia (Lagathu et al., 2019). Dyslipidaemia is more frequent in individuals with impaired fasting glucose or diabetes mellitus than in those with normoglycemia (Jin et al., 2016). HIV patients have high rates of insulin resistance and although the prevalence has decreased with newer ART regimens, it is still a major concern (Araujo et al., 2014). Most PIs as well as some NNRTIs, such as efavirenz, have been associated with insulin resistance and impaired secretion, thus favouring diabetes (Hadigan et al., 2001). Older generations of ART have also been associated with a specific type of body fat redistribution known as HIV-associated lipodystrophy (Lagathu et al., 2019). Newer ART regimes are generally better tolerated with fewer side effects directly linked to treatment, however cardiovascular and metabolic complications are still being reported (Shah et al., 2018). A recent three-year prospective study showed that CIMT increased in HIV ART-naïve patients compared to HIV-negative patients and treatment mitigated the difference (Low et al., 2019). These findings support the argument that HIV itself, rather than the side effects of ART, is the major cause of increased CVD risk in HIV-infected patients.

In summary, the pathophysiology of HIV-related CVD is likely due to a combination of classic CVD risk factors, persistent inflammation and immune activation and the long-term effects of ART. Furthermore, the pathophysiology of HIV-related CVD in SSA may differ from the rest of the world due to a high prevalence of tuberculosis co-infection, high diabetes mellitus rates, socioeconomic differences, variances in ethnic susceptibility to coronary artery disease and a varied ART coverage rate (Manga, 2015; Vachiat et al., 2017) (figure 4). This intensifies the need for the detection of HIV-specific biomarkers to not only detect CVD at an early stage but also to possibly guide both ART regimen choice and the use of lipid-lowering agents such as statins in PLWH (see review, (Hudson et al., 2020)).

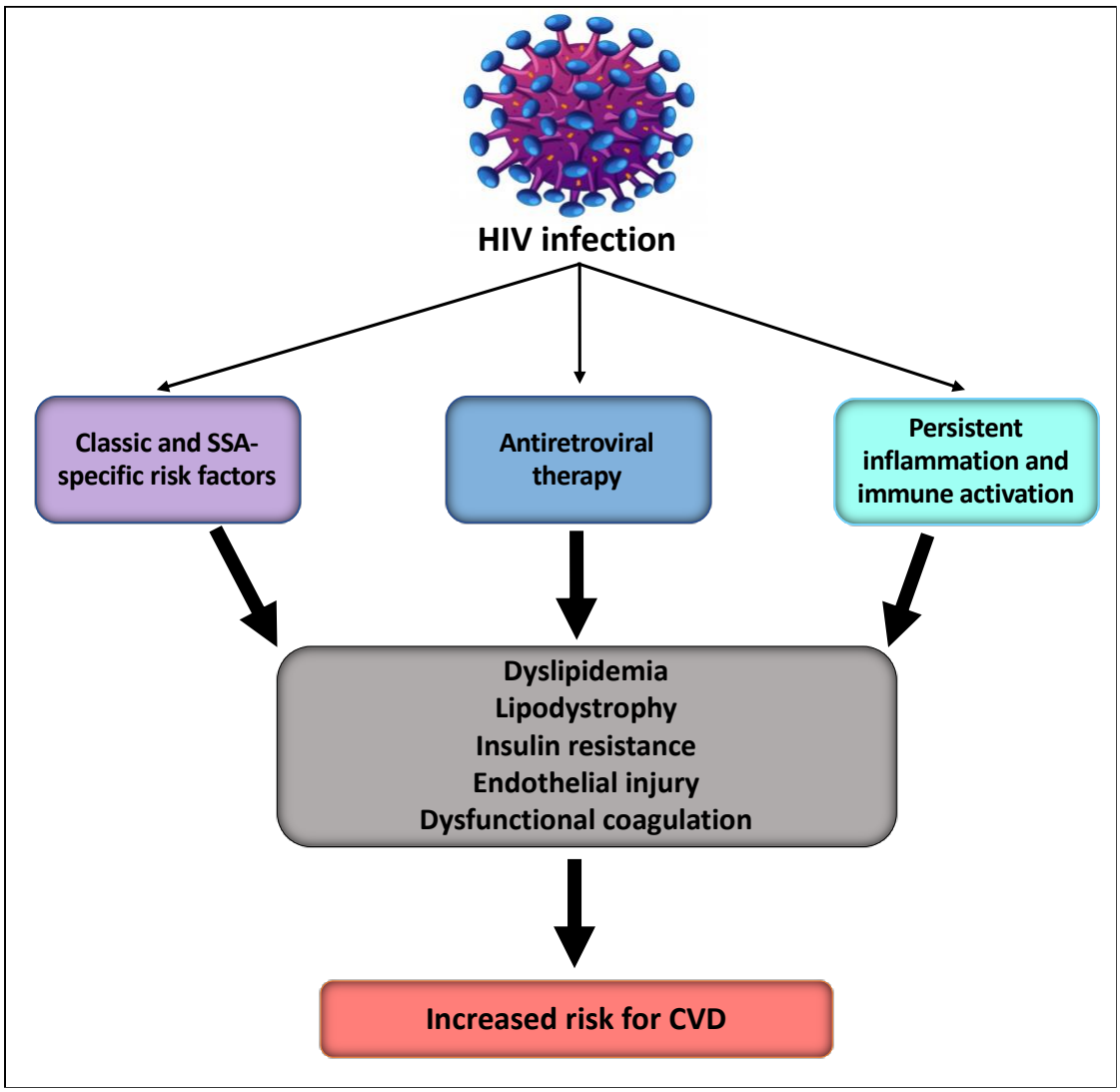


Figure 4. Factors that may increase the risk for CVD in HIV patients from Sub-Saharan Africa.

1.4.3 The role of statins in the prevention of atherosclerosis in PLWH

Statins (3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase enzyme inhibitors) are the current gold standard for treating hypercholesterolemia (Baigent et al., 2010). They are the preferred agents for reducing the risk of CVD among PLWH, based on guidelines extrapolated from the general population (Mosepele et al., 2018). Many studies have reported that statins lower LDL and ox-LDL in HIV-infected patients via multiple mechanisms including the downregulation of inflammatory biomarkers and improving endothelial function, thus slowing down the progression of atherosclerosis (Bernal et al., 2017; Maggi et al., 2017). Despite this, statins are still underutilized and under-dosed in this population possibly due to

varying reasons including concerns for drug-drug interactions, non-adherence and poor access to health care (Mosepele et al., 2018). The ongoing Randomized Trial to Prevent Vascular Events in HIV (REPRIEVE; launched in 2015) is a promising clinical trial with plans to test the ability of statin medication (pitavastatin) in decreasing the risk of HIV-related CVD (Grinspoon et al., 2019). Treatment with newer statins, together with the implementation of lifestyle modifications and switching to newer ART should help lower the risk of CVD in PLWH.

1.5 Early markers for HIV-related CVD

CVD risk assessment tools, such as the Framingham risk score, use clinical risk factors to estimate the risk of a CVD event over a period of time. Unfortunately, the scores derived from these tools are not always an accurate representation of an individual's risk, especially in the case of HIV due to disease-specific burden usually not included in score determination (Parra et al., 2010; Karmali & Lloyd-Jones, 2017). It is therefore critical to find early and reliable markers for HIV-related CVD. Surrogate CVD markers include ultrasound-based measurement of the CIMT, brachial artery diameter and flow-mediated dilation (FMD) (Vos et al., 2017). An increased CIMT is an indicator of conditions such as myocardial infarction and stroke (Lorenz et al., 2008). PLWH have been shown to have higher CIMT and therefore an increased atherosclerotic burden compared to HIV-negative individuals (Gupta et al., 2018). Baseline brachial artery diameter is associated with cardiovascular risk in the general population and HIV-infected individuals (Stein et al., 2013; Maruhashi et al., 2018). A reduced FMD is a well-known marker of endothelial dysfunction and is also associated with generalised cardiovascular risk (Charakida et al., 2010; Maruhashi et al., 2018).

A systematic review from 2017 found that associations between traditional inflammatory markers and surrogate markers of CVD in HIV patients are limited (Vos et al., 2017). These include associations between CIMT and C-reactive protein (CRP), Interleukin-6 and D-dimer (Vos et al., 2017). Single immune markers are non-specific and are therefore unlikely to sufficiently represent the multifaceted process of immune activation and increased arterial wall thickness (Baker & Duprez, 2010). This intensifies the need for a more specific novel biomarker for the early detection of HIV-related CVD. Lp-PLA_2 has been identified as a potential candidate. Lp-PLA_2 is an enzyme that is released into the bloodstream by leukocytes and hepatocytes and binds to LDL (Ji et al., 2008). Lp-PLA_2 hydrolyses phospholipids,

producing metabolically active lipid mediators such as pro-inflammatory free fatty acids, which activate platelets and recruit T-cells and monocytes, all of which are important effectors of the atherosclerotic plaque formation (Gonçalves et al., 2012). There is evidence to suggest that PLWH have higher Lp-PLA_2 levels than uninfected healthy people, irrespective of their triglyceride and LDL levels (Eckard *et al.*, 2014). A recent study conducted in South Africa found that Lp-PLA_2 correlated positively with viral load and inversely with CD4 T cell count in HIV+ patients treated with protease inhibitors, thus suggesting a link between the enzyme and HIV-associated inflammation (Mayne et al., 2019). Interestingly, Lp-PLA_2 is the plasma isoform of platelet-activating factor-acetylhydrolase (PAF-AH) which is an enzyme associated with HDL that greatly contributes to the anti-atherogenic effect of HDL particles (see review, (Karasawa, 2006)).

Dyslipidaemia, characterised by lower HDL-C and raised LDL-C, is a prominent risk factor for CVD, and it is more common in PLWH than in the general population (Gordon et al., 1981; D'Agostino et al., 2001; Friis-Møller et al., 2003; Zhou et al., 2015; Madsen, Varbo & Nordestgaard, 2019). Low HDL-C is a strong independent predictor of CVD and increased LDL-C is a major risk factor for CVD (Bachorik & Ross, 1995; Warnick & Wood, 1995). The analysis of lipid profiles is by no means novel but the analysis of lipoprotein subfractions and their associated functions is. Lipoprotein classes are heterogeneous and consist of multiple subfractions that vary in size, density, and chemical composition (Mahley et al., 1984; Asztalos, Tani & Schaefer, 2011). There is mounting evidence that lipoprotein subfractions, especially HDL subfractions, may be of clinical use as a potential biomarker for the detection of CVD (Ben-Aicha, Badimon & Vilahur, 2020; Lappegård, Kjellmo & Hovland, 2021). Lipoproteins, their associated functions, and their potential use as an early biomarker for the early detection of HIV-related CVD will be discussed further in the following sections.

1.6 CVD and lipoproteins

1.6.1 Lipoprotein structure and composition

Lipids are transported in the blood by water-soluble macromolecules called lipoproteins, composed of an inner triglyceride and cholesterol layer, surrounded by phospholipids and apolipoproteins (Morrisett, Jackson & Gotto, 1975). Lipoproteins are classified according to density and size and include chylomicrons (largest and least dense), very-low-density

lipoprotein (VLDL), intermediate-density lipoprotein (IDL), LDL and HDL (smallest and most dense) (figure 5) (Ridker, 2014).

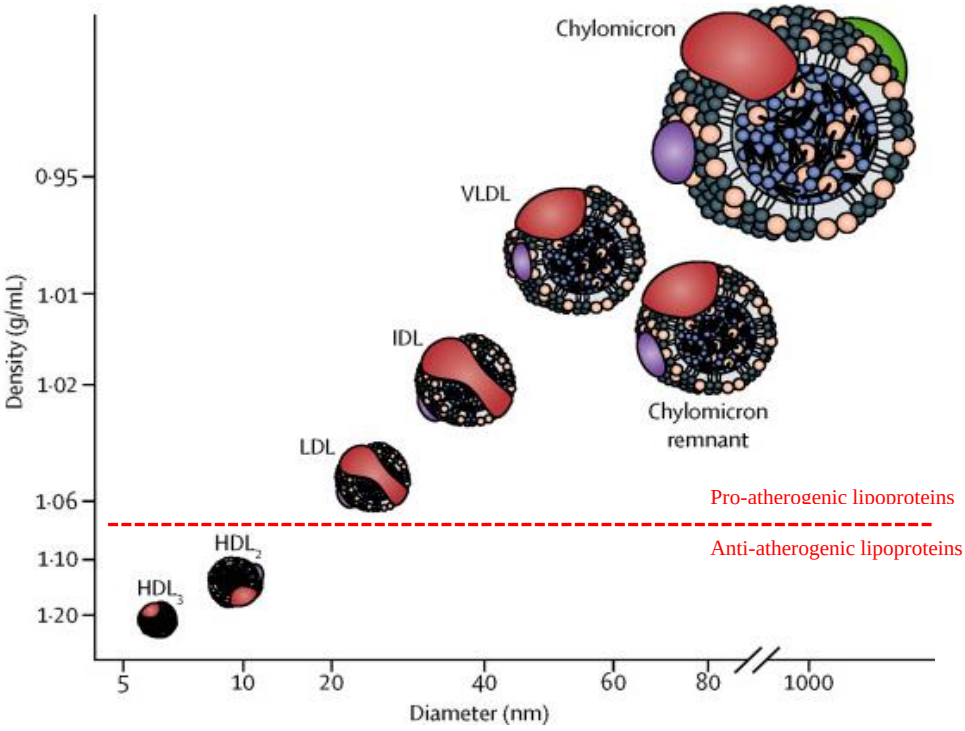


Figure 5. Lipoproteins classified according to their diameter and density. Adapted from (Ridker, 2014).

The exogenous lipoprotein pathway starts when chylomicrons incorporate dietary cholesterol and triglycerides in the intestine and transport them to other locations in the body (Mahley et al., 1984). Triglycerides are metabolised in muscle and adipose tissue by lipoprotein lipase, releasing free fatty acids which are metabolized by the muscle and adipose tissue and used for storage or energy (Mahley et al., 1984). Triglyceride depletion of chylomicrons produces cholesterol-enriched pro-atherogenic remnants (Feingold & Grunfeld, 2000).

The endogenous lipoprotein pathway starts in the liver with the formation of VLDL. These triglyceride-rich particles are large (30-90nm) with a low density (<1.006 g/ml). Triglycerides are metabolized in the peripheries by lipoprotein lipase and this process forms IDL (density = 1.006 – 1.019 g/ml) which are further metabolized to LDL (density = 1.019 – 1.063 g/ml). IDL and LDL are the principal cholesterol transporting lipoproteins and are composed of multiple subfractions (Mahley et al., 1984). HDL, the only anti-atherogenic lipoprotein, is the smallest

(8-12nm in diameter), most dense (> 1.21 g/ml) and most heterogenous of all lipoproteins (Mahley et al., 1984).

Lipoproteins containing apoB are known as apoB-containing lipoproteins. All lipoproteins, except HDL, contain apoB and it is the primary organizing protein component of these particles as it is essential for their formation and stability (Ridgway & McLeod, 2015). Apart from LDL, lipoproteins contain many different apolipoproteins that perform different functions. LDL only contains apoB100 and it acts as a ligand for LDL receptors within the body. ApoB can be measured more accurately and precisely than LDL-C and has recently been suggested to be a more accurate marker of cardiovascular risk than LDL-C (Kohli-Lynch et al., 2020). Although LDL only contains one apolipoprotein, it differs significantly in particle size and density. Lipoprotein heterogeneity has been demonstrated by several analytical methods, the first being agarose gel electrophoresis (Asztalos, Tani & Schaefer, 2011) followed by methods such as nuclear magnetic resonance (NMR) and non-denaturing gradient gel electrophoresis (GGE). Over time, electrophoresis techniques have been modified and refined to include the ability to quantify the relative distribution of each lipoprotein subfraction. The Lipoprint® system (Quantimetrix, Redondo Beach, CA) is a relatively new method of analysis that uses a linear, polyacrylamide gel electrophoresis system. The LDL Lipoprint system separates LDL into seven subfractions ranging from the largest, LDL-1 to the smallest, LDL-7. Genetics and environmental factors, such as diet and smoking, contribute to differences in the degree of LDL heterogeneity (Kulanuwat et al., 2015; Manabe et al., 2015). Patients whose lipoprotein profiles primarily consist of larger, buoyant LDL-1 and LDL-2 have a pattern A profile, while profiles that mainly consist of smaller and denser subfractions, LDL-1 through LDL-7, have a pattern B profile (figure 6) (Quantimetrix, 2005). Small-dense LDL (sdLDL) is the most pro-atherogenic form of LDL, due to enhanced arterial wall permeability and increased sensitivity to oxidation. Additionally, sdLDL circulates in the bloodstream for longer than that of large LDL particles because of their decreased affinity for the LDL receptor (Hurt-Camejo et al., 1990; Ivanova et al., 2017).

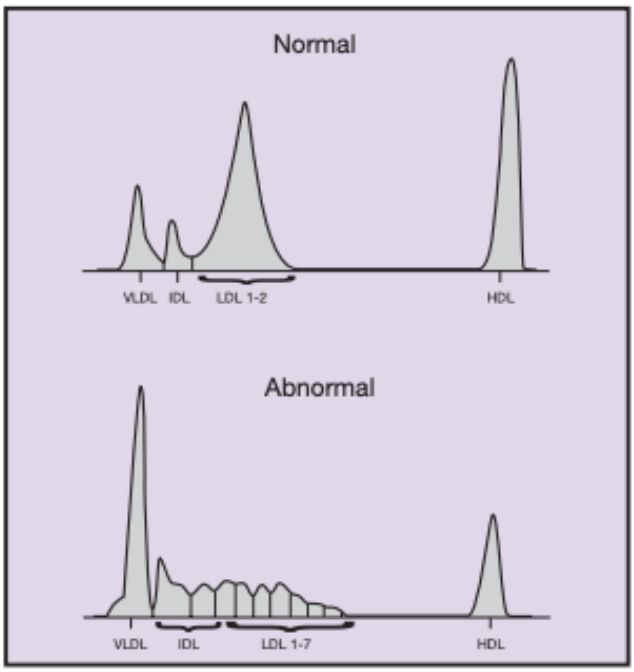


Figure 6. An example of a normal (pattern A) LDL subfraction profile consisting of mainly LDL-1 and LDL-2 and an abnormal (pattern B) LDL subfraction profile consisting of LDL-1 to LDL-7. Adapted from (Quauntrimetrix, 2005).

HDL biogenesis is more complex than that of the other lipoproteins because, unlike the others, it originates as discoidal particles which can either be formed within the liver or produced from lipid and apolipoprotein constituents in the circulation (Hamilton, Williams and Fielding and Havel, 1976). Discoidal HDL is composed of multiple phospholipid molecules, some unesterified cholesterol and at least two apolipoproteins (Rye & Barter, 2014). Apolipoprotein A-I (apoA-I) is the most common apolipoprotein and comprises approximately 70% of total plasma protein. Apolipoprotein A-II (apoA-II) makes up 15-20% of the total plasma protein but it is not ubiquitous in HDL. ApoA-I and apoA-II are known as scaffold proteins because they primarily determine HDL particle structure (Gauthamadasa et al., 2010). The other apolipoproteins associated with HDL are apolipoprotein A-IV (apoA-IV), apolipoprotein C (apoC), apolipoprotein E (apoE) and apolipoprotein M (apoM); they comprise <10% of HDL protein (Phillips, 2013). ApoM is of clinical importance because it allows for the specific binding of sphingosine-1-phosphate (S1P), an important phospholipid (Murata et al., 2000; Christoffersen et al., 2011). ApoA-I, which is mainly synthesized in the liver, is critical for the formation of discoidal HDL with ATP binding cassette transporter (ABC) A1 (Wang et al., 2001). ApoA-I also activates lecithin cholesterol acyltransferase (LCAT) which is the enzyme responsible for converting discoidal HDL into mature, spherical HDL (Temel et al., 2002).

LCAT activity is regulated by several different factors including the availability of apoA-I, the number of phospholipids and the size of the discoidal HDL (Jonas, Kezdy & Wald, 1989; Bolin & Jonas, 1996; Scott et al., 2001). Similarly to apoB, apoA-I can be measured accurately and precisely and it is proposed as a more accurate marker of cardiovascular risk than HDL-C (Barter & Rye, 2006). Since both of these apolipoproteins are individual markers for the risk of CVD, their ratio (apoB/apoA-I) is also commonly used as a predictor for cardiovascular risk (Kohli-Lynch et al., 2020).

Most of the anti-atherogenic function of HDL function is linked to structural association with key lipids and proteins including the apolipoproteins, paraoxonase (PON), PAF-AH and sphingosine-1-phosphate (S1P) (figure 7) (Davidson et al., 2009; Brinck et al., 2018; Woudberg, Pedretti, et al., 2018). PON is the most important enzyme associated with HDL. PON-1, an extracellular isoform of PON, is widely considered as the main contributing factor to the antioxidant potential of HDL (Mackness, Durrington & Mackness, 2004). PAF-AH regulates the metabolism of platelet-activating factor (PAF), a potent platelet, monocyte and leukocyte activator (Stafforini et al., 1987). S1P is a signalling molecule that has been shown to regulate a wide range of biological responses in a variety of organ systems including the cardiovascular system (Maceyka et al., 2012). In the plasma, S1P is transported by all lipoproteins however, it is most commonly bound to HDL via apoM (Murata et al., 2000; Christoffersen et al., 2011). S1P has been shown to protect against ischaemia-reperfusion injury (Swendeman et al., 2017; Wang & Wang, 2017) and to protect endothelial function (Fan et al., 2020).

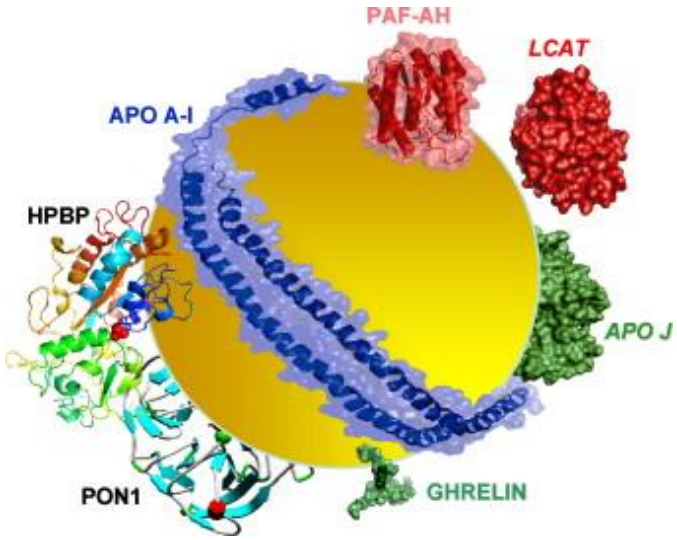


Figure 7. Scaled schematic illustration of HDL with some of its associated proteins. Adapted from (Rochu et al, 2007).

Two key proteins that play an important role in the regulation of HDL are the phospholipid transfer protein (PLTP) and the cholesterol ester transfer protein (CETP) (Clay et al., 1992; Jauhiainen et al., 1993). Together these proteins (along with other regulators) cause HDL to be the most heterogeneous of the lipoproteins because they lead to the production of specific HDL subfractions or subclasses. Indeed, PLTP facilitates the transfer of phospholipids between HDL and other lipoproteins (Jauhiainen et al., 1993) as well as converts HDL into two distinct subpopulations comprising of large and small HDL products (Pussinen et al., 2001; Settasatian et al., 2001). The role of CETP is to transfer cholesterol esters, triglycerides, and phospholipids. The exchanges that occur lead to changes in HDL structure ultimately resulting in significant remodelling of HDL (Clay et al., 1992).

The term, spherical HDL, refers to a spectrum of lipoprotein particles within the density range of 1.063–1.210 g/ml. This range is divided into different types of subfractions depending on the methods used for separation. Through ultracentrifugation, HDL has traditionally been divided into two groups: HDL2 (1.063–1.125 g/ml) and HDL3 (1.125–1.210 g/ml), which themselves can be further separated into additional subgroups (Asztalos & Schaefer, 2003; Rosenson et al., 2016). HDL2 is larger (mean diameter 100 Å and molecular weight 350 kDa) and lipid enriched while HDL3 is smaller (mean diameter of 75 Å and a mean molecular weight of 175 kDa) and protein enriched. Proteomic analysis of the HDL subclasses has revealed that certain proteins are exclusively associated with HDL2 and others with HDL3 (table 1) (Davidson et al., 2009). The exclusive association of these proteins play a role in the specific functions of each of the HDL groups (see review, (Woudberg *et al.*, 2018)). The HDL Lipoprint® system (Quantimetrix, Redondo Beach, CA) works in a similar way to the LDL system. The HDL Lipoprint® system separates HDL into ten different subfractions from HDL-1, the largest subfraction through to HDL-10, the smallest subfraction. The subfractions can also be grouped into subclasses. HDL-1, HDL-2 and HDL-3 are grouped as large HDL, HDL-4, HDL-5, HDL-6 and HDL-7 are grouped as intermediate HDL and HDL-8, HDL-9 and HDL-10 are grouped as small HDL. (Hoefner et al., 2001).

Table 1: Preference distribution of peptides between HDL2 and HDL3. Adapted from (Davidson et al., 2009). See page vii for abbreviations.

HDL2	**HDL3**
apoC-II	apoA-I
apoC-III	PON-1
apoE	PON-3
apoB	apoL-I
apo(a)	apo-IV
	PLTP
	apoD
	apoJ
	apoF
	apoM
	PAF-AH
	SAA 1 and 2

1.6.2 Anti-atherogenic functions of HDL

HDL particles exert their major atheroprotective properties through the removal and transport of cholesterol from peripheral tissues and cells back to the liver for excretion in bile and faeces (Glomset, 1968). This anti-atherogenic process is known as reverse cholesterol transport (RCT) and there are several pathways by which it occurs. The majority of cholesterol efflux is mediated by an active process, with ABCA1 being the most common pathway (Adorni et al., 2007). Other mechanisms of cholesterol efflux to HDL include processes mediated through ABCG1 transporters and scavenger receptor type 1 receptors (SR-B1) (Ji et al., 1997; Oram et al., 2000; Wang et al., 2004). Cholesterol can also move across cell membranes, binding to HDL by passive aqueous diffusion (Yancey et al., 2003; Phillips, 2014). It is these active processes involving the transporters that play a crucial role in maintaining the efflux capacity of HDL. They are either involved through the direct management of cholesterol efflux or they trigger downstream signalling pathways which can then lead to other anti-atherosclerotic effects (see review, (Phillips, 2014)).

Other than its role in RCT, HDL performs several physiological functions that are cholesterol independent. These include anti-inflammatory, antioxidant, anti-thrombotic and anti-apoptotic functions (Nofer et al., 2002; Frias et al., 2010). These supplementary functions mostly focus on controlling the pro-atherogenic impact of oxLDL which, as previously mentioned, is cytotoxic to macrophages and is the main player in generating atherogenic foam cells (Barter et al., 2004).

HDL has known antioxidative properties however, the mechanism by which HDL exerts these effects is not clear. As mentioned previously, PON-1 is the main contributing factor to the antioxidant potential of HDL (Mackness, Durrington & Mackness, 2004). PON-1 and other proteins such as apoA-I hydrolyse lipid hydroperoxides and oxidised phospholipids in LDL (Wang et al., 2013). Oxidative stress and inflammation are two integrated processes therefore the anti-oxidative effect of HDL also contributes to the anti-inflammatory effect of HDL (Bonizzi et al., 2021).

OxLDL triggers the expression of monocyte chemoattractant protein-1 and inflammatory responses in damaged endothelial cells (Assmann & Gotto, 2004; Barter et al., 2004). This causes activated monocytes to adhere to the endothelial layer via adhesion molecules such as VCAM-1. HDL performs anti-inflammatory functions by inhibiting the expression fo adhesion molecules and interacting with inflammatory cells (Cockerill et al., 1995; Diederich et al., 2001). The nitric oxide related suppression of the nuclear factor of kappa-light-chain-enhancer of activated B cells (NF-κB) pathway may facilitate downregulation of adhesion molecule expression (Cockerill et al., 1999; Murphy et al., 2009). HDL activates phosphatidyl-inositol-3 (PI-3) and Akt kinases which trigger nitric oxide (NO) synthesis leading to the inhibition of tumour necrosis factor-alpha (TNF-α) (Schmidt et al., 2006). HDL interacts directly with inflammatory cells by binding to the surface of activated T-cells, therefore, blocking monocyte contact activation with the T cells (Hyka et al., 2001).

Thrombosis is the localized clotting of blood and it is associated with the activation of platelets (Mineo et al., 2006; Mackman, 2008). HDL's anti-thrombotic functions include controlling blood coagulation and inhibiting platelet activation (Griffin et al., 1999; Calkin et al., 2009). The main action of inhibition is via the interaction of prostacyclins with nitric oxide. Indeed, HDL induces the expression of cyclooxygenase-2 which leads to increased endothelial prostacyclin synthesis (Cockerill et al., 1999). S1P is indirectly involved in this process because

it leads to the the production of cyclic adenosine monophosphate (cAMP), thus causing the formation and release of prostacyclins which are potent inhibitors of platelet activation (Damirin et al., 2005). PAF-AH, an HDL associated enzyme mentioned earlier, also plays a major anti-thrombotic role by hydrolysing PAF and rendering it inactive (Stafforini et al., 1987; Durrington, Mackness & Mackness, 2001). HDL can also directly reduce the production of thromboxane A2, another platelet activator (Camont et al., 2013).

The pathophysiology of atherosclerosis involves the breakdown of the endothelial cell layer's structural viability. HDL regulates proliferation and vasorelaxation of endothelial and smooth muscle cells, thus preventing endothelial apoptosis and facilitating their repair (Chen et al., 1986; Tso et al., 2006). HDL stimulates endothelial nitric oxide synthase (eNOS) activity via SR-B1 and S1P receptors 1 and 3, leading to reduced endothelial oxidant stress (Yuhanna et al., 2001; Igarashi et al., 2007). Interactions with these receptors initiate a signalling cascade that involves the activation of sarcoma family kinases PI-3 kinase and Akt, leading to phosphorylation of eNOS and ultimately an increase in enzyme activity (Seetharam et al., 2006; Zhang et al., 2011). Furthermore, PON-1 has been shown to play a role in eNOS activation. PON-1-deficient mice are unable to stimulate endothelial NO production and this leads to impaired endothelium protection and repair (Barter et al., 2004).

In summary, HDL is a highly complex molecule that possesses several protective functions (figure 8), some of which are still not fully understood. Given all the emerging evidence, the focus on HDL has shifted away from simply measuring the concentration of HDL-C to assessing the functional quality of the HDL.

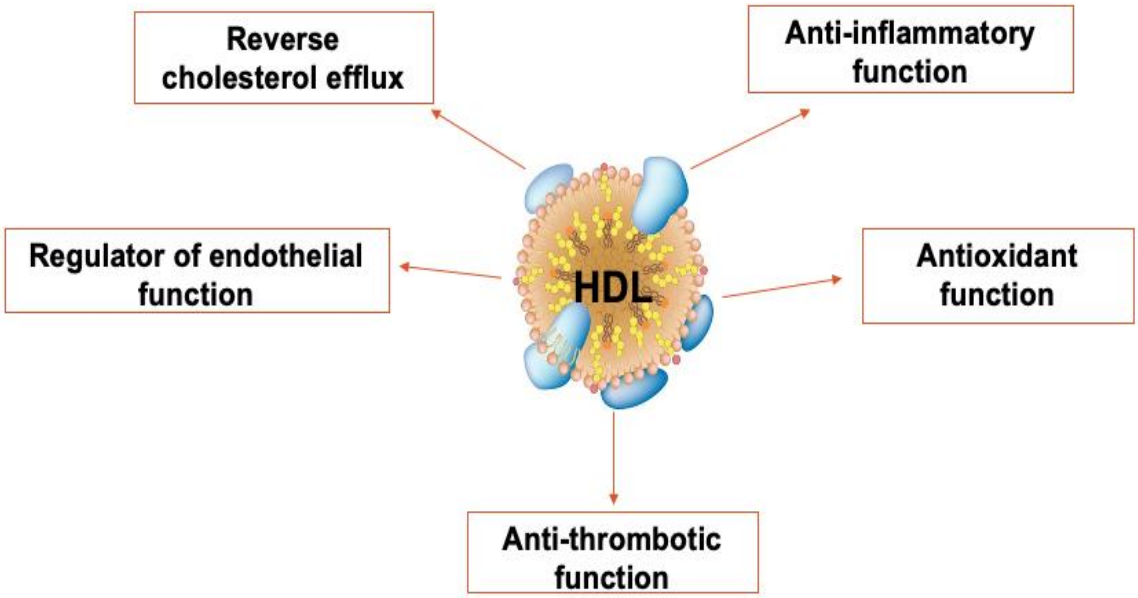


Figure 8. Summary of the major anti-atherogenic functions of HDL.

1.7 Is HDL quality a better indicator of cardiovascular disease risk than HDL quantity?

The Framingham study, which launched in 1948, was the first to show a negative correlation between HDL-C and the risk of CVD and this correlation has since been confirmed in additional epidemiological studies. (Gordon et al., 1981; Castelli et al., 1986; D'Agostino et al., 2001; Madsen, Varbo & Nordestgaard, 2019). It was therefore hypothesized that therapies that can increase HDL-C should theoretically decrease the risk of CVD. However, the results from large-scale clinical trials have shown this therapeutic strategy to be disappointing (Boden et al., 2011; Schwartz et al., 2012; Kühnast et al., 2015). Furthermore, there is evidence to suggest that elevated HDL-C levels are associated with an increased risk of adverse cardiovascular events in an at-risk population. Indeed, a recent large study reported that both low and very high levels of HDL-C were associated with cardiovascular death and all-cause mortality, forming a "U-shaped" association (Allard-Ratick et al., 2018). It is suggested that a complex relationship between HDL-C and cardiovascular risk may be related to the reduced capacity of HDL to receive free cholesterol from triglyceride-rich lipoproteins during lipolysis by LDL when concentrations of HDL are low or extremely high (Feng et al., 2020). The ineffectiveness of therapies aimed at elevating HDL-C levels to reduce cardiovascular risk and the U-shaped association has brought into question the HDL-C concentration hypothesis

(Kühnast et al., 2015). In this regard, it has been shown that HDL particles may change in composition (and therefore in function) in the presence of different cardiovascular risk factors (see review, (Woudberg *et al.*, 2018)). It is, therefore, appropriate to consider whether measurements of HDL function and its subfraction distribution, as opposed to HDL-C concentrations, may prove to be more clinically relevant when assessing CVD risk.

As previously mentioned, HDL is the most heterogenous lipoprotein and it can be separated into different subgroups or subclasses. Differences in HDL subclass composition contribute to differences in function. It is presently under debate as to which subclass is functionally superior. It was initially suggested that HDL2 may be a more accurate risk factor for CVD (van der Steeg et al., 2008; El Harchaoui et al., 2009; Kontush, Lhomme & Chapman, 2013). However, pre-clinical and new epidemiological data suggest that HDL3 may be a better indicator of CVD risk due to structural associations with cardioprotective particles such as S1P (Davidson *et al.*, 2009; Brinck *et al.*, 2018; Woudberg *et al.*, 2018). HDL3 has increased cholesterol efflux capacity and better antioxidant, anti-thrombotic and anti-apoptotic properties when compared to HDL2 (Camont et al., 2013). Evdence suggests that HDL3 inhibits TNF-α-induced inflammation in endothelial cells more effectively than HDL2 (Ashby et al., 1998). PON-1, apoJ and S1P are all components of HDL3 that have cardioprotective effects whereas HDL2 contains apoCIII which is associated with an increased risk for cardiovascular events (Riwanto et al., 2013). A recent study found that dialysis patients who presented with cardio- and cerebrovascular events had lower levels of HDL3 than dialysis patients with no such events (Lee et al., 2021). In another study focusing on end-stage renal disease and haemolysis, the Lipoprint® system was used to show that patients with renal disease had higher levels of large HDL compared to control patients (Gluba-Brzózka et al., 2017). A low level of small HDL was also found to correlate with unfavourable outcomes in stroke patients (Varela et al., 2020).

Routine measurement of HDL-C levels fails to take into account the complexity of HDL structure and function (Harangi et al., 2017). All the findings mentioned above suggest that it is important to consider HDL functionality and composition (quality) instead of the overall quantity of circulating HDL-C.

1.8 HDL and HIV

One of the key drivers for the increased CVD risk in both ART naïve and treated HIV-infected patients is dyslipidaemia (Friis-Møller et al., 2003; Feeney & Mallon, 2011). A large cross-sectional study conducted in South Africa reported that 90% and 85% of the ART-naïve and ART-treated participants respectively, had dyslipidaemia, with low HDL-C being the most common lipid abnormality (Dave et al., 2016). Furthermore, dyslipidaemia was also associated with abnormal glucose metabolism in both ART-naïve and treated participants (Dave et al., 2016).

In addition to its reduced quantity, the HDL particles in HIV patients can also be altered and become impaired or dysfunctional (Ansell, Fonarow & Fogelman, 2007). Lipid rafts are distinct lipid domains found within the plasma membrane and they are crucial to the survival of HIV. Indeed, the budding and assembly of HIV occur at lipid rafts of infected cells and the infection of target cells also involves lipid rafts (Nguyen & Hildreth, 2000). HIV regulates the abundance of lipid rafts via Nef, an HIV protein (Zheng et al., 2001). Nef impairs ABCA-1-dependent cholesterol efflux from human macrophages and can stimulate cholesterol biosynthesis and deliver cholesterol to the plasma membrane leading to an increase in lipid rafts (Zheng et al., 2003; Mujawar et al., 2006). ART significantly reduces levels of Nef which could potentially improve cholesterol efflux capacity, however not to a level similar to that of healthy individuals (Toribio et al., 2017; Ferdin et al., 2018). Nef can cause dyslipidaemia and promotes the formation of foam cells in mouse models of atherosclerosis (Cui et al., 2014). HIV infection favours the redirection of cholesterol to apolipoprotein B lipoproteins which may potentiate atherogenesis (Rose et al., 2008). Furthermore, a recent South African study found that microRNA(miR)-148a, an epigenetic regulator of ABCA-1 expression is differently regulated in HIV patients (Kinoo et al., 2021). HIV has also been associated with the differential expression of several other microRNAs involved in lipid metabolism including miR-27, another regulator of ABCA-1 expression, miR-126, a regulator of lipid-induced inflammation and miR-1307, where its differential expression has been associated with diabetes (Sun et al., 2012; Collares et al., 2013; Goedeke et al., 2015; Low et al., 2019). HDL transports many miRs in the plasma and it is not yet clear if these changes seen in miR abundance are due to HIV infection or changes in HDL structure induced by HIV infection (Low et al., 2019).

HIV-infected patients have reduced PON-1 activity compared to uninfected controls (Pereira et al., 2009; Siegel et al., 2015). Therefore, the enzymatic activity of PON-1 has been identified as a potential marker for evaluating the risk of HIV-related CVD and the progression of the disease (Marsillach et al., 2007). Similarly, systemic inflammation lowers the anti-inflammatory effect of HDL which may transform into a dysfunctional and pro-inflammatory particle (Kelesidis et al., 2011). Dysfunctional HDL in virally suppressed HIV-infected individuals may promote atherosclerosis by promoting monocyte-derived foam cell formation (Angelovich et al., 2017). Dysfunctional HDL has downstream consequences that are specific to CVD, which potentially makes it an attractive, early biomarker compared to inflammatory markers which are non-specific (Kontush, Lhomme & Chapman, 2013). All these findings support the shift in HDL function associated with HIV infection.

Only a few studies have explored the associations between HIV infection and HDL subfractions. American and European HIV-infected patients have larger HDL particles that are less stable and less receptor competent compared to healthy controls (Gotti et al., 2012; Gillard et al., 2013). These results suggest that, similar to other cardiovascular risk factors, HIV infection causes shifts in HDL subclass distribution. Munger et al examined the lipid profile and particle size of ART-treated patients with traditionally normal lipid profiles and reported a decrease in large HDL, an increase in small LDL and reduced reverse cholesterol efflux compared the HIV free patients (Munger et al., 2015). Recently, our group observed alterations in HDL subfractions in a population of HIV-infected individuals in South Africa, with higher distributions of larger HDL subfractions detected in HIV-infected individuals compared to healthy patients (Teer et al., 2019). A detailed characterisation of HDL subfractions, composition and functionality in HIV patients treated with/without ART would be of great interest to evaluate the effect of HIV versus ART on this lipoprotein. Considering this, we therefore aimed to explore the relationships between HDL functionality, composition and subfractions in a population of PLWH in SSA.

CHAPTER TWO: AIMS AND HYPOTHESIS

2.1 Summary

CVD is the leading cause of death globally, with most deaths occurring in low- and middle-income countries (World Health Organization, 2019a). The growing burden of disease in SSA can, at least in part, be attributed to the region's rapid urbanization, population growth and a shift to more westernized lifestyle trends (Mayosi et al., 2009; Mensah et al., 2015). Another potential cause of the increased incidence of CVD is the contraction and management of communicable diseases such as HIV through the use of ART (Sliwa & Ntusi, 2019). ART has improved the life expectancy of PLWH however, this longevity is associated with an increased risk of CVD (Remais et al., 2013). Both ART and HIV viral infection may be potential contributors to the pathophysiology of HIV-related CVD (Freiberg & So-Armah, 2015). The mechanisms behind this remain unclear but it is critical to delineate early biomarkers of cardiovascular risk in the HIV population.

HIV is associated with dyslipidaemia, characterised by lower HDL-C and raised LDL-C (Friis-Møller et al., 2003; Feeney & Mallon, 2011). HDL, the smallest and most dense lipoprotein, performs several anti-atherogenic functions including reverse cholesterol transport, antioxidative and anti-thrombotic functions (Nofer et al., 2002). For years, epidemiological studies have shown a negative correlation between HDL-C concentration and the risk of CVD (Gordon et al., 1981). HDL is a complex molecule, composed not only of cholesterol but also enzymes such as PON-1 and PAF-AH, apolipoproteins, and lipids such as S1P. HDL particles may change in composition and therefore function in the presence of different cardiovascular risk factors (see review, (Woudberg *et al.*, 2018). Failures of large-scale clinical trials that aimed at pharmacologically increasing HDL-C, recent evidence suggesting that HDL can become dysfunctional and findings from population studies have led to the suggestion that measurements of HDL function and its subfraction distribution, as opposed to HDL-C concentrations, may prove to be more clinically relevant when assessing CVD risk. HIV impairs HDL function via many mechanisms which are not yet fully understood. One of the possible mechanisms by which HIV and ART may favour CVD could be by adversely altering HDL subfraction distribution, composition, and functionality.

2.2 Study aims

The aim of the study was therefore to explore whether HIV and/or ART is associated with differences in HDL subfraction distribution, HDL function and composition in a population of 144 HIV-infected (ART-naïve and ART-treated) and uninfected South African men and women.

2.3 Hypothesis

We hypothesize that both HIV infection and ART can alter HDL subfractions and function, thus increasing the risk for CVD. We propose that a shift in the HDL subfraction distribution and a change in HDL composition in HIV patients with/without ART are associated with a decrease in HDL functions such as reverse cholesterol efflux capacity, antioxidant function and anti-thrombotic function.

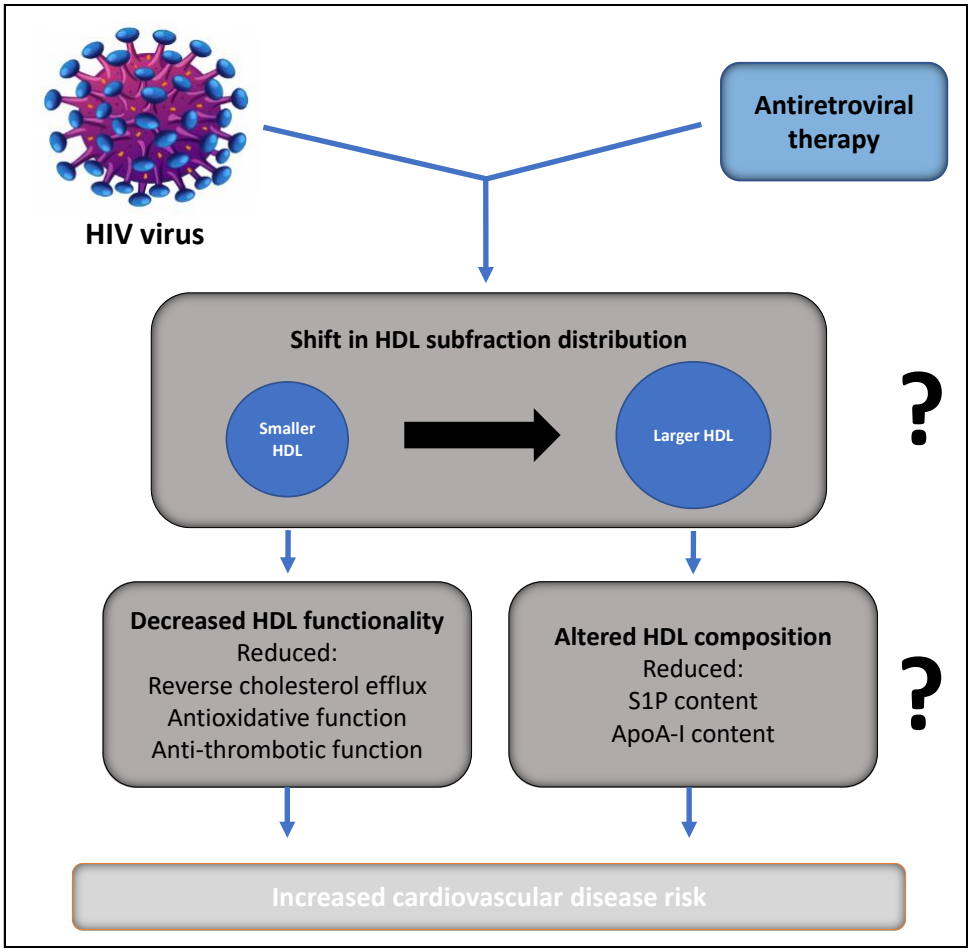


Figure 9: Our hypothesis.

2.4 Objectives

To assess whether HIV with/without ART in South African men and women is associated with differences in HDL composition, function and subfraction distribution, the following objectives will be addressed:

- To assess HDL composition by measuring HDL content in apoA-I and S1P.

- To assess HDL function by measuring the reverse cholesterol efflux capacity, the HDL antioxidative activity (PON-1 activity assay) and the anti-thrombotic activity (PAF-AH activity assay) of HDL particles.

- To assess HDL subfractions using the HDL Lipoprint® system.

CHAPTER THREE: METHODS AND MATERIALS

3.1 Subjects

The sample population consisted of 45 male and 99 female South Africans (144 in total) of black (18%) and mixed-ancestry ethnicity (82%). The patients were recruited on presentation at HIV clinics or community health centres within the Western Cape as a part of the ongoing EndoAfrica study (Strijdom et al., 2017). All participants gave written consent and approval for the study was given by the Health Research Ethics Committee at the University of Stellenbosch (N13/05/064B) and the Research Ethics Committee of the Faculty of Health Sciences of the University of Cape Town (HREC REF: 242/2018). All patients were 18 years or older, not pregnant or with a baby younger than 3 months and not infected with tuberculosis. They were allocated into 3 groups as follows; Group 1: 50 healthy HIV-negative control patients (HIV free control), group 2: 44 HIV-infected patients yet to receive any ART treatment (ART-naïve) and group 3: 50 HIV-infected patients receiving ART (ART-treated). A health questionnaire, anthropometric measurements, cardiovascular measurements, and fasting serum collection were conducted on the day of recruitment. Serum samples were stored at −80° from then onwards. The health questionnaire was completed to collect demographic and lifestyle information. All anthropometric measurements were obtained using standardized procedures (Marfell-Jones, Stewart & De Ridder, 2012). They included height, weight, waist, and hip circumference. Cardiovascular measurements included non-invasive FMD, brachial blood pressure measurements, and CIMT. The Framingham 10-year general CVD risk prediction was calculated using a previously described method (Wilson et al., 1998). Baseline laboratory measurements were performed by the National Health Laboratory Service (NHLS). The following markers were measured: total cholesterol, HDL-C, LDL-C, triglycerides, glucose, glycated haemoglobin (HbA1c), gamma-glutamyl transferase (GGT), highly sensitive C-reactive protein (hsCRP), haemoglobin (Hb), and urine albumin: creatinine ratio. The NHLS also performed the HIV related measurements which included measurements of the viral load and CD4 count. The procedures and rationale behind the EndoAfrica study were discussed in detail in a separate study protocol (Strijdom et al., 2017).

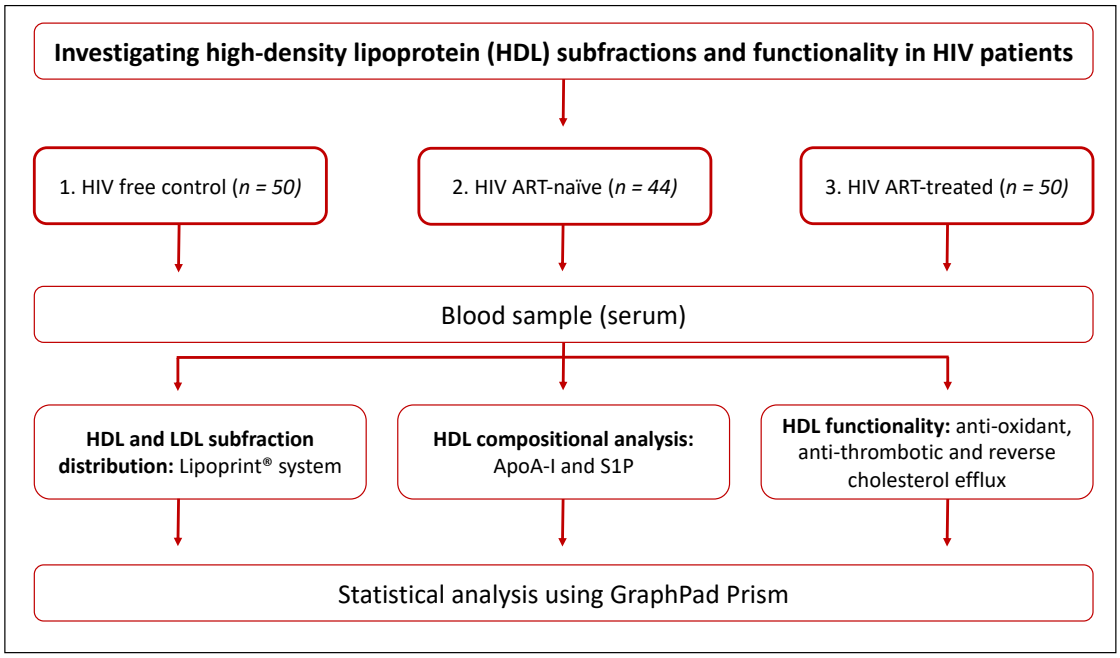


Figure 10. Flow diagram representing the methods of this study.

3.2 ApoA-I and apoB measurements

ApoA-I is the most common apolipoprotein for HDL and apoB is the only apolipoprotein for LDL. They both play a critical role in the function and stability of their associated lipoproteins. For this reason, it is necessary to measure the concentrations of these lipoproteins to assess the composition of these lipoproteins (Phillips, 2013; Ridgway & McLeod, 2015). Furthermore, apoA-I, apoB and their ratio are useful predictors of cardiovascular risk (Kohli-Lynch et al., 2020). ApoA-I and apoB were measured using electrochemiluminescence on the Cobas e501 analyser from Roche Diagnostics (Roche, Rotkreuz, Switzerland) at the University of Geneva, Switzerland.

3.3 S1P measurement

S1P is an important phospholipid and many of the atheroprotective functions of HDL have been linked to it (Maceyka et al., 2012). S1P concentrations in HDL were measured to explore if HIV and/or ART leads to changes in HDL composition. This was done using liquid chromatography tandem mass spectrometry system (LC-MS/MS). Isolated HDL was diluted 1:9 in MeOH containing 10ng/mL internal standard (S1P-d7, Avanti Polar Lipids Inc.

Alabaster, AL, USA) before injection into the LC-MS/MS (Ultimate 3000 LC Series, Thermo Fisher Scientific Inc., Whaltham, MA, USA) and 5500 QTrap® triple quadrupole linear ion trap system equipped with a TurboIon Spray™ interface (AB Sciex, Framingham, MA, USA). Data acquisition and analysis were performed using AnalystTM software (version 1.6.2; AB Sciex, Framingham, MA, USA). For further details, see a detailed description of the measurement of S1P concentration by LC-MS/MS (Brinck et al., 2016).

3.4 HDL isolation

In order to accurately quantify the HDL reverse cholesterol efflux capacity, HDL was isolated from serum using the density-shift ultracentrifugation technique as described previously (Woudberg et al., 2016). Briefly, 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 10000g 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). After which, sodium bromide (275.5 mg/ml of dialysed sample) was dissolved into the 200µl sample and then it was transferred to thick-wall polycarbonate ultracentrifuge tubes (Beckman, 343775) and centrifuged at 223 000g for 20 hours at 4°C. The upper 70 µl layer was extracted and purity was confirmed using reducing 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) stained with Coomassie Blue. The protein concentration of HDL was determined by the modified Lowry method (Markwell et al., 1978).

3.5 Quantification of HDL reverse cholesterol efflux capacity

The primary function of HDL is reverse cholesterol efflux which is the removal and transport of cholesterol from peripheral tissues and cells by HDL to the liver (Glomset, 1968). HDL induced reverse cholesterol efflux was therefore measured using a [^{3}H] cholesterol radioactive method first described in depth by Sankaranarayanan et al and then modified for the use of RAW267.7 by Woudberg et al (Sankaranarayanan *et al.*, 2011; Woudberg *et al.*, 2018). RAW264.7 (macrophage cells derived from mouse blood) cells were cultured in RPMI-1640 media supplemented with 10% foetal calf serum and 1% penicillin/streptomycin. Cells were seeded at a ratio of 100000 cells per 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 incubated in labelling media for 24 hours. Cells were then washed with minimum essential eagle (MEM) in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer before the addition of 25 µg/ml of isolated HDL in MEM-HEPES for 4 hours. Counts per minute (CPM) were measured using TriCarb® Liquid Scintillation Analyzer and QuantaSmartTM 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.

3.6 PON-1 activity assay

PON-1 is the main contributing factor to the antioxidant potential of HDL (Mackness, Durrington & Mackness, 2004), therefore a PON-1 activity assay was performed using the serum samples as a measure of the antioxidant function of HDL. Paraoxon-ethyl substrate (1.0mM; Sigma, D9286) was freshly prepared in phosphate assay buffer containing 2 mmol/L CaCl2 (pH 8). Serum samples were diluted 1:10 (10µl of serum in 90µl of assay buffer). Enzymatic catalysis of the paraoxon-ethyl substrate was quantified by absorbance at A_{405} readings at 30s intervals over 20 minutes. One unit of activity was defined as 1 nmol of substrate hydrolysed per minute (Woudberg et al., 2016).

3.7 PAF-AH activity assay

The PAF-AH enzyme is a structural component of HDL and its role is to regulate PAF, a potent platelet activator (Stafforini et al., 1987). Therefore a PAF-AH activity assay was performed using the serum samples as a measure of the anti-thrombotic function of HDL. Extracellular PAF-AH activity was measured in participant sera using the PAF Acetylhydrolase Assay Kit according to manufacturer instructions (Abcam, ab133088). Briefly, serum was diluted and added to an equal volume of 5,5'-dithio-bis-(2-nitrobenzioic acid) (DTNB; Ellman's Reagent). Catalysis of 20-thio PAF substrate was quantified at A_{412} in 1 minute time intervals for 20 minutes. One unit of activity was defined as 1 µmol of substrate hydrolysed per minute (Woudberg et al., 2016).

3.8 Western blotting

Western blotting was used to assess PON-1 and PAF-AH protein expression. Serum samples from each of the patients were electrophoresed on reducing 12.5% SDS-PAGE gels with 8µg of serum protein loaded per well. Samples were run over multiple separate gels with a control sample repeated in each gel. Blots were transferred onto nitrocellulose membranes (Bio-Rad, 162-0113). To validate equal loading of wells, Ponceau S staining was scanned. Blots were blocked in 5% bovine serum albumin (BSA) in 0.05% Tween in Tris-buffered Saline (TTBS, pH 7.5) before overnight incubation in primary mouse anti-PON-1 antibody (1:200) (James et al., 2010) and mouse anti-PAF-AH (1:2000) (ThermoFisher, MA5-26672). Blots were then washed in tween in tris-buffered saline (TTBS) and incubated in goat anti-mouse-HRP conjugated secondary antibody (1:5000; Bio-Rad, 170 6516) for 1 hour at 4°C. Blots were washed thoroughly in TTBS and then incubated in Amersham TM ECLTM Western Blotting detection reagent (GE Healthcare, RPN2106). The GeneGnome gel imager was used to capture the blots and then densitometry quantification was done using Quantity One software. PON-1 and PAF-AH relative expression data were corrected for the control sample (Woudberg et al., 2016).

3.9 Quantification of HDL subfraction distribution

The Lipoprint HDL system® (Quantimetrix, Redondo Beach, CA) was utilised to quantify the HDL subfractions (Filippatos et al., 2008). The system, which works by polyacrylamide gel electrophoresis, was used following the manufacturer's instructions: 25µl serum was mixed with Lipoprint loading gel. Sudan black dye, a component of the gel, binds proportionally to the relative amount of cholesterol present in each lipoprotein (Muniz, 1977). The mix was placed upon the upper part of the high resolution 3 % polyacrylamide gel. After allowing the loading gel to photopolymerise for 30 min at room temperature, electrophoresis was performed for 50 minutes at 3 mA per gel tube. A quality control was included in each electrophoresis run (Liposure Serum, Lipoprotein Control, Quantimetrix, Redondo Beach, CA.). Gel tubes were scanned and analysed using the Lipoware software. The various stained lipoprotein fractions present in the sample can be determined by their mobility (Retention factor [Rf]). The VLDL and LDL stayed at the origin (Rf = 0.0) while albumin migrated as the leading reference point (Rf = 1.0). 10 HDL subfractions can be detected between these two points. HDL-1, HDL-2 and HDL-3 were grouped as large HDL; HDL-4, HDL-5, HDL-6 and HDL-7 were grouped as intermediate HDL and HDL-8, HDL-9 and HDL-10 were grouped as small HDL. Each subfraction was quantified and expressed as a percentage of total HDL.

3.10 Quantification of LDL subfraction distribution

The Lipoprint LDL system® (Quantimetrix, Redondo Beach, CA) was utilised to quantify the LDL subfractions (Hoefner et al., 2001). The assay procedure is similar to that of the HDL system however, electrophoresis was performed for 60 minutes at 3mA per gel tube. A quality control was included in each electrophoresis run (Liposure Serum, Lipoprotein Control, Quantimetrix, Redondo Beach, CA.). Gel tubes were scanned and analysed using the Lipoware software. The various stained lipoprotein fractions present in the sample can be determined by their Rf. VLDL stayed at the origin (Rf = 0.0) while HDL migrated as the leading reference point (Rf = 1.0) (figure 11). Three midbands, representing intermediate-density lipoprotein are distributed between Rf 0.09 and Rf 0.27 (C = 0.09, B = 0.17, A = 0.27). LDL1 to LDL7 are distributed between Rf 0.32 and Rf 0.64. LDL1 (Rf = 0.32) and LDL2 (Rf = 0.38) are known as large, buoyant LDL and LDL3 up to LDL7 are known as sdLDL. Each subfraction was quantified and expressed as a percentage of total LDL (Quantimetrix, 2005).

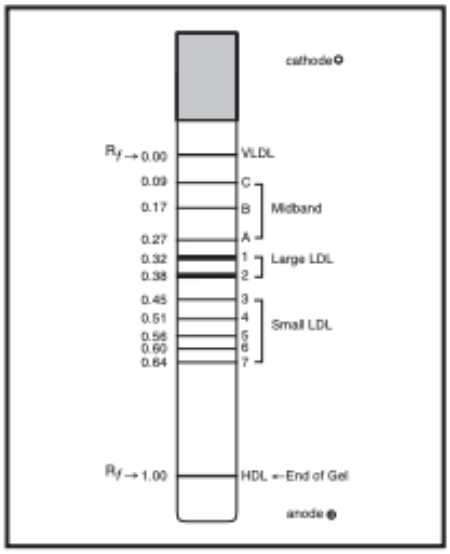


Figure 11. Mobilities of the LDL lipoprotein bands. Adapted from (Quantimetrix, 2005).

3.11 Statistical analysis

Normally distributed data including reverse cholesterol efflux capacity, PON-1 activity and the HDL and LDL subfraction data were presented as means ± standard error of mean (SEM). The Tukey test was used to compare differences between groups. Non-normally distributed data including the patient characteristics, PAF-AH activity were presented as medians ± interquartile range (IQR). K-Wallis and Mann-Whitney nonparametric testing were used to compare differences between groups. Spearman correlation co-efficients were used to explore the relationships between lipoprotein subfraction distribution, HDL function and patient characteristics. All statistical analysis was performed using GraphPad Prism® version 7.0.

3.12 Reagents

Unless it has been otherwise specified, all reagents were ordered from Sigma-Aldrich®.

CHAPTER FOUR: RESULTS

4.1 Patient clinical characteristics

The baseline characteristics of the three participant groups are reported in table 2. The majority of the patients were women representing 68% of the HIV free participants, 68% of the HIV ART-naïve patients and 70% of the HIV ART-treated patients. Patient groups did not differ in age, gender, body mass index (BMI), waist circumference, hip circumference, systolic pressure, diastolic pressure, %FMD, CIMT or Framingham 10-year general CVD risk prediction. The waist/hip ratio of the HIV ART naïve patients was higher than that of the HIV free patients ($p < 0.05$) and HIV ART-treated patients ($p < 0.001$). The majority of the patients were smokers at the time of recruitment representing 74% of the HIV free patients, 63.64% of the HIV ART-naïve patients and 62% of the HIV ART-treated patients. Some of the patients had a history of hypertension representing 8% of the HIV free patients, 14% of the HIV ART-naïve patients and 18% of the HIV ART-treated patients, however these differences were not significant. HIV ART-naïve patients presented with a higher viral load than that of the HIV ART-treated patients ($p < 0.001$) however the two groups had similar CD4 counts ($p = 0.11$) and the duration of HIV infection was similar ($p = 0.17$). 43 patients received first-line ART which included NRTIs and NNRTIs and one patient received second-line ART which was alluvia/tenofovir/lamivudine.

The baseline laboratory characteristics of the three patient groups are reported in table 3. The total cholesterol of the HIV ART-naïve patients was lower than that of the HIV ART-treated patients ($p < 0.05$). The HDL-C levels of the HIV ART-naïve patients were lower than that of the HIV free patients ($p < 0.01$) and the HIV ART-treated patients ($p < 0.01$). In contrast, LDL-C and triglycerides did not differ between groups. Basic biochemical analyses indicated higher HbA1c and urine albumin/creatinine ratio and lower Hb levels in the HIV ART-naïve patients compared to the HIV free patients (all $p < 0.05$). GGT of the HIV ART-treated patients was higher than that of the HIV free patients **(**$p < 0.001$) and the HIV ART-naïve patients ($p < 0.001$). Glucose and hsCRP were higher in HIV ART-treated patients compared to that of the HIV free patients (both $p < 0.05$). In contrast, serum creatinine, urine creatinine and urine albumin, did not differ between the three groups.

Table 2: Baseline characteristics of participants

Parameters	HIV free controls (n = 50)	HIV ART-naive (n = 44)	HIV ART-treated (n= 50)
Age	35.5 (31.0-42.0)	37.0 (28.0-44.8)	37.0 (33.0-45.5)
Sex (female, %)	68	68	70
Anthropometric measurements			
BMI (kg/m2)	21.65 (18.98-28.38)	20.65 (18.25 – 26.86)	21.90 (18.98-28.03)
Hip circumference (cm)	91.50 (82.00-108.30)	89.5 (85.25-103.8)	89.0 (83.5-104.3)
Waist circumference (cm)	81 (68-93)	83 (72-100)	77 (70-92)
Waist/hip ratio	0.86 (0.81-0.89)	0.91 (0.85-0.95) *§§§	0.84 (0.81-0.87)
Cardiovascular measurements			
Mean systolic pressure (mmHg)	115 (108-128)	119 (109-135)	118 (111-130)
Mean diastolic pressure (mmHg)	81 (74-92)	86 (78-91)	86 (76-91)
% FMD	7.05 (4.99-11.09)	4.68 (2.57-9.18)	6.42 (2.84-9.12)
Average CIMT (um)	566 (503-672)	596 (540-659)	602 (559-708)
Framingham 10-year general CVD risk prediction (%)	2.15 (1.40-3.45)	2.10 (1.40-5.20)	2.00 (1.45-6.05)
Current smoker , n (%)	37 (74)	28 (64)	31 (62)
Hypertension, n (%)	4 (8)	6 (14)	9 (18)
HIV-related data			
CD4 (cells/ul)		448 (286-619)	513 (386-711)
Viral load (RNA copies/ml)		25047 (4710-70161) §§§	20 (10-94)
HIV duration (weeks)		104 (88-164)	162 (106-234)

BMI: Body Mass Index. FMD: Flow-mediated dilation. CIMT: Carotid intima-media thickness. HIV: Human immunodeficiency virus. Results presented as medians ± interquartile range (IQR). * *p*-value <0.05 was considered significant vs HIV free subjects, §§§ <0.001 was considered significant between HIV ART-naive and HIV ART-treated.

Table 3: Baseline laboratory characteristics of participants

Parameters	HIV free controls (n = 50)	HIV ART-naive (n = 44)	HIV ART-treated (n= 50)
Total cholesterol (mmol/L)	4.22 (3.60-4.57)	3.79 (3.21-4.39) §	4.27 (3.87-4.80)
HDL-C (mmol/L)	1.33 (1.22-1.61)	1.05 (0.92-1.38) **§§	1.31 (1.04-1.78)
LDL-C(mmol/L)	2.08 (1.90-2.67)	2.23 (1.64-2.61)	2.36 (2.00-2.76)
Triglycerides (mmol/L)	0.85 (0.67-1.07)	0.86 (0.58-1.38)	0.89 (0.71-1.17)
Glucose (mmol/L)	4.60 (4.20-4.95)	4.40 (4.10-5.10)	4.90 (4.40-5.35) *
HbA1c (%)	5.10 (4.88-5.40)	5.40 (5.00-5.60) *	5.15 (4.93-5.48)
GGT (U/L)	26.00 (15.75-33.25)	24.00 (17.00-32.00)	42.00 (28.75-88) ***§§§
Hb (g/dl)	13.60 (12.75-14.40)	12.60 (11.70-14.00) *	13.10 (11.80-14.15)
Serum creatinine (umol/L)	63.00 (58.75-75.50)	59.00 (48.25-68.00)	62.5 (52-74.25)
Urine creatinine (mmol/L)	13.35 (7.9-20.1)	11.6 (8.5-16)	13.9 (8.03-19.23)
Urine albumin/creatine ratio (mg/mmol creatinine)	0.60 (0.35-1.10)	1.00 (0.47-3.20) *	0.80 (0.37-2.23)
Urine albumin (mg/L)	6.90 (1.50-19.50)	14.20 (4.00-44.40)	8.55 (4.35-28.28)
hs-CRP (mg/L)	4.10 (1.30-8.30)	5.60 (1.20-15.63)	6.85 (2.73-15.93) *

HDL-C: High-density lipoprotein cholesterol. LDL-C: Low-density lipoprotein cholesterol. HbA1c: Glycosylated haemoglobin. GGT: Gamma-glutamyl transferase. Hb: Haemoglobin. Hs-CRP: High-sensitivity C-reactive protein. HIV: Human immunodeficiency virus. Results presented as medians ± interquartile range (IQR). * *p*-value <0.05, ** <0.01, * <0.001 was considered significant vs HIV free subjects, § p value <0.05, §§ <0.01, §§§ <0.001 was considered significant between HIV ART-naive and HIV ART-treated.**

4.2 S1P and apolipoproteins

S1P did not differ between the groups. ApoA-I of the HIV ART-naïve patients was lower than both the HIV free patients ($p < 0.01$) and the HIV ART-treated patients ($p < 0.05$). ApoB and the apoB/ apoA-I ratio did not differ between groups.

Table 4: HDL compositional analysis

Parameters	HIV free controls (n = 50)	HIV ART-naive (n = 44)	HIV ART-treated (n= 50)
S1P (pmol/mg)	172.20 (141.90-200.50)	176.90 (145.80-210.80)	183.40 (137.20-215.60)
apoA-I (umol/L)	45.50 (41.00-49.125)	38.50 (35.60-43.10) **§	43.80 (37.88-51.25)
apoB (umol/L)	1.47 (1.24-1.78)	1.47 (1.15-1.76)	1.55 (1.40-1.80)
apoB/ apoA-I ratio	0.61 (0.51-0.74)	0.69 (0.50-0.86)	0.62 (0.51-0.80)

HDL: High-density lipoprotein. S1P: Sphingosine-1-phosphate. ApoA-I: Apolipoprotein A-I. ApoB: Apolipoprotein B. HIV: Human immunodeficiency virus. Results presented as medians ± interquartile range (IQR). * *p* ** <0.01, was considered significant vs HIV free subjects, § p-value <0.05 was considered significant between HIV ART-naive and HIV-ART treated.

4.3 Reverse cholesterol efflux capacity of isolated HDL

The cholesterol efflux capacity of the HDL did not differ between groups (1.75 ± 0.06 vs 1.93 ± 0.08 vs 1.87 ± 0.07% respectively, $p = 0.23$) (figure 12).

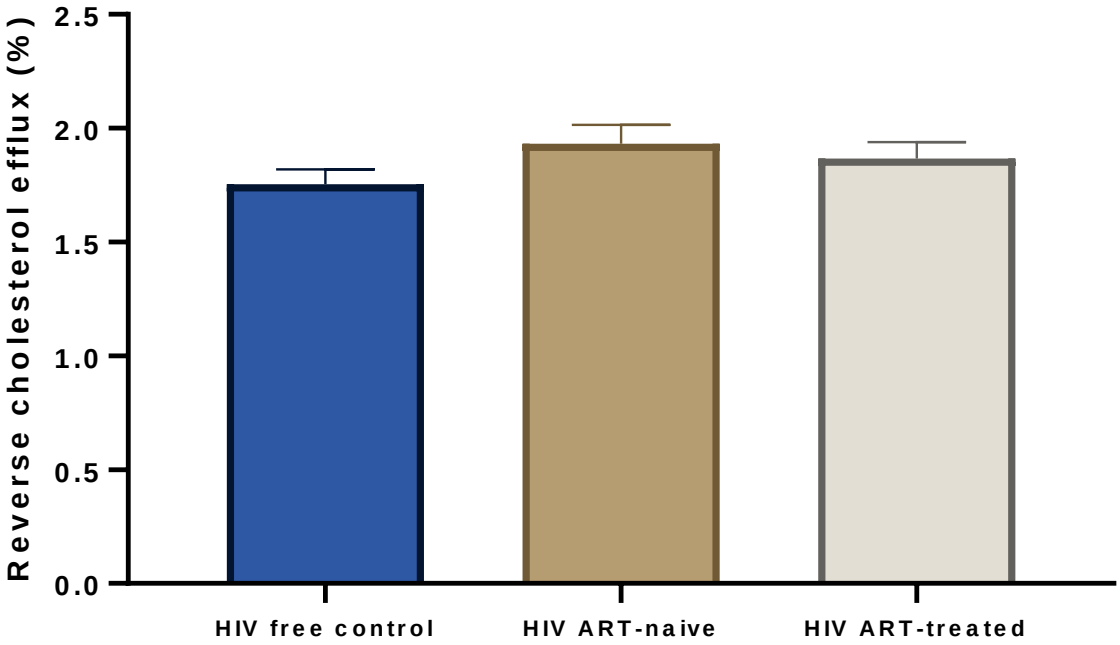


Figure 12. Reverse cholesterol efflux capacity in HIV free controls, HIV ART-naive and HIV ART-treated patients. [^{3}H-Cholesterol] was effluxed from RAW264.7 cells exposed to isolated HDL from human sera for 4 hours before scintillation counting. Cholesterol efflux represents the mean radiolabel present in culture media relative to that of an untreated control and is expressed as a percentage (%). Results represent means ± SEM.

4.4 PON-1 activity and protein expression

The serum activity of PON-1 was quantified as a measure of the antioxidative function of HDL (figure 13). PON-1 activity in HIV free control patients was 0.90 ± 0.05 U/L, which was higher than the range previously reported for white Europeans (Kunutsor et al., 2016) but in a similar range to data previously reported in the literature for black and mixed-race Africans (Macharia, Kengne, Blackhurst, Erasmus & Matsha, 2014; Woudberg et al., 2016). PON-1 activity did not differ between the groups (0.90 ± 0.05 vs 0.79 ± 0.06 vs 0.85 ± 0.04 U/L respectively, $p = 0.30$, figure 2A). Western blotting was performed on the serum to determine if there were any differences in PON-1 protein expression. Changes in serum PON-1 protein expression did not differ between the groups (0.80 ± 0.47 vs 0.82 ± 0.51 vs 0.68 ± 0.45 units respectively, $p = 0.510$, figure 2B &C). The association between PON-1 activity and expression was explored in all participants and serum PON-1 activity was not associated with PON-1 protein expression ($r = 0.16$, $p = 0.07$).

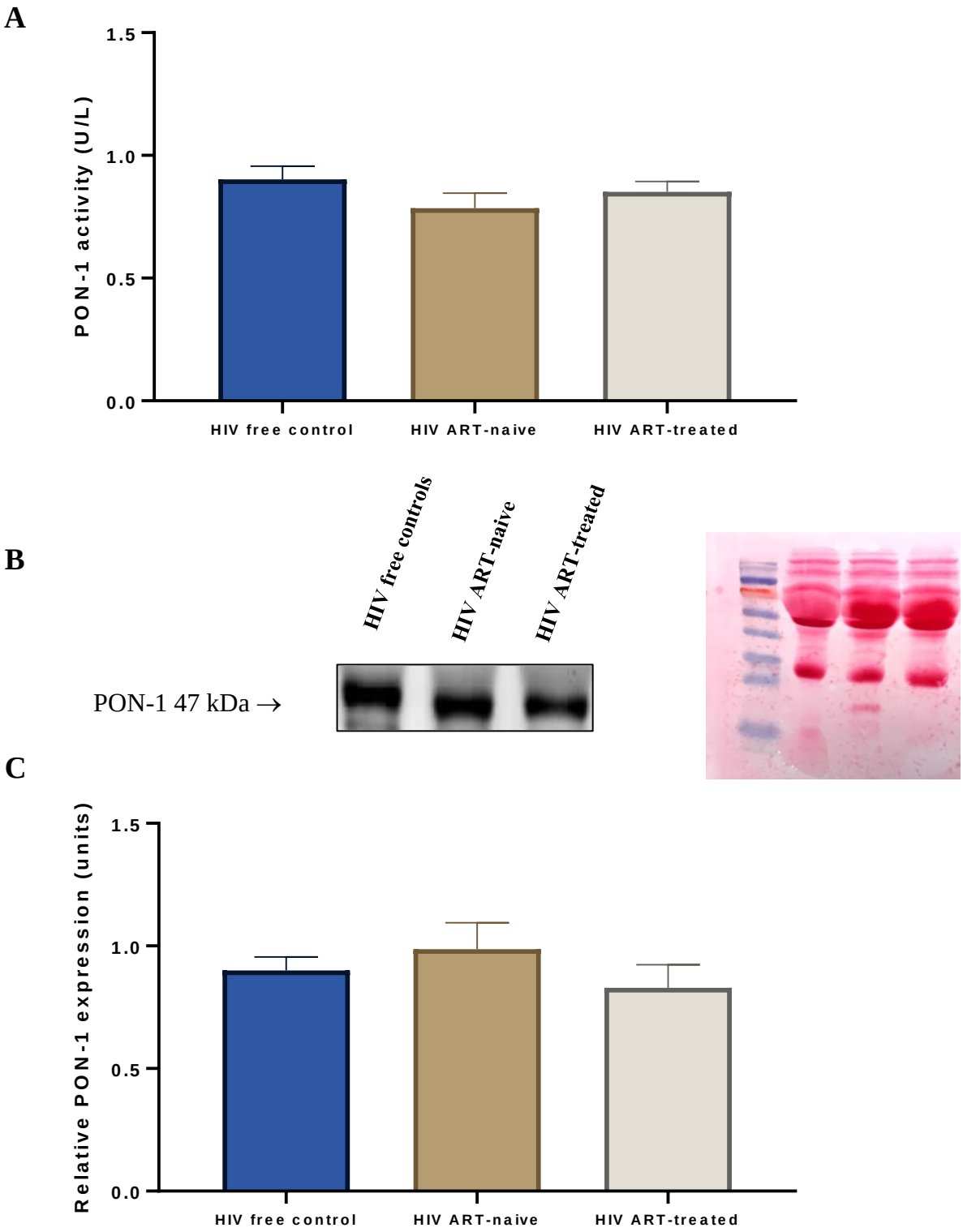


Figure 13. PON-1 activity and expression in HIV free controls, HIV ART-naive and HIV ART-treated patients (A). Patient sera (B-C) were run on reducing 12.5% SDS-PAGE gels and transferred to nitrocellulose membranes. Ponceau S staining confirmed equal loading. Blots were probed with mouse anti-PON1 antibody. Results are representative of one of the experiments (B). Densitometry of PON-1 expression in patient sera (C). Results represent means ± SEM.

4.5 PAF-AH activity and protein expression

The serum activity of PAF-AH was quantified as a measure of the anti-thrombotic function of HDL (figure 14). Serum PAF-AH activity did not differ between the groups 1.29 ± 0.40 vs 1.24 ± 0.64 vs 1.21 ± 0.47 U/L respectively, p = 0.70, figure 3A). Western blotting was performed on the serum to determine if there were any differences in PAF-AH protein expression. Changes in serum PAF-AH protein expression did not differ between the groups (1.70 ± 1.35 vs 1.16 ± 1.01 vs 1.17 ± 1.20 units respectively, p = 0.17 , figure 3B & C). The association between PAF-AH activity and expression was explored in all participants and serum PAF-AH activity was positively associated with PAF-AH protein expression (r = 0,21, p = 0.018).

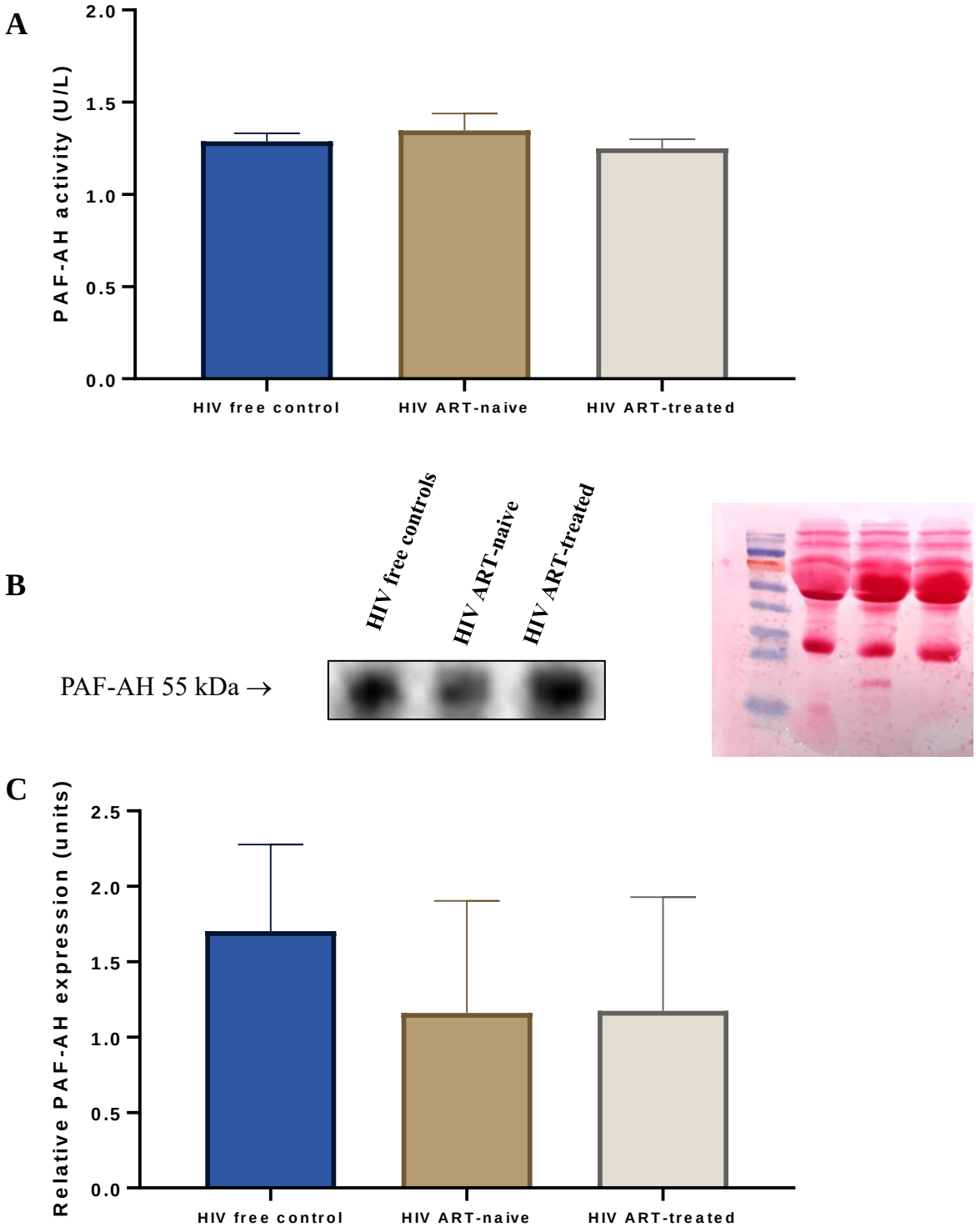


Figure 14. PAF-AH activity and expression in HIV free controls, HIV ART-naive and HIV ART-treated patients (A). Patient sera (B-C) were run on reducing 12.5% SDS-PAGE gels and transferred to nitrocellulose membranes. Ponceau S staining confirmed equal loading. Blots were probed with mouse anti-PAF-AH antibody. Results are representative of one of the experiments (B). Densitometry of PAF-AH expression in patient sera (C). Results represent medians ± IQR.

4.6 HDL subfraction distribution

Figures 15 and 16 show the distribution of HDL subfractions, quantified using the Lipoprint® System. By quantifying scans (figure 15A), unique HDL subfraction profiles were produced (figure 15B-E). The distribution of large, intermediate and small HDL did not differ between the groups (figure 16A) however there were differences in certain subfractions in ART-naïve patients compared to control patients (figure 16B-D). The percentage of HDL-1, the largest HDL subfraction (figure 16B) was higher in ART-naïve patients compared to control patients (12.46 ± 0.95 vs 9.43 ± 0.62%, $p < 0.05$) and there was a tendency of ART-treated patients to have more HDL-1 compared to control patients (11.97 ± 0.82 vs 9.42 ± 0.62%, $p = 0.06$). The percentage of HDL-5, an intermediate subfraction (figure 16C), was higher in ART-naïve patients compared to control patients (12.77 ± 0.39 vs 11.49 ± 0.27%, $p < 0.05$). The ratio of HDL-10/HDL-1 (figure 16E), was significantly lower in ART-naive patients compared to control patients (0.26 ± 0.40 vs 0.45 ± 0.69 units, $p < 0.05$). No difference in HDL subfractions between ART-treated and ART-untreated patients was observed.

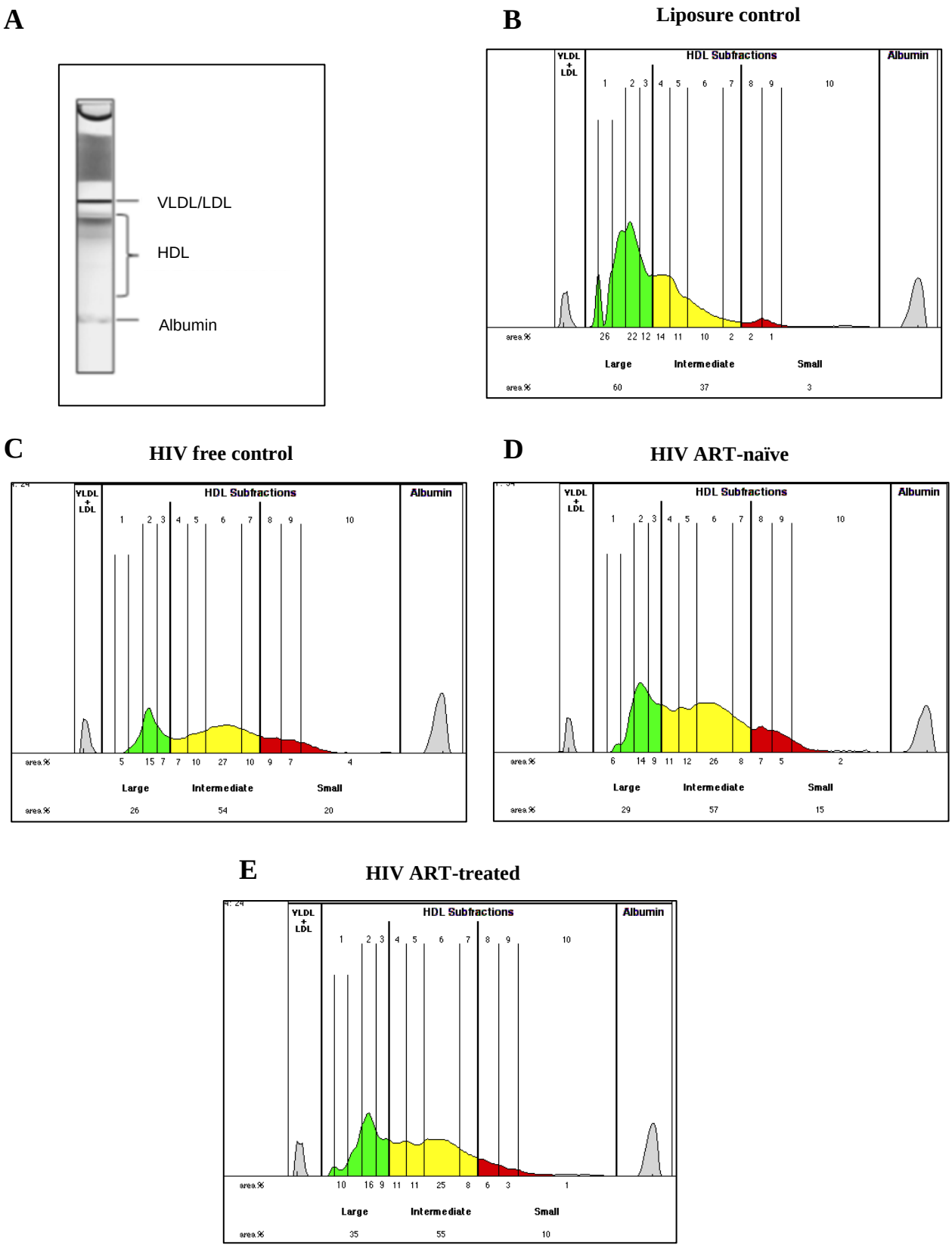


Figure 15. Examples of the scan results of patient HDL subfraction distributions from each group. The patient sera were analysed using the Lipoprint® system and Lipoware software. Representative scan (A) and scan result (B) of Liposure control. Representative scan results from HIV free control, HIV ART-naïve and HIV ART-treated patients (C-E, respectively).

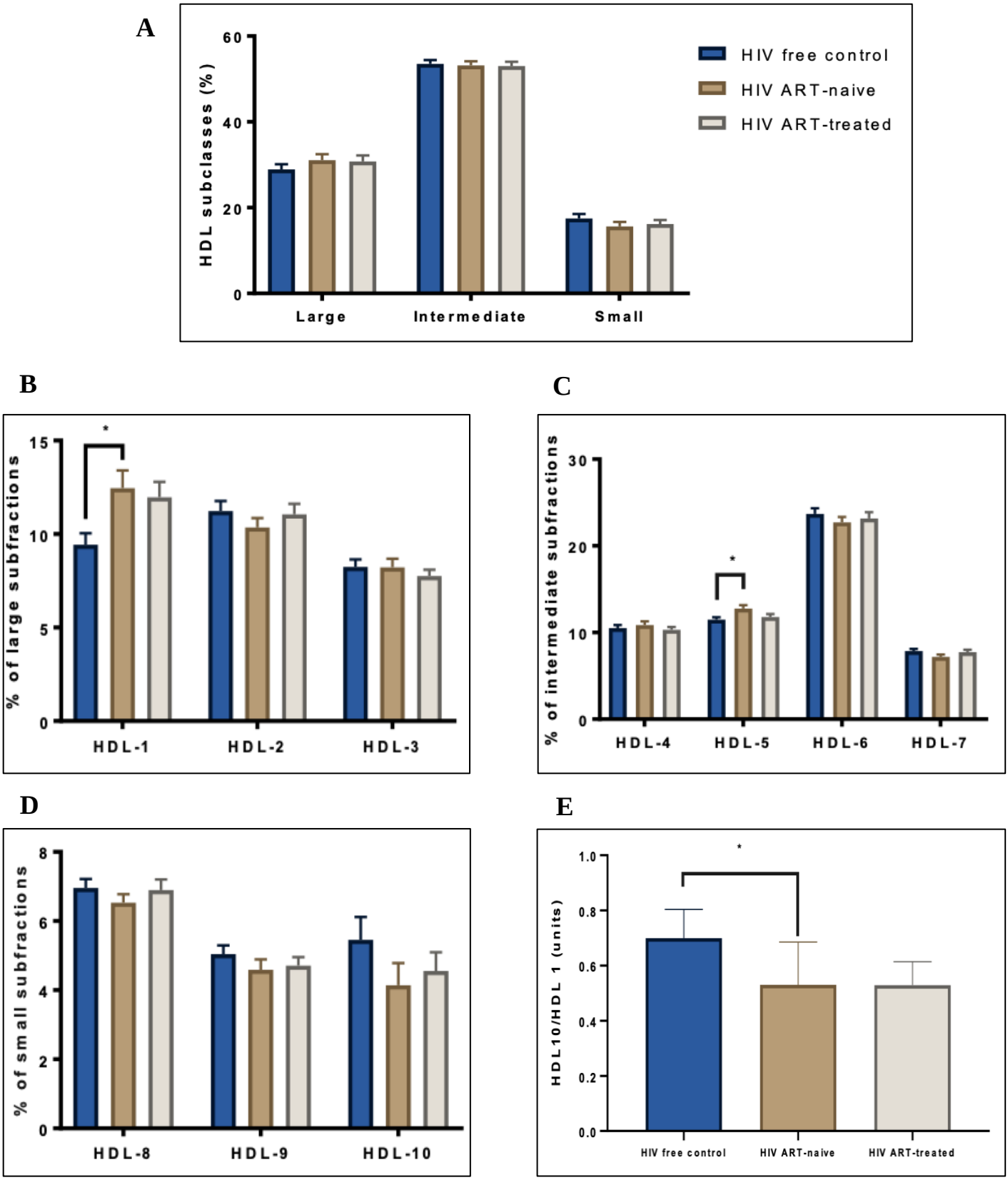
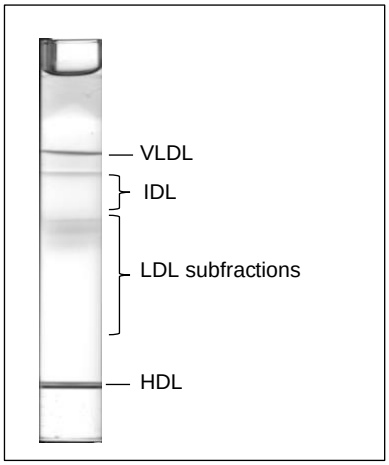


Figure 16. HDL subfraction distribution. Percentages of large, intermediate and small HDL subclasses (A). Percentages of large HDL subfractions (B), intermediate HDL subfractions (C) and small HDL subfractions (D). The ratio of HDL-10/HDL-1 in arbitrary units (E). Results represented as means ± SEM for A, B, C. D and medians ± IQR for E. * $p < 0.05$.

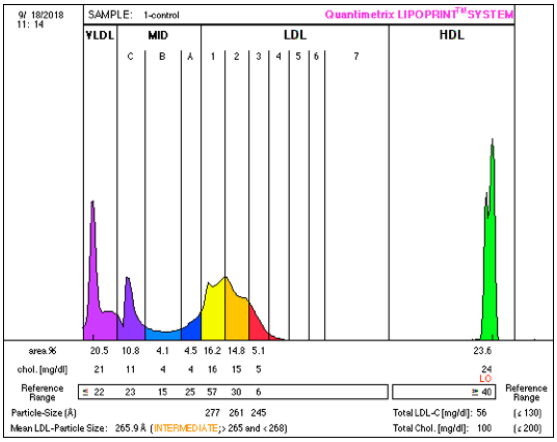
4.7 VLDL, LDL and IDL subfraction distribution

Figures 17 and 18 show the distribution of LDL subfractions, quantified by the Lipoprint® System. By quantifying scans (figure 17A), unique LDL subfraction profiles were produced (figure 17B-E). Although the distribution of VLDL (figure 18A) did not significantly differ between groups, there was a tendency of HIV ART naïve patients to have a higher percentage of VLDL compared to control patients (19.77 ± 0.72 vs 17.96 ± 0.44%, $p = 0.07$). The distribution of IDL-A and IDL-C did not differ between groups however the percentages of IDL-B in the HIV ART-naïve participants were higher compared to HIV free control patients (6.7 ± 3.2 vs 5.2 ± 1.75%, $p < 0.001$) and HIV ART-treated patients (6.6 ± 3.25 vs 5.2 ± 1.75%, $p < 0.05$). The percentages of LDL-1 (the largest and most buoyant LDL subfraction) were higher in HIV free controls compared to both the HIV ART-naïve group (18.89 ± 0.47 vs 16 ± 0.57%, $p < 0.01$) and the HIV ART-treated group (18.89 ± 0.47 vs 17.06 ± 0.62%, $p < 0.05$). The distribution of LDL-2 did not differ between the groups ($p = 0.24$). The particle size of the LDL did not differ between the groups ($p = 0.25$). Only a small number (n = 7) of participants presented with pattern B lipoprotein profiles (predominantly LDL-3 to LDL-7), of those one participant was from the HIV free control group, two were from the HIV ART-naïve group and four were from the HIV ART-treated group.

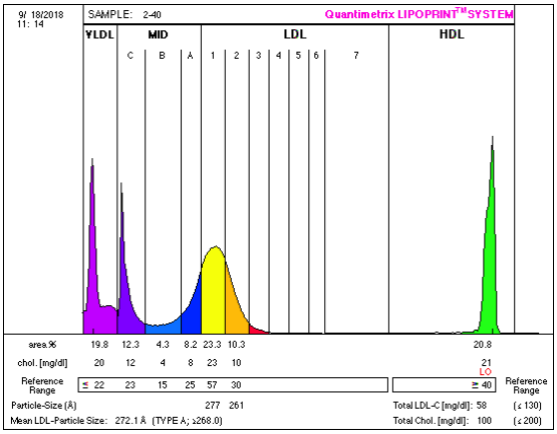
A



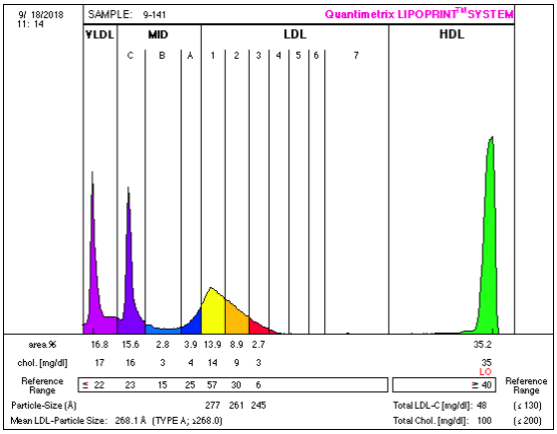
B **Liposure control**



C **HIV free control**



D **HIV ART-naïve**



E **HIV ART-treated**

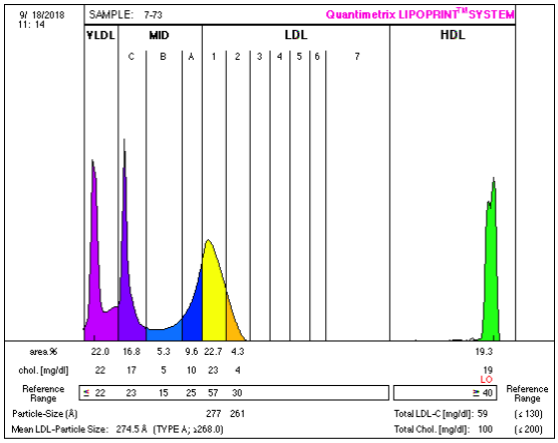


Figure 17. Examples of the scan results of patient LDL subfraction distributions from each group. The patient sera were analysed using the Lipoprint® system and Lipoware software. Representative scan (A) and scan result (B) of Liposure control. Representative scan results from HIV free control, HIV ART-naïve and HIV ART-treated patients (C-E, respectively).

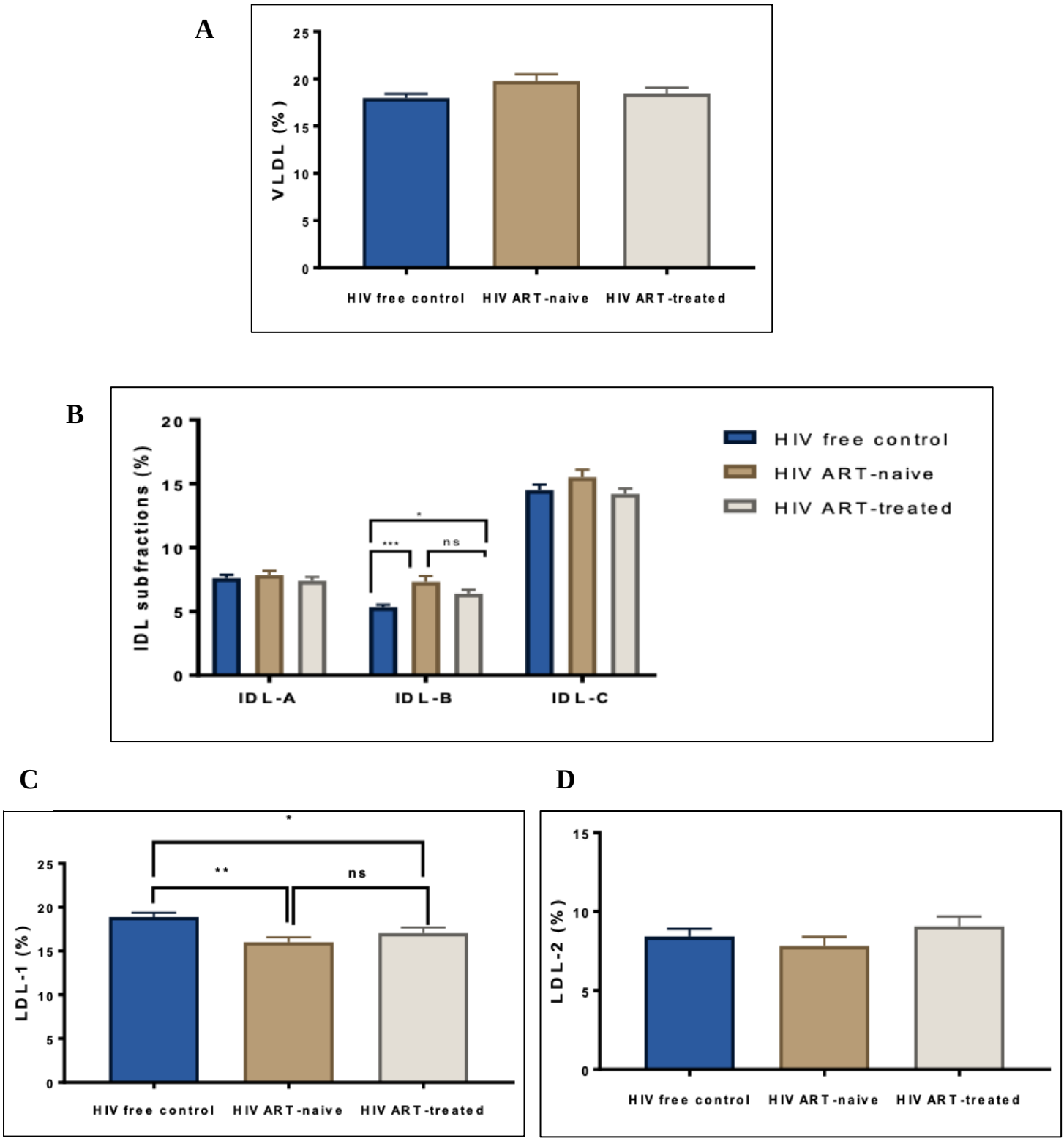


Figure 18. LDL subfraction distribution. The percentages of VLDL (A), LDL-1 (B) LDL-2 (C) and IDL subfractions (D). Results represented as means ± SEM. * p < 0.05. ** p < 0.01. *** p < 0.001

4.8 Relationships between measures of HDL composition, functionality and subclass distribution and patient characteristics

To explore if HDL subclass/subfractions, composition and functionality are associated with any differences in patient characteristics, all patients were combined and Spearman correlation coeffecients were calculated and presented in a matrix (table 4). Increased age was associated with increased BMI (r = 0.181, $p < 0.05$), CIMT (r = 0.483, $p < 0.001$), GGT (r = 0.230, $p < 0.01$), CRP (r = 0.205, $p < 0.05$) , apoA-I (r = 0.211, $p < 0.05$) and an increase in the percentage of small HDL (r = 0.179, $p < 0.05$). Increased BMI was associated with decreased HDL-C (r = -0.165, $p < 0.05$), S1P (r = -0.215, $p < 0.01$), viral load (r = -0.223, $p < 0.05$), reverse cholesterol efflux capacity (r = -0.245, $p < 0.01$) and large HDL (r = -0.309, $p < 0.001$) as well as increased CD4 count (r = 0.329, $p < 0.01$) and small HDL (r = 0.296, $p < 0.001$). Increased CIMT was associated with increased GGT (r = 0.232, $p < 0.01$), CRP (r = 0.268, $p < 0.01$) and PON-1 activity (r = 0.184, $p < 0.05$). Increased HDL-C was associated with decreased CRP (r = -0.172, $p < 0.05$) and viral load (r = -0.259, $p < 0.05$). Increased S1P content was associated with decreased apoA-I (r = -0.222, $p < 0.01$) and reverse cholesterol efflux capacity (r = -0.185, $p < 0.05$) as well as increased HIV duration (r = 0.334, $p < 0.05$). Increased apoA-I was associated with a decrease in viral load (r = -0.249, $p < 0.05$) and in the percentage of intermediate HDL (r = -0.510, $p < 0.001$) as well as an increase in ART duration (r = 0.309, $p < 0.05$), the percentage of large HDL (r = 0.290, $p < 0.001$), reverse cholesterol efflux capacity (r = 0.218, $p < 0.05$) and PON-1 activity (r = 0.177, $p < 0.05$). Increased CD4 counts were associated with a decrease in the percentage of large HDL (r = -0.279, $p < 0.01$) and an increase in the percentage of small HDL (r = 0.333, $p < 0.01$). Increased PON-1 activity was associated with decreased viral load (r = -0.223, $p < 0.05$) and an increase in the percentage of small HDL (r = 0.178, $p < 0.05$).

On further investigation of individual HDL subfractions, increased viral load was associated with a decrease in the percentage of HDL-7 and HDL-8 (r = -0.241 and r = -0.210, $p < 0.05$) and increased CRP was associated with a decrease in HDL-8 and HDL-9 (r = -0,220 and r = -0,192, $p < 0.05$) (data not shown in table 5).

Table 5. Associations between HDL subclass measures, composition and functionality with patient characteristics

	Age	BMI	CIMT	HDL-C	GGT	CRP	S1P	apoA-I	CD4	Viral load	HIV duration	ART Duration	Large HDL	Int HDL	Small HDL	RCE	PON-1	PAF-AH
Age		**0,181***	**0,483*****	0,072	**0,230****	**0,205***	-0,017	**0,211***	0,006	-0,055	0,103	**0,330***	-0,153	0,023	**0,179***	-0,056	0,010	0,012
BMI			0,043	**-0,165***	-0,091	0,109	**-0,215****	-0,059	**0,329****	**-0,223***	0,061	0,186	**-0,309*****	0,087	**0,296*****	**-0,245****	0,092	-0,096
CIMT				-0,090	**0,232****	**0,268****	0,048	-0,089	-0,021	-0,097	-0,081	0,099	-0,104	0,035	0,057	-0,048	**0,184***	0,078
HDL-C					0,121	**-0,172***	**-0,224****	**0,893*****	0,052	**-0,259***	-0,139	0,283	**0,496*****	**-0,559*****	**-0,140***	**0,233***	0,169*	-0,164
GGT						**0,307*****	-0,015	**0,173***	**-0,212***	**-0,257***	-0,110	0,179	-0,102	0,045	0,047	0,153	0,119	0,058
CRP							0,052	-0,143	**-0,248***	0,003	0,042	0,065	-0,038	0,156	-0,086	-0,112	-0,151	-0,045
S1P								**-0,222****	-0,003	0,188	**0,344***	-0,054	0,012	0,057	-0,077	**-0,185***	-0,064	0,042
apoA-I									0,079	**-0,249***	0,010	**0,309***	**0,290*****	**-0,510*****	0,053	**0,218***	**0,177***	-0,168
CD4										**-0,432*****	0,072	-0,112	**-0,279****	0,037	**0,333****	-0,140	0,156	-0,107
Viral load											-0,013	-0,122	0,136	-0,036	-0,144	-0,077	**-0,223***	-0,095
HIV duration												0,207	0,042	0,057	-0,020	-0,281	-0,042	0,194
ART Duration													0,045	-0,152	0,069	-0,233	0,006	-0,084
Large HDL														**-0,627*****	**-0,704*****	0,112	-0,119	-0,009
Int HDL															-0,033	-0,124	-0,045	0,085
Small HDL																-0,020	**0,178***	-0,072
RCE																	0,005	-0,058
PON-1																		-0,149
PAF-AH																		

Values are Spearman correlation coefficients. BMI: Body mass index. CIMT: Carotid intima-media thickness. HDL-C: High-density lipoprotein cholesterol. GGT: Gamma-glutamyl transferase. CRP: C-reactive protein. S1P: Sphingosine-1-phosphate. ApoA-I: Apolipoprotein A-I. HIV: Human immunodeficiency virus. ART: Antiretroviral therapy. RCE: Reverse cholesterol efflux. PON-1: Paraoxonase-1. PAF-AH: Platelet-activating factor acetylhydrolase. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

CHAPTER FIVE: DISCUSSION

The aim of this proof-of-concept study was to explore whether HIV and/or ART is associated with differences in HDL subfraction distribution, function and composition in a South African population. Our data highlight that, HIV ART-naïve patients displayed a shift in HDL subfraction distribution, with increased percentages of HDL-1 and HDL-5 and a decrease in the ratio of HDL-10-/HDL-1 compared to HIV free patients. They also displayed a change in HDL composition, with decreased levels of apoA-I compared to both HIV ART-treated patients and HIV free patients. Although there were no significant differences in HDL function, small HDL was associated with increased CD4 count, increased PON-1 activity and decreased viral load.

CVD is becoming increasingly prominent within the growing HIV-infected population (Keates et al., 2017). Even though Africa is the epicentre of HIV infection, there is a lack of information from this continent (Hyle et al., 2017). Possible reasons for their increased CVD risk include a higher presence of traditional risk factors, chronic immune activation, inflammation and oxidative stress (Fitch et al., 2013; Haser & Sumpio, 2017). HIV itself and ART are associated with dyslipidaemia which is a common risk factor for CVD (Friis-Møller et al., 2003; Yusuf et al., 2004; Feeney & Mallon, 2011). A meta-analysis conducted in 2013 on data from PLWH in SSA found that HIV was associated with lower HDL-C levels but ART treatment was associated with higher HDL-C levels (Dillon et al., 2013). Similarly, within our cohort, it was only the HIV ART-naïve patients that showed abnormal lipid levels. The HDL-C levels of the HIV ART-naive patients were lower than 1.28 mmol/L, which is traditionally considered to suggest an increased risk for CVD (Miller et al., 1977; F. Piepoli, 2017). Higher levels of HDL-C were detected in the ART-treated patients and it has been suggested that HDL-C levels may return to baseline as patients improve clinically (Dave et al., 2016). This may explain why the HDL-C of our ART-treated patients were similar to that of our control patients. Furthermore, increased HDL-C was associated with a decrease in viral load and CRP levels, thus suggesting that increased HDL-C is linked to reduced systemic inflammation and virological control. Indeed, HDL has been shown to inhibit the inflammatory effect of CRP via its anti-inflammatory properties (Wadham et al., 2004). Therefore, the likely reason for the negative association seen between HDL-C and CRP in this study is not simply due to an increase in the quantity of HDL-C but rather a result of its anti-inflammatory function. However, this hypothesis would need further testing as we were unable to assess the anti-inflammatory function of HDL in this study.

The majority of ART-treated patients were on first-line treatment in the form of a fixed-dose combination drug that consisted of tenofovir, emtricitabine and efavirenz. Tenofovir (an NRTI) is not associated with dyslipidaemia (Kelesidis & Currier, 2014), whereas Efavirenz (an NNRTI), on the other hand, is known for its adverse effects on lipid and glucose metabolism (Sinxadi et al., 2016). Therefore, it is important in this study to assess for any differences in the lipid profile and lipoprotein subfraction distributions in the HIV ART-treated patient group compared to HIV ART-naïve and HIV free control groups. Although the HIV ART-treated patients had no differences in their lipid profile, there were other differences in their biochemical markers that did indicate potential chronic, low-grade inflammation and oxidative stress. Indeed, HIV ART-treated patients had higher CRP levels compared to HIV free patients which indicate chronic, systemic inflammation. CRP is associated with CVD risk and all-cause mortality both in the general population and in HIV-infected patients (Guimarães et al., 2008; Li et al., 2017). The HIV ART-treated patients also had higher GGT compared HIV free patients and HIV ART-naive patients. GGT is a liver enzyme that mainly acts to maintain glutathione homeostasis and is a well-known marker of generalized oxidative stress (Drozdz et al., 1998). Interestingly, the Prospective Urban and Rural Epidemiology (PURE) study with HIV patients in the North West Province of South Africa monitored over a period of 10 years, also reported marked increased levels of CRP and GGT in HIV ART-treated patients (Phalane et al., 2019). These findings indicate that ART may cause chronic, low-grade inflammation and oxidative stress. Of note, GGT is also a known marker of alcohol-related liver disease, therefore it is possible that alcohol use may have an effect on these results (Lucien et al., 2010).

HDL is highly heterogeneous (Mahley et al., 1984; Camont, Chapman & Kontush, 2011) and recent evidence suggests that the quality of HDL is more important than its cholesterol concentration (see reviews, (Rizzo *et al.*, 2014; Santos-Gallego, 2015; Woudberg *et al.*, 2018)). Therefore, it is surprising that relatively few studies have considered that HIV and ART may lead to a shift in HDL subfraction distribution and an associated reduction in HDL function. It is also necesscary to examine whether compositional remodelling has an effect on the loss of function of HDL. Alterations in the content and/or conformation of important apolipoproteins such as apoA-I and key lipids such as S1P may be associated with decreased HDL functionality. Many of HDL's atheroprotective functions have been shown to be associated with S1P. The lipid's protective effects include preventing damage against ischaemic injury (Theilmeier et al., 2006; Frias et al., 2012), reducing drug-induced cytotoxicity (Kimura et al., 2001; Kontush et al., 2007), preventing LDL oxidation (Kontush et al., 2007) and protecting

endothelial function (Fan et al., 2020). In this study, there was no difference in the levels of S1P between groups and the levels were in a similar range to data previously reported in the literature (Brinck et al., 2016). Although S1P concentration did not significantly correlate with any of the HDL functions measured in this study, the physiological levels observed in our study may be one of the reasons why there were no differences seen in HDL functionality. Similarly, apoA-I is the most common apolipoprotein associated with HDL and it is also the most important in terms of structural integrity and function (Gauthamadasa et al., 2010). As such, it has been proposed as a more accurate measure of cardiovascular risk than HDL-C. Similar to that of HDL-C, the levels of apoA-I were lower in HIV ART-naïve patients compared to both HIV ART-treated patients and HIV free control patients. Higher levels of apoA-I were associated with a lower viral load and a higherc reverse cholesterol efflux capacity and PON-1 activity suggesting that it does play a role in moderating HIV and HDL functionality.

Cholesterol efflux capacity, the major function of HDL *in vivo*, did not differ between groups. The HIV protein, Nef, disrupts the efflux of cholesterol by HDL, thus allowing for more cholesterol to be redirected to pro-atherogenic, apoB containing lipoproteins (Mujawar et al., 2006; Rose et al., 2008). However, ART, especially newer first-line treatment, reduces levels of Nef which may explain why ART-treated individuals have relatively normal efflux capacity (Toribio et al., 2017; Ferdin et al., 2018). It is also still unclear how long one needs to be infected with HIV for a significant reduction in function. In our study, the ART-naïve participants had only tested positive for HIV approximately 104 weeks before serum collection. Nef binds to ABCA1 causing a cascade resulting in its downregulation (Zheng et al., 2003), and it would be useful to include measurements of the levels of Nef and the expression of ABCA1 in future studies assessing HIV and cholesterol efflux.

Similarly, there were no differences in PON-1 activity or PAF-AH activity between groups nor any difference in the expression of these proteins between groups. Small HDL is enriched in PON-1 and PAF-AH which may confer greater function in comparison with larger subclasses (Davidson et al., 2009; Gugliucci & Menini, 2015; Perségol et al., 2018). This is confirmed in our study as we observed a positive correlation between small HDL and PON-1. PON-1 is a powerful antioxidant and decreased activity of this enzyme has been associated with HIV, obesity, renal failure and other oxidative stress-related situations (Ferretti et al., 2005; Siegel et al., 2015; Chistiakov et al., 2017). However, the exact mechanism by which PON-1 function is altered in these situations is still unknown. There have only been a few studies that have

explored the association between HIV and PON-1 and none of them were conducted in PLWH in Africa. Previous studies have reported decreased PON-1 activity in HIV positive patients, especially those who are ART-naïve (Siegel et al., 2015) and some have shown that ART-treated patients show partially recovered or normal PON-1 levels compared to HIV free control patients (Pereira et al., 2009; Marinho et al., 2016).

Interestingly, the PON-1 activity of all the groups in this study was higher than the range previously reported for white Europeans (Kunutsor et al., 2016). Higher PON-1 activity levels have also been reported in black South African women compared to white South African women (Woudberg et al., 2016). Furthermore, in a Portuguese study, the PON-1 activity of HIV positive black patients was higher than that found in white patients regardless of treatment (Pereira et al., 2009). Polymorphisms in the PON-1 gene, can cause a reduction in PON-1 activity however, this is less frequent in black and Asian populations (Phuntuwate et al., 2005; Mackness & Mackness, 2015). Genotyping and Western Blotting of isolated HDL would also need to be performed to support this data. Therefore it has been hypothesized that the lower incidence of myocardial infarction in black African populations compared to white populations (Sliwa et al., 2012) may be explained by higher PON-1 activity levels. However, this hypothesis still needs to be tested and may not apply to all South African populations especially since a study in a mixed-race South African population (similar to this study's cohort) found that PON-1 activity was not associated with CVD risk (Macharia, Kengne, Blackhurst, Erasmus, Hoffmann, et al., 2014). Furthermore, since PON-1 activity was associated with small HDL and there was no difference observed in the percentages of small HDL may further explain why there was no difference seen in PON-1 activity in this study.

PAF catabolism is moderated by PAF-AH and Lp-PLA_2. PAF is a potent pro-inflammatory mediator, mainly via its actions as a platelet, monocyte, and leukocyte activator (Stafforini et al., 1987; Demopoulos, Karantonis & Antonopoulou, 2003). The biosynthesis of PAF is known to be induced by the HIV viral protein, Tat and its action has been related to AIDS dementia (Sorbo et al., 2001; Anderson et al., 2002). Reduced HDL-associated PAF-AH activity is associated with increased risk of CVD (Kakafika et al., 2003) however this association has yet to be shown in HIV-related CVD. One study reported that PAF-AH levels of HIV ART-naïve participants did not change over 12 months (Papakonstantinou et al., 2016). Furthermore, PON-1 activity modulates HDL-associated PAF-AH activity (Kakafika et al., 2003), therefore the higher PON-1 activity seen in this study's cohort may circumvent reductions in PAF-AH

activity. The levels of PAF were not measured in this study but if they were potentially increased by HIV itself it is hypothesized that this may lead to increased or normal PAF-AH activity rather than reduced activity.

The current study is the first to examine how HDL subfraction distribution may differ in both ART-naïve and ART-treated HIV-infected patients compared to healthy patients. HDL subfraction distribution shifts have been associated with obesity, hypertension, CKD and diabetes (Magkos, Mohammed & Mittendorfer, 2008; Li et al., 2016; Woudberg et al., 2016; Gluba-Brzózka et al., 2017; Woudberg, Lecour & Goedecke, 2019). CKD patients displayed a greater percentage of large HDL and a lower percentage of small HDL compared to healthy control patients (Gluba-Brzózka et al., 2017). Our data show a similar shift from small to large HDL subfractions in HIV ART-naïve patients compared to control patients. The Lipoprint® system separates HDL into 10 subfractions allowing for accurate quantification (Filippatos et al., 2008). HIV ART-naïve patients had significantly more HDL-1 (the largest subfraction) and HDL-5 (an intermediate subfraction) compared to control patients. Although there was no difference in HDL-10 (the smallest subfraction) in the ART-naïve patients compared to control patients, the ratio of HDL-10/HDL-1 was significantly lower. Therefore, extreme subfractions may play an important role in the functionality of HDL. There was no shift observed in the HIV ART-treated group, therefore ART may reverse the shift seen in the untreated group. Recent evidence suggests that small HDL offers more protection than less dense intermediate HDL and that large HDL offers the least protection. Thereofore, the shift in HDL subfractions seen in the HIV ART-naïve group may indicate that they are at greater risk for cardiovascular disease compared to the HIV-ART-treated patients and control patients (Perségol et al., 2018). Furthermore, since there was a shift observed in the HIV ART-treated group and no associated changes in function or composition, ART in this case be protective against the risk for CVD instead of being a risk factor for it. A potential reason for this is that ART regimens have shifted towards drugs with fewer side effects over the last decade (Domingo et al., 2018). Small HDL subfractions were inversely correlated with viral load and CRP which suggests that it may be protective against HIV (data not shown). Furthermore, the positive correlation between small HDL and CD4 count and negative correlation between large HDL and CD4 count are exciting novel findings and suggest that HDL subfraction distribution could be a biomarker to assess early cardiovascular risk in the HIV population.

Although HDL was the focus of this study, the other lipoproteins, especially LDL, are also of clinical importance and can also be separated into subfractions by the Lipoprint® system. We observed a higher percentage of LDL-1 in HIV free control patients compared to the HIV positive groups. LDL-1 is composed of larger and more buoyant particles than the following subfractions (Tribble et al., 1992) and is generally considered to be less pro-atherogenic than the latter subfractions (LDL-2 - LDL-7) (Hurt-Camejo et al., 1990; Ivanova et al., 2017). We also observed a higher percentage of IDL-B in HIV ART-naïve patients compared to HIV ART-treated patients and HIV free control patients. Evidence suggests that high IDL concentrations could be associated with angiographic coronary atherosclerosis or increased CIMT (Mack, Krauss & Hodis, 1996; Hodis et al., 1997) however, IDL-B did not correlate with any of the cardiovascular measurements performed in this study including CIMT. There is limited data on the distribution of lipoprotein subfractions and functionality in an African population. To our knowledge, this is the first study the examine the role of HIV and/or ART on lipoprotein subfractions and functionality. Much of the research performed on HIV-related CVD has been out of Africa despite the fact that SSA is the world's epicentre of HIV. Therefore, this study is particularly relevant since it explores HDL functionality, composition and subfractions and their potential use as an early, specific biomarker for CVD in PLWH in Africa.

CHAPTER SIX: LIMITATIONS AND CONCLUSIONS

6.1 Limitations

This study had a relatively small cohort from the Western Cape of South Africa where a large portion of the population is of mixed ancestry, while the majority of HIV-infected people in SSA are ethnic Black Africans. Therefore, this study is not fully representative of the overall HIV infected population of SSA. Furthermore, recent evidence suggests that ethnicity may affect HDL functionality and HDL subclasses (Woudberg et al., 2016) and that ethnicity should also be considered when studying the effects of ART on PON-1 activity (Pereira et al., 2009). There is limited data regarding people of mixed ancestry from South Africa and their risk for CVD. The limited data regarding the exact type of first-line treatment the patients were receiving and the relatively low sample size make the study 'preliminary' in nature. Despite this, the novel findings from this study remain relevant especially since there is a need for the detection of HIV-specific biomarkers to not only detect CVD early but also guide both ART regimen choice and the use of lipid-lowering agents such as statins. Furthermore, this study also improves the knowledge regarding people of mixed ancestry from South Africa and their risk for CVD.

The patients in this study were recruited as a part of the ongoing EndoAfrica study (Strijdom et al., 2017). The serum that was collected on the day of recruitment was needed for several sub-studies and this, therefore, limited us with regards to the number of analytical tests we could perform. We initially planned to assess the anti-inflammatory function of the patient HDL by quantifying the TNF-α induced expression of VCAM in HUVEC cells but due to limited available serum, this was not possible. HDL downregulates the expression of adhesion molecules such as VCAM and ICAM in endothelial cells (Cockerill et al., 1995; Calabresi, Gomaraschi & Franceschini, 2003; Gomaraschi et al., 2008) and it has been shown that HDL3 (smaller HDL) inhibits TNF-α induced expression of VCAM in HUVEC cells to a greater extent than HDL2 (larger HDL) (Ashby et al., 1998).

The activities of the HDL-associated enzymes, PON-1 and PAF-AH, were quantified in serum only. Both enzymes are associated *in vivo* with HDL, PON-1 is almost completely HDL bound (James & Deakin, 2004) whereas PAF-AH is mainly associated with LDL but HDL is responsible for much of its beneficial aspects (Tselepis & Chapman, 2002). However, it is difficult to attribute the activities of specific enzymes as strict HDL 'functions'. Therefore, it would have been useful to perform western blotting not only in serum but also in isolated HDL

so that we could have attributed the activities of the enzymes as HDL-specific or not HDL-specific. Furthermore, ethnicity-specific polymorphisms in the PON-1 gene allow for a genetic approach towards the research. Future work on HDL-associated antioxidant activity may be improved by examining genetic abnormalities in the PON-1 gene in large cohorts of patients as well as measuring the total antioxidant capacity using a test such as the oxygen radical absorbance capacity (ORAC) assay.

The Lipoprint® System has only been utilized a few times in African sample populations. This study designated HDL subclasses as large, intermediate and small and focused on the individual subfractions within those subclasses however, the two principal HDL subclasses described in the literature are HDL2 and HDL3. This is due to there being different methods of quantification and separation. Pure HDL2 and HDL3 has been previously separated using the Lipoprint® System in our laboratory (Woudberg, 2017). HDL2 was largely represented by large HDL but HDL3, which one expects would be represented by intermediate and small HDL, was not clearly defined. Therefore, data obtained from the Lipoprint® system can only be accurately compared with similar data. However, the system is being utilized more frequently and it has been approved for clinical diagnosis in the United States. This will make it easier to compare findings from the Lipoprint® System with others in the literature in the future.

6.2 Conclusions

Our data strongly suggest that HIV is associated with a shift in HDL subfraction distribution and an alteration of HDL composition however, it is still unclear if this is translating into reduced HDL functionality. HIV ART-naïve patients displayed a shift in HDL subfraction distribution, with increased percentages of HDL-1 and HDL-5 and a decrease in the ratio of HDL-10/HDL-1 compared to HIV free control patients. HIV ART-naïve patients also displayed a change in HDL composition, with decreased levels of apoA-I compared to both HIV ART-treated patients and HIV free patients. HIV ART-treated patients showed no significant shift in HDL subfractions and no differences in HDL function or composition suggesting that treatment may alleviate the shift seen in HIV ART-naïve patients. In addition, decreased PON-1 activity was associated with a decreased CD4 count, decreased percentage of small HDL and an increased viral load. This data add to the suggestion that the pathophysiology of HIV-related CVD in SSA differs from that of the rest of the world and support that HDL subfractions may be considered as a potential early biomarker to assess the risk for cardiovascular disease in PLWH. More in-depth studies should therefore be conducted to better understand the exact role of HIV and/or ART on the modification of HDL.

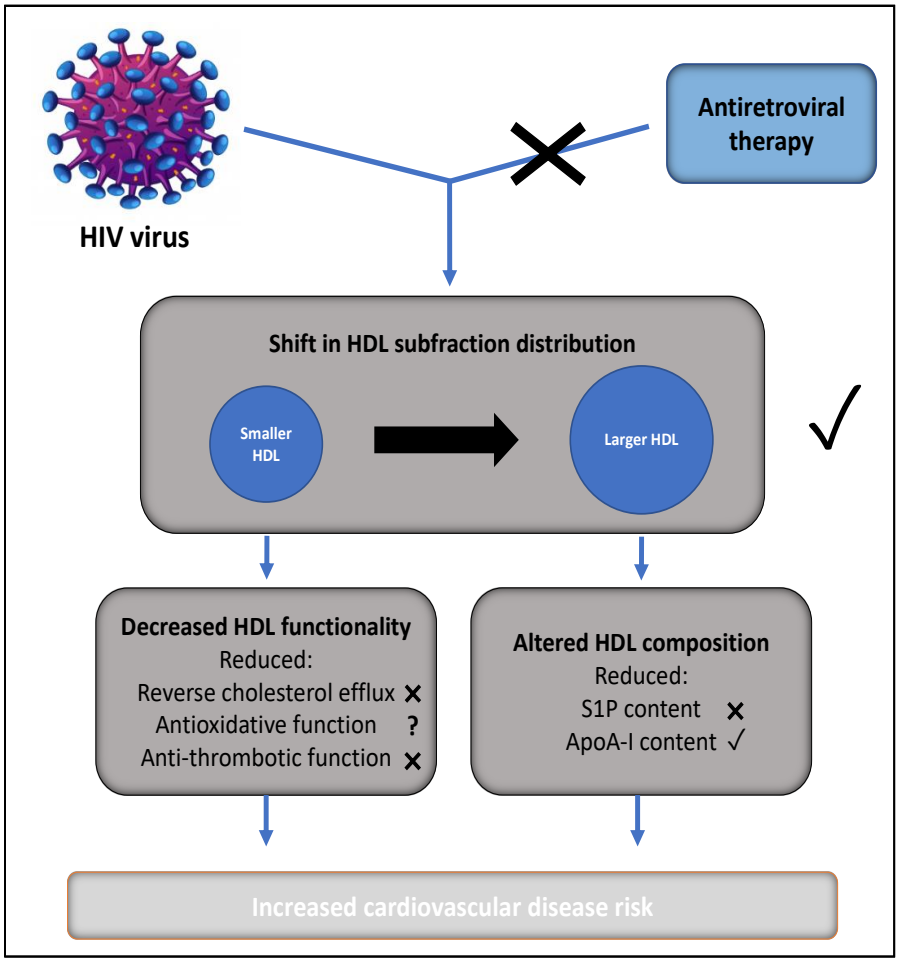


Figure 19. Summary of major findings. HIV viral infection leads to a shift in HDL subclass distribution which may contribute to the increased risk of CVD in HIV patients.

Publications and abstracts

Poster presentations:

1. Department of Medicine Research Day, University of Cape Town, 2019. Investigating high-density functionality, composition and subclass in HIV patients.

2. Hudson, P., Woudberg, N.J., Abrahams, C., Kamau, F., Vuilleumier, N., Strijdom, H., Frias, M. and Lecour, S., 2021. High-density lipoprotein (HDL) subclasses and functionality in HIV patients. European Atherosclerosis Society annual meeting (virtual), June 2021. Published in *Atherosclerosis*, *331*, p.e125. DOI: https://doi.org/10.1016/j.atherosclerosis.2021.06.370.

3. Hudson, P., Woudberg, N.J., Abrahams, C., Kamau, F., Vuileumier, N., Strijdom, H., Frias, M.A., Lecour, S., 2021. Shift of subfractions of lipoproteins in HIV patients. *Frontiers in Vascular Physiology*, *ISHR 2021* 36th Annual Meeting of the International Society for Heart Research European Section (Virtual)., pp. 120-121. DOI: 10.3389/978-2-88971-002-7.

Oral presentations:

1. Biomedical Research & Innovation Platform Symposium, 2020. Investigating high-density functionality, composition and subclass in HIV patients. Invited speaker.

Peer reviewed publications:

1. Hudson, P., Woudberg, N.J., Kamau, F., Strijdom, H., Frias, M.A. and Lecour, S., 2020. HIV-related cardiovascular disease: any role for high-density lipoproteins?. *American Journal of Physiology-Heart and Circulatory Physiology*, *319*(6), pp. H1221-H1226. DOI: https://doi.org/10.1152/ajpheart.00445.2020.

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