An evaluation of the role of platelet activation in HIV-related cardiovascular diseases onset

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Declaration

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Abstract

Background: Although combined anti-retroviral treatment (cART) lowered HIV-AIDS onset, HIV-positive individuals display increased cardiovascular disease (CVD) onset. Enhanced myocardial fibrosis has emerged as a crucial mediator of HIV-induced heart failure (HF). This study hypothesized that in HIV-infection there is immune dysregulation that can trigger persistent platelet activation and the release of mediators, which contributes to an increased risk of CVD. Markers of platelet activation were investigated in a South African population. These parameters were correlated with clinical tests of cardiac function (blood pressure [BP], electrocardiogram [ECG] and flow-mediated dilation [FMD]), markers of disease progression (CD4 and viral load) and a pro-fibrotic marker transforming growth factor-β [TGF-β].

Aim: The aim of this study was to investigate platelet activation through the expression of platelet activation markers in HIV-positive individuals and its association with CVD.

Methods: Thirty-six male and female participants between the ages of 18-55 years were recruited at People's Healthcare clinic in Worcester in the Western Cape province: n=13 HIV-negative, n=23 HIV-positive on cART. Patients with tuberculosis co-infection and pregnancy formed part of the exclusion criteria and were excluded from this study. Medical history and lifestyle questionnaires were completed while BP, ECG and FMD readings were recorded. Fasted blood samples were collected by a registered research nurse. Flow cytometry was conducted to evaluate platelet activation markers, such as CD62P, latent associating protein (LAP), glycoprotein A-repetitions predominant (GARP) and TGF-β.

Results: Our results showed a significant lower systolic and diastolic BP amongst HIV-positive patients, $p<0.0001$ and $p<0.05$, respectively. This displayed a moderate, negative correlation with

platelet activation markers, such as CD62P and LAP ($p<0.05$). GARP exhibited significant, positive correlations with diastolic BP ($p<0.05$), TGF-β ($p<0.0001$) and viral load ($p<0.05$). Additionally, a significantly lower FMD ($p<0.05$) with shorter QT interval was also observed in HIV-positive patients/subjects ($p<0.05$).

Conclusion: The major findings of this study for HIV-positive individuals are: a) the observation of lower BP (systolic and diastolic), b) GARP upregulation and its strong correlation with disease progression (CD4 and viral load) and fibrosis (TGF-β) markers, c) the identification of a moderate negative association between platelet activation markers and BP, d) lower FMD and e) shorter QT intervals. We propose that these factors contribute to an increased risk of HF/CVD in HIV-positive individuals. Our findings warrant future investigation to elucidate the exact role of platelet activation and the risk of HF/CVD in PLHIV, especially in SSA.

Opsomming

Agtergrond: Alhoewel gekombineerde anti-retrovirale behandeling (kARK) die aanvang van MIV-VIGS verlaag het, vertoon MIV-positiewe individue ‘n verhoogde risiko van kardiovaskulêre siektes (KVS). Miokardiale fibrose kom voor as 'n belangrike faktor van MIV-geïnduseerde hartversaking. Hierdie studie hipoteseer dat daar immuunstelselregulering in MIV-positiewe individue is wat aanhoudende bloedplaatjie-aktivering en die vrystelling van bemiddelaars kan veroorsaak, en bydra tot 'n verhoogde risiko van KVS. Merkers van bloedplaatjie-aktivering in 'n Suid-Afrikaanse MIV-positiewe groep is ondersoek. Hierdie parameters korreleer met kliniese toetse van hartfunksie (bloeddruk (BD), elektrokardiogram (EKG),vloei-gemedieerde dilatasie (VGD)), merkers van siekteprogressie (CD4 en virale lading) en pro-fibrotiese merker transformerende groeifaktor beta-β (TGF-β).

Doelwitte: Die doel van hierdie studie was om bloedplaatjie-aktivering te ondersoek deur die uitdrukking van bloedplaatjie-aktivering merkers in MIV- positiewe individue en die assosiasie met kardiovaskulêre siektes.

Metodes: Ses-en-dertig deelnemers tussen die ouderdomme 18-55 jaar is in People’s Healthcare kliniek in Worcester in die Wes-Kaap provinsie gewerf: n = 13 MIV-negatief, n = 23 MIV-positief op kARK. Pasiënte met tuberkulose-infeksie en swangerskap , vorm deel van die uitsluitingskriteria en is nie by die studie ingesluit nie. Mediese geskiedenis en 'n leefstylvraelys is voltooi terwyl BD, EKG en VGD opgeneem is. Bloedmonsters is deur ‘n geregistreerde navorsings verpleegster versamel tydens vas. Vloeisitometrie ontledings is gebruik om plaatjie-aktivering merkers,CD62P, latente assosierende proteïen (LAP), glikoproteïen A-herhalings oorheersend (GARP) en TGF-β te evalueer.

Resultate: Hierdie studie toon ‘n beduidende verlaagde sistoliese en diastoliese BD in MIV-positiewe pasiënte, $p<0.0001$ en $p<0.05$ onderskeidelik. Dit toon ‘n negatiewe korrelasie met plaatjie-aktivering merkers, CD62P en LAP ($p<0.05$). GARP toon ‘n beduidende positiewe korrelasie met diastoliese BD ($p<0.05$), TGF-β ($p <0.0001$) en virale lading ($p<0.05$). Verlaagde VGD ($p<0.05$) met 'n korter QT-interval is in die MIV-positiewe groep waargeneem ($p<0.05$).

Gevolgtrekking: Die belangrikste bevindings van hierdie studie in MIV-positiewe individue is as volg: a) die waarneming van verlaagde BD (sistoliese en diastoliese), b) GARP-toename en die sterk korrelasie daarvan met die progressie van MIV (CD4 en virale lading) en merkers van fibrose (TGF-β) , c) ‘n negatiewe assosiasie tussen plaatjie-aktivering merkers en BD, d) verlaagde VGD en e) korter QT intervalle. Ons stel voor dat hierdie faktore waarskynlik bydra tot 'n verhoogde risiko van hartversaking /KVS in MIV-positiewe individue. Verdere navorsing is dus nodig om die rol van bloedplaatjie-aktivering en die risiko van hartversaking/KVS in MIV-positiewe pasiënte, veral in SSA, te bepaal. .

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HIV	Human immunodeficiency virus
ARV	Antiretroviral
PLHIV	People living with HIV
cART	Combined antiretroviral treatment
CVD	Cardiovascular diseases
AIDS	Acquired immunodeficiency syndrome
CAD	Coronary artery disease
SCD	Sudden cardiac death
HF	Heart failure
SSA	Sub-Saharan Africa
TGF-β	Transforming growth factor-β
Th1	T helper 1
Th2	T helper 2
VL	Viral load
RTV	Ritonavir

PI	Protease inhibitor
TF	Tissue factor
TRAF6	TNF receptor associated factor-6
ECG	Electrocardiogram
GARP	Glycoprotein A-repetitions predominant
LAP	Latent associated protein
SBP	Systolic blood pressure
DBP	Diastolic blood pressure
WHO	World health organization
LV	Left ventricle
DD	Diastolic dysfunction

Chapter 1 – Introduction

It is estimated that 37.9 million individuals live with human immunodeficiency virus (HIV) with 24.5 million people receiving antiretroviral treatment (ARV) as of June 2019 (WHO 2019). Increased access to combination antiretroviral therapy (cART) has significantly improved the lifespan of people living with HIV (PLHIV), reduced viral replication with a relatively maintained immune system, less opportunistic infections and associated mortalities (Hileman & Funderberg et al., 2017). With the successful implementation of cART, the main cause of death amongst PLHIV has shifted from acquired immunodeficiency disease (AIDS)-related immunocompromised states to non-AIDS age-related deaths, such as cardiovascular diseases (CVD). Over the past decade, the proportion of deaths due to CVD in PLHIV increased from 2.5% to 4.6% (Feinstein et al., 2016; Alonso et al., 2019). Patients present with a variety of cardiac and vascular co-morbidities, however certain cardiac disorders manifest with greater frequency amongst PLHIV and vary with geographic locations. In developed countries, PLHIV present with hypertension, coronary artery disease (CAD), atherosclerosis and the metabolic syndrome (Feinstein et al., 2016; Gopal et al., 2009; Friis-Moller et al. 2016; Lekakis & Ikonomidis, 2010; Worm et al., 2009). In contrast, heart failure (HF)/sudden cardiac death (SCD) due to HIV-associated cardiomyopathy and tuberculosis (TB)-associated pericarditis is more prevalent in developing counties such as sub-Saharan Africa (SSA) (Triant, 2013; Tseng et al., 2012).

Despite this imminent health threat, the underlying mechanisms driving HIV-mediated CVD onset remain relatively poorly understood. Currently, an emerging concept – i.e. chronic upregulation of inflammatory activity (even with cART) - plays a crucial role in CVD onset in PLHIV. For example, our laboratory recently found that there is a strong interplay between immune activation, coagulation and lipid subclass alterations in South African HIV-positive patients (Teer et al., 2019).

Of note, chronic inflammation is a characteristic feature of numerous CVD and was recently identified as a crucial contributor to diastolic dysfunction (DD), HF and SCD (Hsue et al., 2016). HIV infection causes immune dysregulation which due to downstream effects leads to the excessive release of pro-coagulant, pro-fibrotic and pro-inflammatory cytokines. This leads to an increased thrombotic state, endothelial dysfunction and myocardial fibrosis. The culmination of such factors can lead to systolic and DD which puts the patient at risk for HF/CVD (Figure 1.1). While the pathogenesis behind this is multi-factorial, myocardial fibrosis is emerging as a key mediator underlying HIV-induced systolic and DD.

Myocardial fibrosis elicits profound effects on myocardial function and can lead SCD/HF; thus, understanding its pathogenesis may help identify promising therapeutic targets. The following chapter will introduce the complexity of HIV-related CVD with a specific focus on HF/SCD and considering the contribution of a chronic inflammatory state and persistent immune activation in this setting. We will also discuss the role of platelets in the context of the diagnosis and therapeutic monitoring together with a consideration of potentially novel biomarkers.

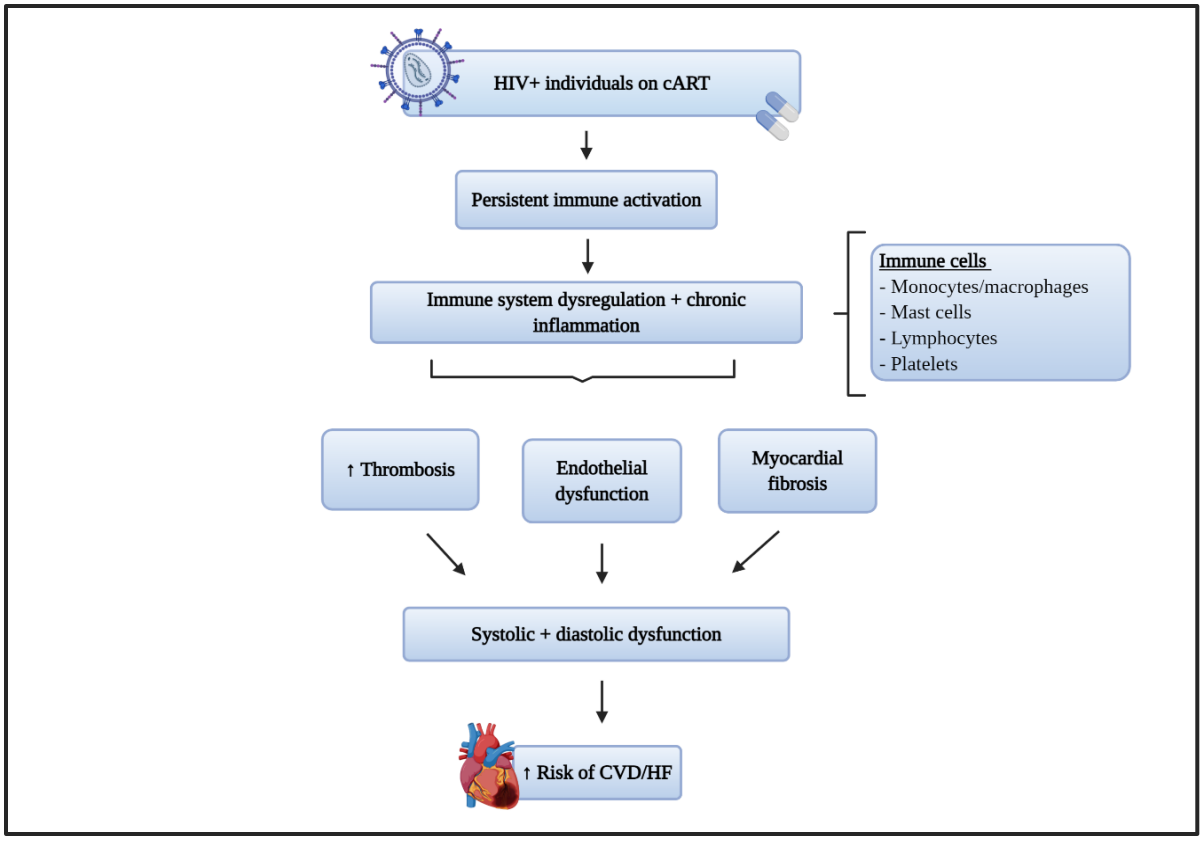

Figure 1.1: The role of persistent immune activation in HIV-related CVD onset. HIV infection with persistent immune activation results in immune system dysregulation and chronic inflammation. This leads to an increased thrombotic state, endothelial dysfunction and myocardial fibrosis. The culmination of such factors can lead to systolic and diastolic dysfunction which puts the patient at risk for HF/CVD. CVD: cardiovascular diseases, cART: combined antiretroviral treatment, HIV: human immunodeficiency virus, HF: heart failure.

Chapter 2 – Literature review

2.1. Human immunodeficiency virus (HIV) – A global epidemic

It is estimated that there are currently 38 million PLHIV globally (WHO, 2019). Since the peak of infections in 1997, new infections have decreased by 40% with ~ 1.7 million newly infected individuals in 2018 compared to 2.9 million in 1997 (WHO, 2019). Approximately three quarters of the population infected with HIV live in SSA, with two-thirds of new infections also originating from this area and thus making Africa the most severely HIV-burdened continent (Dwyer-Lindgren et al., 2019).

2.2. HIV/AIDs and cardiovascular disease - a double-edged sword

Since the start of the epidemic the prognosis and lifespan of PLHIV has improved tremendously due to effective and timeously cART administration (Friis-Moller et al., 2015). This has shifted the cause of death from AIDS-related morbidities, due to severely immunocompromised states, to non-AIDS related co-morbidities such as CVD. Several studies have consistently demonstrated elevated rates of CVD amongst PLHIV with the main findings summarized here (Table 2.1).

Table 2.1. Cardiovascular complications that occur in PLHIV (Dominick et al., 2020)

Cardiovascular complication	**Prevalence (%)**	**Reviewed by**
Coronary artery disease	≤57	Barnes et al., 2017; Hanna et al., 2016
Pulmonary arterial hypertension	<50	Almodovar et al., 2011; Almodovar et al., 2010
Myocarditis	40-52	Almodovar et al., 2011; Ntusi, 2017
Hypertension	35	Al-Nozha et al., 1997; Gaziana et al., 2010; Kearns et al., 2017
Stroke	>30	Benjamin et al., 2012
Heart failure	20-30	Al-kindi et al., 2016
Vasculopathy	10-30	Benjamin et al., 2012
Endocarditis	6-35	Ntusi, 2017
Arrhythmias	13	Ntusi, 2017
Pericarditis	11-12	Sani, 2008; Syed & Mani, 2013

Cardiovascular diseases are the leading causes of death globally, responsible for ~ 31% of total mortalities with >75% occurring in low- and middle-income countries (WHO, 2019). Together this presents as a doubled-edged sword causing a major burden on the economic and health care systems of both developed and developing countries (Feinstein et al., 2016; Hanna et al., 2016).

2.2.1. Sudden cardiac death

An increase in SCD in PLHIV due to HF is observed in developing countries already overburdened with chronic diseases such as HIV/CVD (Hsue et al., 2010; Butler et al., 2018). People living with HIV have a 4.5-fold increased risk of SCD (Tseng et al., 2012) and it is responsible for 5-15% of mortalities in the United States (Tseng et al., 2012). While the mechanisms contributing to this are not well understood, it is likely related to HF and CAD that are increasingly prevalent amongst PLHIV (Myerburg & Junttila, 2012). As most SCD cases occur in patients with previously undiagnosed CVD, identification of populations at high risk is essential for screening and prevention. A study by Tseng et al. (2012) observed that in an urban HIV cohort, SCD accounted for 13% overall deaths and 86% mortalities related to CVD (Tseng et al., 2012). SCD also occurred in patients already on treatment and that displayed better disease control as measured by viral load (VL) (low) and CD4 count (within normal range) (Tseng et al., 2012). Evidence shows that SCD in PLHIV is linked to arrhythmias, cardiomyopathies, HF, hypertension and hyperlipidemia (Hsue et al., 2004; Myerburg & Junttila, 2012). This is unlike the general population where CAD is well-known as the major cardiovascular complication (Hsue et al., 2004; Dominick et al., 2020).

Growing evidence suggests that SCD in PLHIV is linked to DD (Barnes et al., 2017). Diastolic dysfunction is characterized by an abnormality of the left ventricle (LV) to fill with an adequate volume of blood at normal diastolic filling pressures (Fontes-Carvalho et al., 2015). Subclinical DD is recognized as an independent predictor of HF development and long-term mortality (Fontes-Carvalho et al., 2015; Bursi et al., 2006). Studies in PLHIV on cART observed elevated rates of DD compared to age matched controls (Table 2.2). This is of concern given the link between DD, HF with preserved ejection fraction and mortality (Butler et al., 2018; Hsue et al., 2010). Previous research show that DD development is related to conditions with increased inflammatory

cytokines, endothelial dysfunction – as observed in HIV patients -, obesity, hypertension and the metabolic syndrome (Remick et al., 2014). In support, Fontes-Carvalho *et al*. (2015) found that HIV-infection was associated with alterations in myocardial structure and function, with reduced diastolic reserve. The changes in diastolic function occurred independently of cART as patients presented with this complication *before* starting therapy (Fontes-Carvalho et al., 2015). However, despite such findings it remains unclear why HIV-positive individuals are at a higher risk for DD and related pathophysiology.

Table 2.2. **Diastolic dysfunction amongst HIV-infected patients on cART in comparative studies.** ART, antiretroviral therapy; BSA, body surface area; DD, diastolic dysfunction; HIV, human immunodeficiency virus; LV, left ventricular. (Butler et al., 2018)

Study	Population	Findings
Schuster et al., 2008	30 HIV-positive males and 26 age-matched HIV-negative subjects	HIV-positive patients had higher prevalence of DD (64% vs 12%; $p < 0.001$). Lower left ventricular (LV) systolic function indexes and higher pulmonary arterial pressure were observed in HIV-positive cohort.
Hsue et al., 2010	196 HIV-positive adults and 52 age-matched HIV-negative subjects	LV mass was higher in HIV-positive vs HIV-negative (77.2 vs 66.5 g/m^2; $p <0.001$). HIV-positive patients presented with 50% mild DD vs 29% in HIV− ($p = 0.008$). HIV-positive patients had a 2.4 higher risk of DD compared to control subjects ($p = 0.019$).
Luo et al., 2014	325 HIV-positive patients initially ART-naïve with repeated evaluation after 48 weeks of ART. Additionally 97 age	HIV-infected patients had an increased prevalence of DD vs control subjects (16.5% vs 7.2%; $p= 0.027$) including LV systolic dysfunction (7.3% vs 2.1%; $p= 0.056$). DD increased from baseline measurements to

	matched HIV-negative control subjects were recruited.	week 48 (23.3%; p = 0.056 vs baseline) in HIV-positive patients.
Fontes-Carvalho et al., 2015	206 HIV-positive patients (88 ART-naive and 116 on ART) and 30 HIV-negative control subjects.	Prevalence of DD in HIV-infected patients 23% vs 3.3% in control subjects (p = 0.01).

Myocardial fibrosis characterized by an accumulation of collagen with reduced ventricular compliance is the major physiological mechanism contributing to most cardiac pathologic conditions such as DD and SCD/HF (Thiara et al., 2015). Continuous mechanical stress caused by chronic overstraining of cardiac tissues together with immune activation can result in negative cardiac remodeling. In support, PLHIV on cART and with no CVD history exhibited a 6-fold higher rate of myocardial fibrosis than uninfected age-matched uninfected controls (Hollaway et al., 2013). Moreover, HIV infection (with and without cART) is linked to an increased incidence of myocardial fibrosis and also systolic and diastolic LV dysfunction (Holloway et al., 2013; Utay et al., 2015; Ahamed et al., 2016). Others also found a significantly higher prevalence of myocardial fibrosis in PLHIV who suffered mortality due to SCD (Tseng et al., 2012). Furthermore, a study on 90 HIV-positive patients on cART versus 39 uninfected controls (with no history of CVD) found that HIV-positive patients displayed a 6-fold higher rate of patchy myocardial fibrosis after controlling for age, gender and CAD (76% vs 13%, respectively, p <0.001) (Holloway et al., 2013). Others also found that HIV-positive patients exhibited greater evidence of myocardial fibrosis than their negative counterparts, despite relatively normal ejection fractions (Thiara et al., 2015). However, the underlying mechanisms responsible for the

development of myocardial fibrosis during HIV-infection, the associated risk factors and clinical consequences of such pathology still require further elucidation. Of note, persistent immune activation and systemic inflammation (as typically manifesting in PLHIV) can contribute to myocardial fibrosis and subsequent adverse cardiac remodeling (Fukunaga et al., 2007; Yndestad et al., 2003; Pitulli et al., 2016) and possibly cause fatal arrhythmias (Tseng et al., 2012).

2.2.2. The role of myocardial fibrosis in the pathogenesis of sudden cardiac death

In order to understand the role fibrosis plays in SCD it is essential to understand the physiology of the heart. Myocytes in the ventricles are tightly arranged and coupled with cardiomyocytes which include adjacent layers that are separated by clefts (Weber, 1989). The myocardium contains extracellular matrix (ECM) proteins comprised of fibrillar collagen. The cardiac matrix is divided into three parts: the epi-, peri- and endomysium (Weber, 1989). Type I collagen comprises ~ 85% of total myocardial collagen whereas type III comprises 11% (Weber, 1989; Jugdutt, 2003; Kong et al., 2013). Fibroblasts, one of the many distinct cellular components in the cardiac interstitium, regulate collagen turnover by controlling the synthesis and degradation of matrix proteins (Ieda et al., 2009; Banerjee, 2007). The ECM forms a link between intracellular cytoskeletal proteins and intercellular proteins. This allows biochemical signals to transmit from the heart through mechanosensation which play a significant role in activating and differentiating myofibroblasts (Weber et al., 2013; Liu et al., 2017).

There are two types of myocardial fibrosis; reactive and replacement fibrosis. Reactive fibrosis, characterized by excessive ECM deposition in interstitial or perivascular spaces, is associated with pathological conditions (Kong et al., 2013). When the regulation of collagen metabolism (synthesis and degradation) is disturbed, structural abnormalities occur (Janicki & Brower, 2002). These structural abnormalities cause the disruption of myocardial excitation and contraction in the systole

and diastole. This leads to profound impairment of systolic and diastolic function (Janicki & Brower, 2002; Kong et al., 2013). Excessive fibrosis can cause mechanical stiffness which result in impairment in electric conduction and cardiac DD as it forms a barrier between cardiomyocytes leading to impaired cardiac systolic function. (Kong et al., 2013). Thus, myocardial fibrosis can cause SCD in the following manner: loss of fibrillar collagen which impairs the transduction of cardiomyocyte contraction resulting in uncoordinated contraction of cardiomyocyte bundles (Kong et al., 2013). Additionally, fibrosis can cause sliding displacement of cardiomyocytes will decrease the number of muscular layers in the ventricular wall and LV dilation (Beltrami et al., 1994).

In contrast, replacement fibrosis occurs due to loss of viable myocardium and results in scar formation (Kong et al., 2013; Khan et al., 2006). However, structural integrity of ventricles remains. For the prevention of cardiac dysfunction, a balance between replacement and reactive fibrosis is required (Khan et al., 2006; Beltrami et al., 1994). Myocardial fibrosis can elicit profound effects on myocardial function and potentially lead to SCD/HF (Figure 2.1), thus understanding its pathogenesis may help identify promising targets for therapeutic interventions.

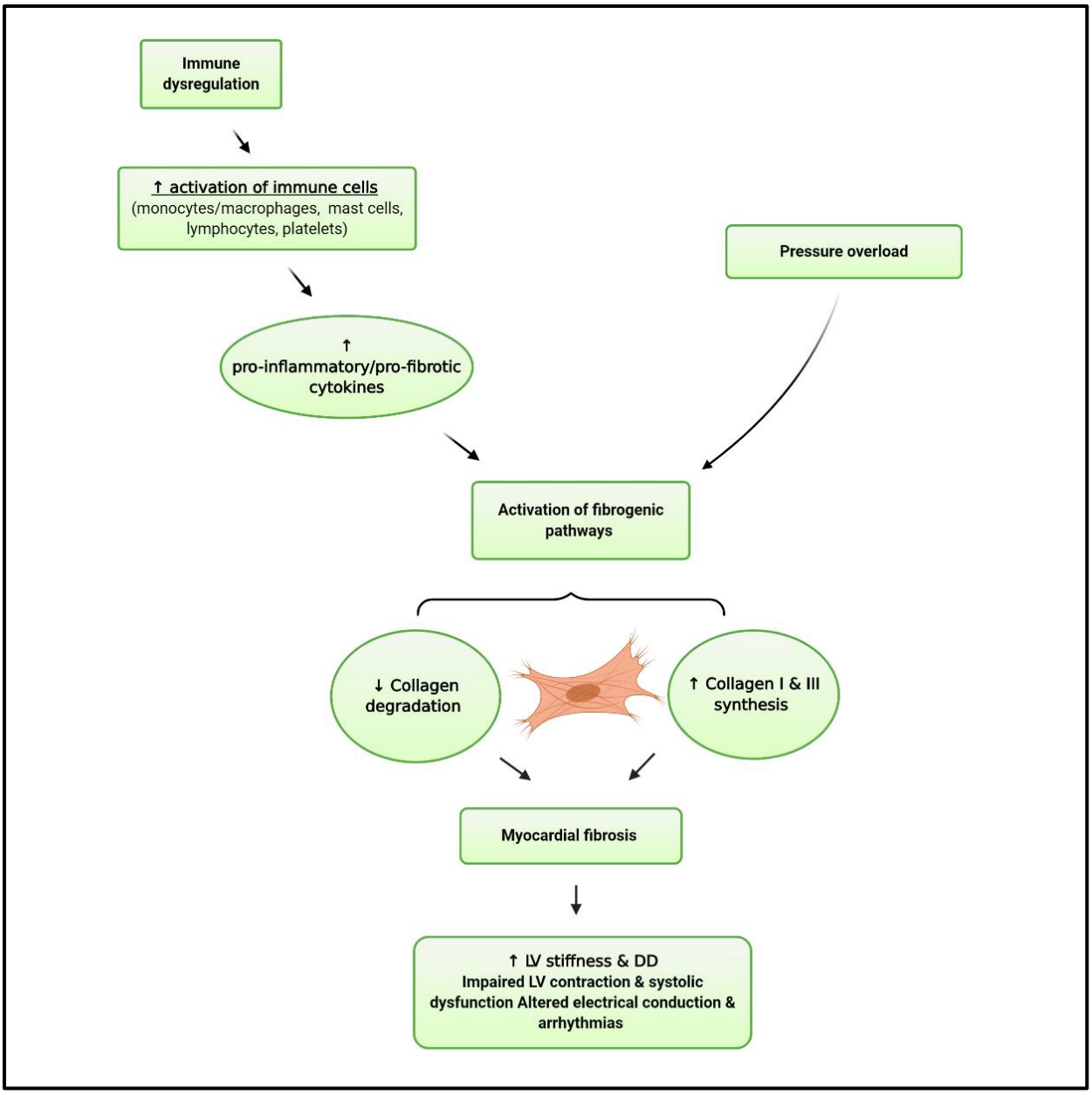

Figure 2.1. The pathology and role of myocardial fibrosis in the pathogenesis of HF/CVD. CVD: cardiovascular disease, DD: diastolic dysfunction, HF: heart failure, LV: left ventricle.

Although the mechanisms that render PLHIV more susceptible to HF onset remain relatively unclear, several hypotheses have been put forward in this regard. This includes an interplay of risk factors such as cART side-effects, lifestyle risk factors and persistent immune activation (Figure 2.2). Thus, an improved understanding of the pathogenesis, various cellular and molecular pathways should help with the identification of promising novel targets for therapeutic intervention. For example, a central role for inflammation in HF development has been postulated and the phenomenon of persistent immune activation (despite effective cART and viral suppression) should also be considered for PLHIV.

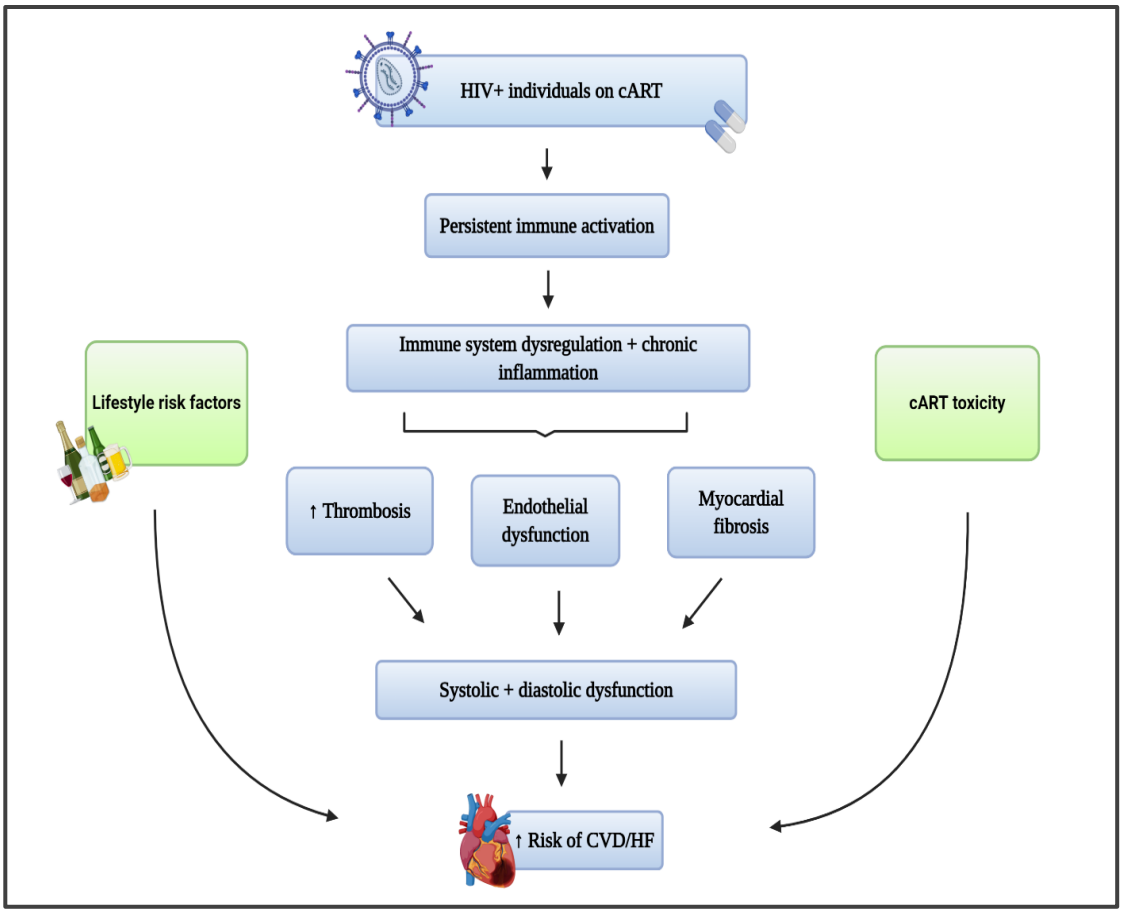

Figure 2.2 Mechanism central to the development of HF/CVD in PLHIV. cART- combined antiretroviral treatment, CVD: cardiovascular disease, HF: heart failure, PLHIV: people living with HIV.

2.3. The role of immune activation and chronic inflammation in HF/SCD in HIV

2.3.1. HIV and the immune response

HIV infection activates the innate and adaptive immune systems enabling a chronic infection which forms the basis of ongoing immune activation and immunodeficiency (Deeks et al., 2013). The inflammatory response can be categorized into two phases - acute and chronic (Deeks et al., 2013).

Inflammation is essential in resolving infections, tissue damage and maintaining a state of hemostasis (Hsue et al., 2010). The innate immune system consists of granulocytes (neutrophils, basophils, eosinophils), mast cells and antigen presenting cells, and macrophages and dendritic cells. Pathogen-associated molecular patterns and damage-associated molecular patterns can bind to cell surface toll-like receptors which subsequently result in their activation (Deeks et al., 2013; Pereyra et al., 2012). The activated cells of the innate immune response will produce pro-inflammatory cytokines and interferons to further amplify the inflammatory response. The acute inflammatory response thus starts rapidly, becomes severe over short periods of time and may last few days. If the pathogen-induced stimulation persists then the inflammatory process acquires new characteristics associated with chronic inflammation. This is a slow, long-term inflammation lasting for prolonged periods. This is induced by cytokines such as interferon gamma which promotes the activation of the adaptive immune system (Pereyra et al., 2012). T-cells play a significant role in the adaptive immune response and differentiate into either CD4 or CD8 cells. CD4 T-helper cells, primary target of HIV, manage the immune response, whereas CD8 cytotoxic T-cells destroy cells infected with the virus (Deeks et al., 2013; Pereyra et al., 2012). The aforementioned cells, macrophages and natural killer cells are responsible for cell-mediated immunity. B-cells produce antibodies and is responsible for humoral immunity. The inflammatory response is the result of a complex interplay between multiple immune cells.

2.3.2. HIV and persistent immune activation

Persistent immune activation and chronic inflammation occurs in HIV-infection despite cART adherence and suppressed viremia (Pereyra et al., 2012). The inflammatory response is attenuated when not required and becomes chronic if there is a persistent source of activation and/or due to defective control mechanisms (Appay & Sauce, 2008). The harmful consequences of persistent immune activation and inflammation with HIV-infection have been extensively reviewed in the literature (Fahey, 1998; Paiardini & Muller-Trutwin, 2013; Sokoya et al., 2017; Appay & Sauce, 2008; Hsue et al., 2012). For example, studies on simian chimpanzee hosts infected with simian immunodeficiency virus indicate that chronic immune activation significantly contributes to pathogenic infection (Pandrea et al., 2008; Veazey et al., 2000). Chronic inflammation can lead to inflammatory-associated complications that are associated with complications such as CVD (Kaplan et al., 2011). There are several pathological mechanisms that contribute to persistent immune activation including microbial translocation, chronic viral replication, CD4 T-cell depletion/altered balance of T-cell subsets, viral proteins, and a pro-inflammatory milieu (Appay & Sauce, 2011; Dillon et al., 2014, Krikke et al., 2014, Mutlu et al., 2014; Sereti & Altfeld, 2016).

2.3.2.1. Persistent immune activation and CVD

Chronic inflammation and immune dysfunction increase the risk of cardiovascular morbidities and mortalities through, endothelial dysfunction, hypercoagulation and myocardial fibrosis (Chu, 2005; Pereyra et al., 2012; Sinha et al., 2016, Witkowski et al., 2016; Vachiat et al., 2017). Two large studies found a robust correlation between HIV and myocardial infarction, with an increased risk of 44% to 48% (independently of traditional risk factors) (Hanna et al., 2013; Marcus et al., 2014).

In support, the Strategies for Management of Antiretroviral Therapy study revealed that HIV and associated inflammation are significant risk factors for CVD onset (Siedner, 2016). Moreover, another study found a significant link between increased markers of immune activation and subclinical atherosclerosis (Sainz et al., 2014). Together these findings show an association between chronic inflammation and an increased CVD risk. Thus, it is essential to consider the pathophysiology thereof in PLHIV (Sainz et al., 2014; Siedner, 2016).

The persistent activation of the innate and adaptive immune systems (monocytes/macrophages and T cells, respectively) result in increased circulating pro-inflammatory and pro-fibrotic cytokines (Mogensen et al., 2010, Serhan et al., 2008; Bernberg et al., 2012, Frystyk et al., 2007; Mooney et al., 2015, Sico et al., 2015, Tchernof & Despres, 2013; Hansson, 2005, Longenecker et al., 2016, Sinha et al., 2016). These cytokines contribute to hypercoagulation, endothelial dysfunction and fibrotic remodeling which increase the risk of CVD onset in PLHIV (Sager et al., 2017; Thiara et al., 2015; Nou et al., 2016; Vachiat et al., 2017; Arildsen et al., 2013; Marincowitz et al., 2019; Nou et al., 2016). Fibrotic remodeling as a result of immune dysfunction is a significant area of research due to its detrimental effects on cardiac function and links to SCD/HF in PLHIV (Butler et al., 2018). Myocardial fibrosis is a major contributor to SCD, especially in PLHIV on cART (Hsue & Tawakol., 2016). The role of inflammation in myocardial fibrosis involves the secretion of pro-fibrotic cytokines from inflammatory cells (Krikke et al., 2014). More recently, studies show that persistent activation of the innate and adaptive immune responses leads to fibrosis in the myocardium of PLHIV (Figure 2.1) (Sager et al., 2017; Thiara et al., 2015).

2.4. The role of persistent immune activation and chronic inflammation in myocardial fibrosis

Fibrosis and ECM formation with proliferation and activation of myofibroblasts can occur as a result of different pathological conditions such as inflammation (Kong et al., 2013; Hsue and Tawakol, 2016). Varying cardiac diseases present with different pathologies. However, the cells and molecular pathways involved in fibrotic remodeling remain the same (Kong et al., 2013). Although cardiomyocyte death is usually the cause of activation of fibrogenic signals, certain stimuli such as inflammation (as seen in HIV) or pressure overload may activate pro-fibrotic remodeling of the heart (Kong et al., 2013). Several cell types are implicated in such fibrotic remodeling. This may occur directly by matrix proteins production or indirectly by the secretion of fibrogenic mediators (Kong et al., 2013). The role and contribution of myofibroblasts, monocytes/macrophages, mast cells and lymphocytes in this context will now be briefly discussed.

2.4.1. Monocytes/Macrophages

Cardiac remodeling is regulated by two different macrophage subsets that are classified as M1 and M2 (Wynn and Barron, 2010). M1 macrophages are pro-inflammatory and secrete pro-inflammatory cytokines (IL-1, TNF), whereas M2 macrophages follow the pro-inflammatory cells and exhibit an anti-inflammatory response. M2 macrophages play a crucial role in fibrosis (Liu et al., 2011; Hulsmans et al., 2016) by releasing pro-fibrotic mediators such as IL-10, TGF-β, platelet derived growth factor, and chemokines that recruit fibroblasts (Hulsmans et al., 2016).

In support, a study found that HIV-positive women on cART displayed diffused myocardial fibrosis with reduced DD (Zanni et al., 2019). These women exhibited increased systemic immune

activation and increased levels of sCD163, a monocyte activation marker, which correlated with myocardial fibrosis (Hulsmans et al., 2016; Zanni et al., 2019). This indicates that monocytes can be recruited to the myocardium to propagate both myocardial inflammation and fibrosis (Hulsmans et al., 2016; Zanni et al., 2019). Such monocytes can differentiate into macrophages, with the M2 subpopulation able to secrete anti-inflammatory cytokines, triggering collagen production by neighboring fibroblasts (Hulsmans et al., 2016; Zanni et al., 2019). While differentiation of M2 macrophages is associated with the progression of myocardial fibrosis (Yang et al., 2012), they can also inhibit fibrosis by phagocytosing apoptotic myofibroblasts and regulating the balance of matrix metalloproteinases and tissue inhibitors of metalloproteinases (Hulsmans et al., 2016; Liu et al., 2011). However, the contribution of monocytes/macrophages to the fibrotic response depends on the pathophysiological nature of cardiac fibrosis.

2.4.2. Mast cells

Mast cells are essential participants in fibrosis (Liu et al., 2017). Myocardial fibrosis is associated with increased accumulation of mast cells that contribute to cardiac remodeling and myocardial fibrosis through the release of pro-fibrotic cytokines, histamine, tryptase and chymase (Liu et al., 2012; Levi-Schaffer & Rubinchick, 2012; Levick, 2012). Histamine can stimulate the proliferation of fibroblasts and collagen synthesis (Befus et al., 1988; Kong et al., 2015; Hatamochi et al., 1985). In support, administration of a histamine H2 receptor inhibitor improved ventricular remodeling in HF patients, reflecting the pro-fibrotic effects of histamine (Kim et al., 2006). Chymase is a protease that can enhance the fibrogenic activity by elevating concentrations of angiotensin II and TGF-β, both significant contributors to fibrotic signaling pathways (Liu et al., 2017). Others also found that chymase may contribute to myocardial fibrosis through the activation of the TGF-β/Smad pathway as opposed to angiotensin II. In this case, inhibition of the angiotensin II receptor

did not elicit any effects on chymase-induced production of TGF-β (Liu et al., 2017). In agreement, animal models of myocardial fibrosis demonstrated the significant role of chymase signaling in fibrotic ventricle remodeling (Matsumoto et al., 2003; Oyamada et al. 2011). Tryptase activates the extracellular signal-regulated kinase/mitogen-activated protein kinase signaling pathway in cardiac fibroblasts and promotes the expression of collagen through activation of the protease-activated receptor-2 (Mclarty et al., 2011). Tryptase can also contribute to connective tissue breakdown and as a result the activation of pro-collagenase and the induction of a matrix metalloproteinase cascade can occur. The connective tissue subsequently becomes more permeable allowing for the infiltration of leukocytes during inflammation. Tryptase also induces fibroblast proliferation by stimulating the synthesis of cyclooxygenase and prostaglandins (Rao & Brown, 2008; Levi-Schaffer & Piliponsky, 2003). Moreover, activated mast cells also release a wide variety of granule-stored cytokines and growth factors such as TNFα, TGF-β, platelet derived growth factor that can stimulate cardiac fibroblast proliferation and collagen synthesis (De Almeidea et al., 2002; Frangogiannis et al., 1998; Kanellakis et al., 2012). However, the exact contribution of mast cells in cardiac fibrosis is unknown as these cytokines and growth factors are also released by various other cells.

2.4.3. Lymphocytes

T helper 1 cells mediate tissue damage and suppress the development of fibrosis through the release of IFN-γ and IL-12 (Wynn, 2008). In contrast, T helper 2 cells are pro-fibrotic through the release of IL-4 and IL-13 which are both potent stimulators of fibroblast-derived collagen synthesis. Additionally, T helper 2 cells drive macrophage differentiation towards an M2 phenotype that further enhances the fibrotic response (Kong et al., 2015). While increased expression of IL-4 and IL-13 is associated with myocardial fibrosis, the precise role of T helper 2 cells in cardiac fibrotic

remodeling is unknown (Wynn, 2008; Wei, 2011). Other T cell subpopulations are also involved in myocardial fibrosis and associated with persistent T cell-mediated inflammation (Kong et al., 2015). A large body of evidence implicated regulatory T cells in fibrotic remodeling, especially considering their increased TGF-β expression and IL-10 secretion, both potent regulators of fibrosis (Tang et al., 2012). Moreover, T helper 17 through IL-17 generation stimulates collagen production and thereby contributes to myocardial fibrosis (Liu et al., 2017).

Thus, the role of inflammatory cells in myocardial fibrosis and downstream outcomes such as SCD is well-established and has been extensively reviewed (Kong et al., 2015; Hulsmans et al., 2016; Zanni et al., 2019; Liu et al., 2011; Liu et al., 2011; Tang et al., 2012; Wynn et al., 2010; Mantovani et al., 2012; Yang et al., 2012; Kanellakis et al., 2012; Barron et al., 2011).

However, the crucial role of HIV-mediated platelet activation with the induction and maintenance of pro-fibrotic pathways has not received sufficient attention thus far (Figure 2.3).

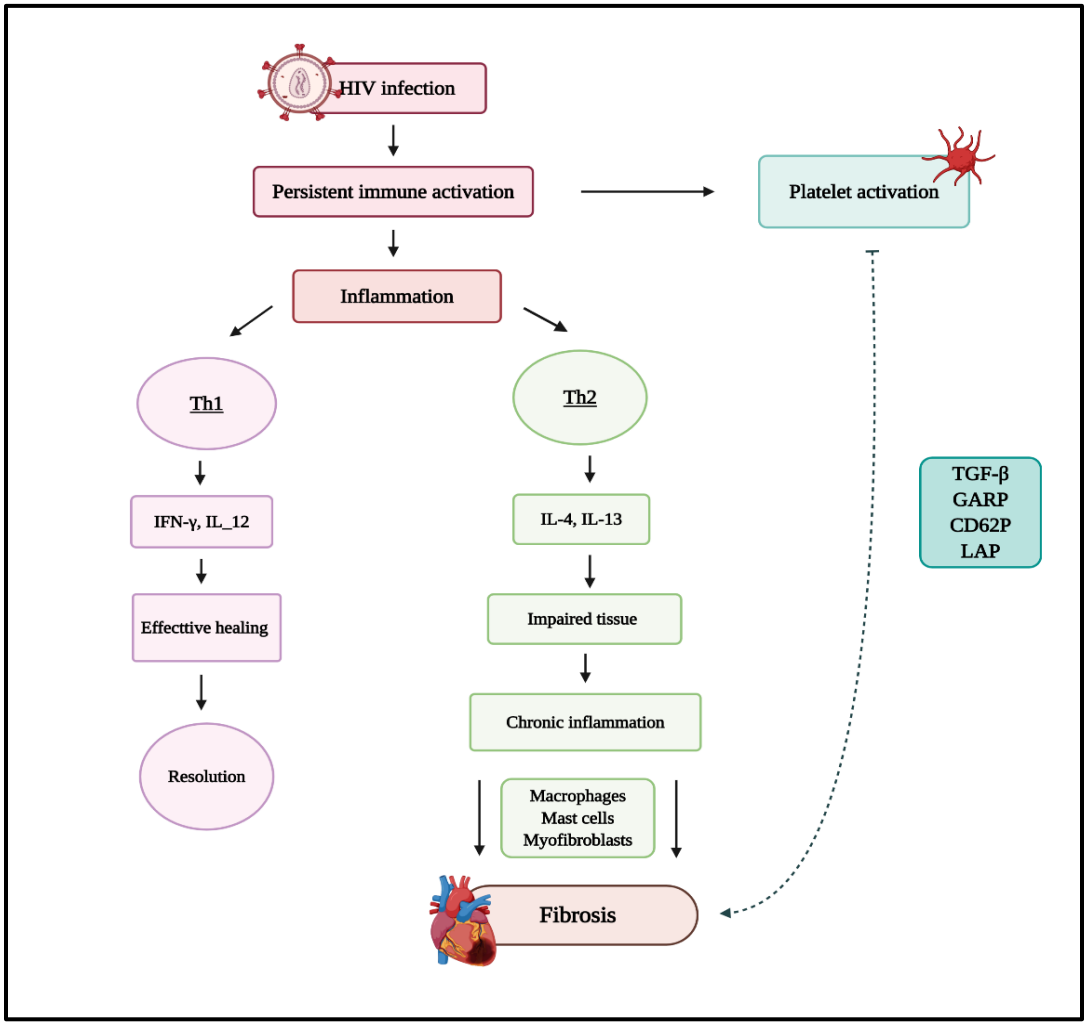

Figure 2.3. HIV infection causes the persistent activation and dysfunction of the immune and repair mechanisms. Effective healing is usually characterized by a dominant Th1 response, whereas a shift of the balance towards Th2 cells leads to chronic inflammation which can ultimately result in fibrosis. GARP: glycoprotein A repetitions predominant, HIV: human immunodeficiency virus, IFN-γ: interferon-γ, IL- interleukin, LAP: latent associate protein, SCD: sudden cardiac death, Th: T helper, TGF-β: transforming growth factor-β.

2.5. The crucial role of platelets in the induction of HIV-related myocardial fibrosis

The contribution of platelets to myocardial fibrosis in the context of HIV remains poorly understood. However, it is well established that upon activation platelets are an essential source of pro-fibrotic cytokines and growth factors that directly or indirectly, stimulate a fibrotic response. This occurs through the activation of fibroblasts or by promoting a fibrotic phenotype in macrophages and/or lymphocytes (Ahamed et al., 2016). Although persistent platelet activation is well documented in the HIV/cART setting its contribution to myocardial fibrosis is less emphasized (Van der heijdin et al., 2017; Ahamed et al., 2016; Mayne et al., 2012; Satchell et al., 2014). Of note, both cART-treated and cART-naïve patients display elevated plasma TGF-β1 concentrations that directly correlate with VL (Liovat et al., 2012), indicating that fibrosis may occur independently of cART. While platelets contain a multitude of factors that may contribute to fibrosis, recent studies indicate that platelet-derived TGF-β play a significant role in the pathogenesis of myocardial fibrosis (Meyer et al., 2012; Laurence et al., 2017; Varshney et al., 2019).

2.5.1. Morphology, structure and function

Platelets, small anucleate cellular fragments, are derived in the bone marrow from megakaryocytes (Rendu et al., 2001). Platelets are complex fragments that consist of functional organelles and various zones (peripheral, sol-gel and organelle) that contribute to the essential and multifactorial functions of platelets (Rendu et al., 2001; Blair and Flaumenhoft., 2009; Cimmino and Golino., 2013). The peripheral zone consists of coagulation factors and endogenous platelet agonists (Rendu et al., 2001), while the sol-gel zone contains the open canicular and dense tubular system (Blair &

Flaumenhoft., 2009; Cimmino & Golino., 2013). The organelle zone contains numerous granules, chemokines and mitogenic factors. The platelet granule content consists of various chemokines, coagulation proteins and immunological molecules that contribute to its function (Blair & flaumerhof., 2009; Cimmino & Golino., 2013). Platelets contain three types of granules (alpha, dense, and lysosomes) that each contains abundant chemokines (Blair & Flaumehaft, 2009; Fitch-Tewfik et al., 2013). Alpha granules are the most abundant type found in the platelets (Blair & Flaumehaft., 2009), while the dense granules contain high levels of calcium and phosphate (Rendu et al., 2001). Lysosomes contain hydrolytic enzymes that are active constituents of the ECM (Rendu et al., 2001). The contents of such granules are secreted when platelets are activated.

2.5.2. Platelet activation

Platelet activation can be initiated by numerous agonists (Blair & Flaumenhoft., 2009; Cimmino & Golino., 2013). During platelet activation its morphology undergoes physical changes, i.e. from a discoid shape to a spherical form with filopodia extrusions (Blair & Flaumenhoft., 2009). The activation process is complex and involves several intracellular signaling pathways including increased calcium, phosphoinositide metabolism and phosphorylation, and nuclear proteins. Activated platelets subsequently secrete the contents of their granules (Cimmino & Golino., 2013) and this can result in severe complications if excessively activated.

2.5.3. The role of platelet as a pro-fibrotic mediator in HIV infection

While platelets contain a wide variety of profibrotic cytokines and growth factors that can stimulate a fibrotic response through the direct activation of fibroblasts, or indirectly through the promotion of a fibrotic phenotype in M2 macrophages and lymphocytes (Kong et al., 2013), recent studies portray a significant role for platelet-derived TGF-β in the pathophysiology of myocardial fibrosis (Varshney et al., 2019; Meyer et al., 2012; Laurence et al., 2017).

2.5.3.1. Transforming Growth Factor-β

Transforming growth factor-β is one of the most significant fibrogenic growth factors that is persistently activated in animal models of cardiac remodeling and fibrosis (Dobaczewski et al., 2011; Frangogiannis, et al., 1998). Transforming growth factor-β is a pro-fibrotic cytokine that stimulates ECM protein production in different organ systems. Transforming growth factor-β exists in three isoforms in mammals, i.e. TGF-β_1, TGF-β_2 and TGF-β_3. Transforming growth factor-β is expressed in myofibroblasts, vascular smooth muscle cells, endothelial cells and macrophages

(Agrotis et al., 2005). While TGF-β plays a significant physiological role, its overexpression results in increased ECM protein and collagen synthesis, which may lead to fibrosis. The myocardium contains TGF-β as a biologically inactive molecule bound to latent-associated peptides (Pedrozo, 1998). Following injury or activation by cell–cell interaction, acidification and enzymatic cleavage (Linjen et al., 2000), there is an increase in extracellular TGF-β concentrations. This occurs through *de novo* synthesis, release from latent stores and via secretion from several cellular sources such as macrophages, fibroblasts, vascular cells, cardiomyocytes and platelets (Dobaczewski et al., 2011).

2.5.3.2. Molecular mechanisms of TGF-β action and implication in myocardial fibrosis

Several studies implicate TGF-β in myocardial fibrosis (Dobaczewski et al., 2011). For example, some found that TGF-β1 deficient mice exhibited attenuated age-associated fibrosis (Dobaczewski et al., 2011). In agreement, the inhibition of TGF-β prevented myocardial fibrosis in a rat model of cardiac pressure overload (Varshney et al., 2019). More recently, the genetic deletion of the TGF-β receptors in fibroblasts reduced myocardial fibrosis in an animal model of ventricular pressure overload (Varshney et al., 2019). Together these studies highlight the significant role of TGF-β in cardiac fibrotic remodeling (Dobaczewski et al., 2011).

After TGF-β1 synthesis and release into the extracellular space, it may bind to two serine-threonine kinase receptors namely TGF-β1 receptor 1 (TβRI) and 2 (TβRII) (Dobaczewski et al., 2011). Binding of TGF-β1 to TβRI results in the phosphorylation of Smad proteins and the formation of a heteromeric complex that regulates DNA transcription. In the heart, the effects of TGF-β1 are mediated through Smad2 phosphorylation (Greene et al., 2003). After Smad2 phosphorylation, a complex is formed with Smad3 and Smad4 which translocates to the nucleus of the target cells and binds to oligonucleotides in the regulatory regions of specific genes (Greene et al., 2003). Here the

complex regulates the expression of genes involved in fibrogenesis (Greene et al., 2003) that include connective tissue growth factor and periostin (Greene et al., 2003). The regulation of gene expression results in the production of pro-fibrotic matricellular protein and secretion into the ECM. This modulates intercellular and cell-matrix interactions that can enhance ECM protein synthesis (Lijnen & Petrov, 2002). The TGF-β1-Smad pathways can also activate collagen-gene promoter sites to enhance DNA transcription of collagen type I. In contrast, Smad proteins 6 and 7 inhibit the phosphorylation of Smad2 and disrupt the Smad complex formation (Lijnen & Petrov, 2002). An alternative pathway for TGF-β1-induced fibrosis exists and involves the TGF-β1 activated kinase (TAK1) pathway that is activated when TGF-β1 binds to TβRII (Greene et al., 2003). TGF-β1 activated kinase is a major downstream modulator of the TGF-β1 superfamily and is a member of the mitogen-activated protein kinase family (Lijnen & Petrov, 2002). In support of these notions, the administration of TGF-β1 to cardiac fibroblasts resulted in a 200-400% increase in TAK1 activity together with enhanced cardiac mass and accompanied by significantly decreased systolic and diastolic cardiac functioning (Lijnen & Petrov, 2002).

The various cellular TGF-β sources make it difficult to pinpoint the exact source that contributes most to myocardial fibrosis. However, three recent studies indicated that platelet derived TGF-β plays a significant role in the pathogenesis of myocardial fibrosis (Meyer et al., 2012; Laurence et al., 2017; Varshney et al., 2019) (Table 2.3).

2.5.3.3. Platelet contributions to plasma TGF-β and myocardial fibrosis

Following constriction of the transverse aorta, plasma TGF-β levels were significantly decreased in thrombocytopenic mice with a megakaryocyte-specific deletion of the TGF-β1 gene ($Tgfb1^{flox}$). Thrombocytopenia in these mice were induced by injecting mice with an anti-αIIbβ3 monoclonal antibody. Of note, these mice also did not develop cardiac hypertrophy, fibrosis and systolic

dysfunction in response to aortic constriction (Meyer et al., 2012). This highlights the significant contribution of platelet-derived TGF-β in myocardial fibrosis and cardiac dysfunction. The mice with the megakaryocyte-specific deletion of TGF-β1 ($Tgfb1^{flox}$) survived into adulthood without abnormalities, unlike other studies where non-specific targeted deletion of the TGF-β1 gene resulted in early morbidities (Meyer et al., 2012). The platelet counts, mean platelet volume and function (measured by platelet aggregation by ADP/thrombin) in the $Tgfb1^{flox}$ mice were similar to controls (Meyer et al., 2012). This indicates a possible therapeutic intervention as mice with a targeted megakaryocytic deletion of TGF-β can still function optimally without the risk of cardiac dysfunction.

A recent murine study showed that TGF-β deletion in platelets attenuated aortic stenosis, a condition characterized by myocardial fibrosis and narrowing of aortic valves, further implicating the role of platelet-derived TGF-β in fibrosis (Varshney et al., 2019). Additionally, this study revealed the significant role of platelets in the progression of aortic stenosis. Here scanning electron microscopy and immunostaining imaging of aortic valves revealed the presence of activated platelets attached to valvular endothelial cells which expressed high levels of phosphorylated Smad2 (Varshney et al., 2019). In mice with targeted deletions of platelet-TGF-β1 (TGF-$\beta1^{plateletKO}$-LDLR); reduced aortic stenosis progression with lower Smad2 phosphorylation was observed compared to controls.

Some also found that fibrosis can be linked to platelet activation and TGF-β1 release (Laurence et al., 2017). This study provided more context into the HIV field as the investigators treated mice with pharmacological daily doses of ritonavir, a potent PI, for 8 weeks (Laurence et al., 2017). Here mice with a targeted TGF-β deletion in megakaryocytes were partially protected from ritonavir-induced cardiac dysfunction and fibrosis (Laurence et al., 2017). In contrast, mice

without the deletion demonstrated fibrosis and decreased cardiac function in response to ritonavir. The fibrosis correlated with plasma TGF-β levels and the activation of Smad2/3 and TAK1/MKK3/p38 pathways in the heart (Laurence et al., 2017). The significant contribution of platelet-derived TGF-β in myocardial fibrosis may be attributed to the fact that platelets contain 40-100x the physiological TGF-β levels that are released upon platelet activation as compared to other cells that also secrete this growth factor.

Table 2.3. Contribution of platelet-derived TGF-β to pathological myocardial fibrosis

Reference	Model	Study design	Study Outcome
Meyer et al., 2012	Mice	Thrombocytopenic mice induced by injection of anti-αIIbβ3 monoclonal antibody.	No cardiac hypertrophy, fibrosis and systolic dysfunction development.
Meyer et al., 2012		Deletion of platelet specific TGF-β gene (TGF-^{flox}).	Platelet count, function and mean platelet volume similar to controls. No sign of cardiac hypertrophy, fibrosis & systolic dysfunction.
Varshney et al., 2019	Mice	Deletion of platelet specific TGF-β gene (TGF-β1$^{PlatletKO-LDLR}$).	Reduced aortic stenosis (defined by myocardial fibrosis), decreased Smad2 phosphorylation.
Varshney et al., 2019		Mice without targeted deletion of platelet specific TGF-β gene.	Increased activated platelets & elevated expression of phosphorylated Smad2.
Laurence et al., 2017	Mice	Targeted deletion of platelet-TGF-β1 Treated with daily dose of Ritonavir for 8 weeks.	Partial protection against Ritonavir-induced cardiac dysfunction & fibrosis.
Laurence et al., 2017		Mice without the targeted deletion of platelet TGF-β.	Decrease cardiac function with fibrosis, increase plasma TGF-b1 and increased activation of

			Smad 2/3 & TAK1/MKK p38 pathways.

2.6. The role of platelet-derived TGF-β in HIV-related myocardial fibrosis

With HIV-infection there are three mechanisms of platelet activation that can result in TGF-β release, i.e. a) binding of the HIV viral envelope to dendritic cell-specific ICAM-grabbing non-integrin, a pathogen receptor expressed on platelets (Assinger, 2014; Ahamed et al., 2017), b) stimulation by inflammatory cytokines (IL-6, IL-8, and IL-1β) that are elevated due to the chronic state of inflammation (Assinger, 2014; Ahamed et al., 2017), and c) thrombin generation that is mediated through monocyte-derived tissue factor (TF) which is significantly increased in HIV-positive patients (both in its soluble state as well as its expression on monocytes) (Funderburg et al., 2010). Furthermore, certain types of cART – especially PI – can also promote platelet activation (Loelius et al.,2017). Therefore, platelet activation persists due to the persistent immune activation and chronic state of inflammation. This in turn can induce the secretion of platelet-derived TGF-β (Ahamed et al., 2017; Ahamed et al., 2005; Ahamed et al., 2006).

While platelets contribute to ~ 80% of TGF-β in terms of development of cardiac fibrosis, HIV-related fibrosis is multi-factorial and other inflammatory cells can further exacerbate this pathology. Due to the infection, endothelial cell injury along with activated monocytes/macrophages and platelets lead to the production of reactive oxygen species production and oxidative stress (Ahamed & Laurence et al., 2017). Reactive oxygen species is a potent activator of TGF-β and its generation occurs relatively early-on during HIV infection and despite effective cART (Ahamed & Laurence et al., 2017). This creates a positive feedback loop with

platelet activation and the transition of latent TGF-β to its active, pro-fibrotic form (Ahamed & Laurence et al., 2017). Protease inhibitors such as ritonavir and abacavir can further exacerbate platelet activation and fibrotic signaling. This can directly activate platelets or indirectly through the induction of oxidative stress (Van der Heijden et al., 2017; Loelius et al., 2017; Ahamed et al., 2016; Mayne et al., 2012; Laurence et al., 2017; Satchell et al., 2016; Kort et al., 2011).

Ahamed et al., (2017) hypothesized a positive feedback loop where HIV infection and particular protease inhibitors can increase oxidase stress that in turn promotes platelet activation and TGF-β1 signaling via activation of TNF receptor associated factor-6 (TRAF-6) (a nuclear signaling adapter protein) and ubiquitin E3 ligase (Ahamed et al., 2016; Laurence et al., 2017). In support, others also implicated this pathway in the exacerbation of pathological cardiac hypertrophy via TAK1/MKK3/p38-dependent signaling (Ji et al., 2016). However, the signaling activity (and not plasma TGF-β concentrations) may be elevated due to cART-mediated facilitation of TRAF-6 activity through the inhibition of immunoproteasome function. The latter suppresses TRAF-6 degradation that is essential for activation of collagen regulatory pathways. The inhibition of immunoproteasome function prevents the degradation of TRAF6. This will increase the activation of cytokine pathways regulated by TRAF6 such as IL-6, IL-8 and TGF-β (Laurence et al., 2007).

2.7. Diagnosis of myocardial fibrosis

The present gold standard of diagnosing myocardial fibrosis is an endomyocardial biopsy which allows for the detection and quantification of interstitial collagen content (Jellis et al., 2010; Martos et al., 2007). There are non-invasive techniques that can identify fibrotic tissue and the most common is cardiac magnetic resonance imaging which allows for the determination of LV volume and mass. Cardiac magnetic resonance can also identify focal and diffuse myocardial fibrosis, inflammation and edema (Puntmann et al., 2016; Wong et al., 2012). The assessment of

replacement cardiac scar fibrosis, which generally occurs after a myocardial infarction, employs the use of gadolinium-based contrast agents. The quantification of extracellular volume and characterization of cardiac tissue composition can be completed using T1 mapping (Graham-Brown et al., 2017). However, this method is expensive and requires significant skills for acquisition and analysis of the acquired images (Graham-Brown et al., 2017).

Echocardiography (ECG) is another diagnostic tool utilized where 'speckles' can be detected with ultrasound natural acoustic reflections. This shows various characteristic patterns throughout the myocardium (Graham-Brown et al., 2017). While quantitative and functional assessments are possible, this depends on image quality that varies from patient to patient and also between operators handling the equipment (Jellis et al., 2010; Graham-Brown et al., 2017).

Collagen volume fraction (CVF) presents the total amount of myocardial tissue occupied by collagen fibers. This can be determined using automated image analyses with collagen-specific staining or monoclonal antibodies specific for collagen fibers (Hoyt et al., 1984). Single photon emission computed tomography or positron emission tomography can be used to detect perfusion defects (Disertori et al., 2017). There are currently only two confirmed biomarkers associated with myocardial fibrosis; carboxy-terminal propeptide of procollagen type I and the amino-terminal pro-peptide of procollagen type III (Prockop & Kivirikko, 1995; Jellis et al., 2010).

Although the methods discussed are routinely employed to diagnose myocardial fibrosis, none fulfills all the requirements to identify myocardial fibrosis. A combination of imaging, biomarker assessment, histological and histochemical staining is required to characterize myocardial fibrosis (Graham-Brown et al., 2017). Although the endomyocardial biopsy procedure is safe, the difficulty in performing it in clinical practice for research purposes must be recognized. Thus, the current

status reveals that there is scope for the development of novel and non-invasive methods for the detection of myocardial fibrosis *in vivo*.

2.8. Research question, motivation, aims and objectives

Combined antiretroviral treatment has prolonged and enhanced the quality of life of PLHIV by improving immunological recovery and by suppressing viremia. However, despite such advances immune activation persists and subsequent immune dysregulation can occur. This in turn can lead to a variety of complications due to downstream effects of activated immune cells. For example, it can lead to the induction of pro-thrombotic and pro-fibrotic states, endothelial dysfunction and increased risk for CVD onset and progression. This study therefore focuses on the less studied role of platelets in this context, with an emphasis on myocardial fibrosis.

While we understand that platelet activation contributes to fibrosis through various pathways as discussed, the detection methods are limited as many studies for example reported decreased plasma TGF-β levels in HIV-positive patients. Furthermore, the current methods to assess fibrosis are of an invasive nature (endomyocardial biopsy) and also relatively expensive (cardiac magnetic resonance). In light of this, we set out to pursue a novel way of screening for myocardial fibrosis and potential HF/CVD risk in HIV-positive individuals:

1) Blood pressure and ECG

2) Flow cytometric analyses of increased platelet activation through the following markers:

    - CD62P and LAP – classic markers of platelet activation

    - GARP and intracellular TGF-β – linked to fibrotic signaling

2.8.1. Hypothesis

We hypothesize that due to the immune dysregulation in HIV-infection there is hyperactivation of platelets. This results in the excessive release of mediators that promote a pro-thrombotic and pro-fibrotic state in addition to endothelial dysfunction. These factors culminate and increase the risk for HF/CVD.

To test this hypothesis, the following objectives were set:

a) Evaluate platelet activation markers and indices

b) Correlate platelet activation with TGF-β, a pro-fibrotic marker

c) Correlate platelet activation with clinical characterization of HF and CVD, BP, ECG and flow mediated dilation (FMD)

d) Determine the relationship between platelet activation and HIV disease progression (VL CD4 count).

Chapter 3 -Methodology

3.1. Study population and design

This thesis forms part of a larger longitudinal investigation, namely the EndoAfrica study, set in Worcester that is located within the Cape Winelands region of the Western Cape (South Africa). This particular thesis is a cross-sectional study investigating the association between platelet activation and the onset and/or elevated risk of HF/SCD in HIV-positive patients on cART. Ethical approval was obtained from the Human Research Ethics Committee of Stellenbosch University and the Department of Health (Western Cape Government, South Africa), reference number N19/02/029. Prior to the study, all participants were informed about the various procedures and consent forms were signed by all the study participants included. After the completion of informed consent, fasted blood samples were obtained from participants in addition to the completion of various evaluations (detailed later in this section). The inclusion criteria were HIV-positive and HIV-negative participants, 18-55 years; while HIV/TB infected patients, pregnant patients were excluded from the study. Women were given a rapid pregnancy test. Study subjects recruited were divided into groups: HIV-negative and HIV-positive on cART (Figure 3.1). The status of HIV negative controls was confirmed by a rapid HIV test.

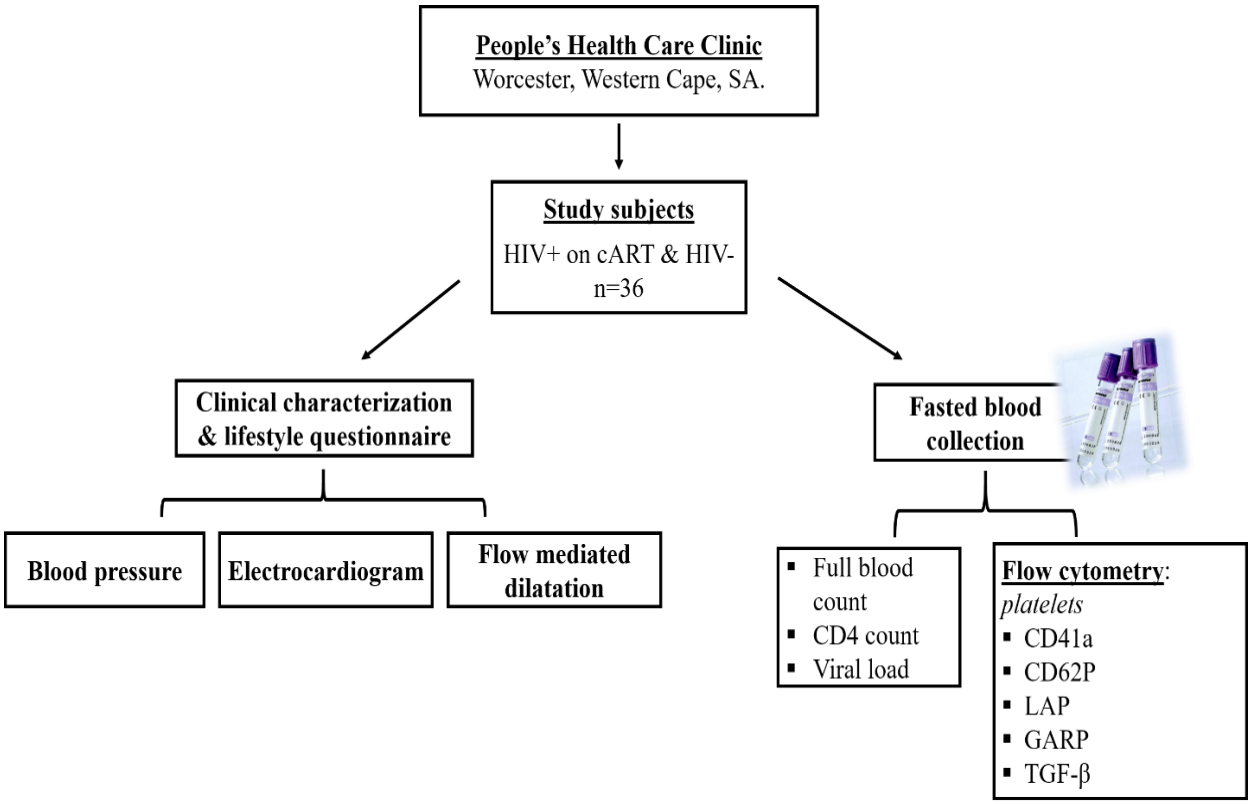

Figure 3.1. Layout of research methodology. Summary of study recruits and various assessments completed. cART-combined antiretroviral treatment, GARP – glycoprotein-A repetitions predominant, LAP – latent associated protein, TGF-β – transforming growth factor-β, SA – South Africa.

3.1.1. Clinical visitations

The study was conducted at the People's Healthcare Clinic located in Worcester (Western Cape, South Africa). Participants were originally recruited at the Worcester Community Health Centre which they attended for HIV testing and to receive cART. As this is a follow-up study, the patients recruited for this study had also previously been recruited by the registered research nurse.

3.1.2. Clinical history and patient characterization

All participants were recruited from the same communities within the Worcester region (South Africa) to ensure similar CVD risk and socio-economic factors. A formal interview was conducted and during this process a comprehensive lifestyle questionnaire was completed for all patients by Dr. Ingrid Webster (a co-worker on the larger study). However, the main history and characterization profile of participants were recorded as follow:

- Age
- Duration of infection (if HIV-positive)
- History of cART – including the duration, first line or second line and regularity of treatment
- Viral load
- CD4 count
- Blood pressure, heart rate
- CVD history – including family history
- Record of any other medication
- Information of smoking, alcohol consumption and diabetes

3.1.3. Clinical examinations

Blood pressure and heart rate

Blood pressure measurements were obtained before blood was drawn, and were obtained twice on the left arm with an automated BP reader Omron M6 (OMRON Healthcare, Netherlands) with 2-5-minute intervals of rest between. For BP measurements, participants sat upright with their legs uncrossed and arm supported at heart level.

Electrocardiogram

The OMRON Heart Scan HCG-801 ECG monitor (OMRON Healthcare, Netherlands) was used to evaluate resting ECG of patients.

Flow-mediated dilation

Endothelial dysfunction occurs early in CVD and FMD is a non-invasive test that can evaluate it. It is based on the ability of the endothelium to release nitric oxide in response to a stress stimulus (Solages et al., 2006). Here the brachial arterial diameter can be measured by ultrasound. This test evaluates the baseline brachial artery diameter (at rest) after which the maximum diameter is taken after five minutes of ischemia once the BP has been deflated. The subsequent vasodilation can be imaged and quantitated as an index of vasomotor function (Solages et al., 2006). This procedure was performed at sample collection by one of our collaborators from the Division of Medical Physiology (Faculty of Medicine and Health Sciences, Stellenbosch University, South Africa).

3.1.4. Blood collection

Whole blood was collected by the research nurse in the correct order of draw to avoid additive cross contamination. Approximately seven tubes were drawn per patient (35.5 mL in total). Blood samples were sent to National Health Laboratory service (NHLS) laboratories at Tygerberg Hospital (Western Cape, South Africa) for CD4 counts. 1x EDTA tube was used for peripheral blood film preparation and analyses. The second sodium citrate tube collected was utilized for platelet evaluations by flow cytometry (refer section 3.2) – this was to avoid excessive platelet activation. Of the whole blood collected, 1x EDTA-containing and 1x red top tube (clotted blood) tubes were immediately centrifuged at 2000g for 10 minutes. Plasma and serum were subsequently isolated and samples stored at -80◦C until thawed for subsequent analyses.

3.1.5. CD4 Count

The CD4 count was performed at NHLS, Tygerberg hospital, Western Cape (South Africa). The standard CD4 count methodology employed used TruCOUNT tubes (BD Biosciences, San Jose CA) together with MultiTEST™ CD3 FITC/ CD8 PE/ CD45 PerCP/ CD4 APC Reagent (BD Biosciences, San Jose CA). MultiTEST CD3-FITC/ CD8-PE/ CD45-PerCP/ CD4-APC reagent was added to the TruCOUNT tube followed by addition of 50 µL EDTA blood and gentle mixing. The samples were then incubated for 15 minutes at room temperature (in the dark) whereafter FACS lysing solution (BD Biosciences, San Jose, CA) was added to the tube. This was followed by a 15-minute incubation at room temperature (in the dark), after which the cells were analyzed on a BD FACS Calibur four-color flow cytometer with designated software (BD Biosciences, San Jose, CA).

3.1.6. Viral load (HIV-1 quantitative assay)

The evaluation of VL is an important assessment of HIV status and disease progression. Approximately 1 mL of frozen plasma (previously collected) was used to determine VL. This *in vitro* nucleic acid amplification test allows the quantitation of HIV RNA in human plasma using the COBAS® AmpliPrep Instrument and TaqMan® Analyzer amplification and detection (Roche Molecular Systems, Inc., Branchburg, NJ). These tests were completed at a South African National Accreditation Services-accredited laboratory based within the Division of Medical Virology, Faculty of Medicine and Health Sciences (Stellenbosch University, South Africa).

3.2. Flow cytometry

These studies were performed at the Central Analytical Facilities (CAF) Fluorescent Imaging Unit of Stellenbosch University. The BD FACS Aria I cell sorter (BD FACS Aria I, BD Biosciences, San Jose, CA) was used and the following strategy employed (Figure 3.2):

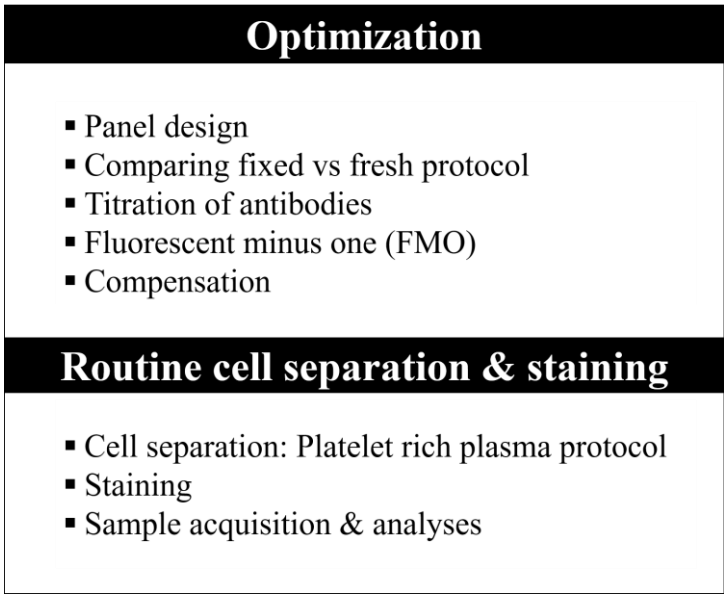

Figure 3.2. **Flow diagram of flow cytometry protocol**. This is a broad road map of the strategy employed and will be explained in detail for the rest of this chapter.

3.3. Optimization of flow cytometry set up

3.3.1. Panel design and gating strategy for flow cytometry work

Multicolor flow cytometry is a powerful tool to detect and analyze multiple parameters at a cellular level. However, the quality of the results depends on the proper design and optimization of the instrument set up and here we employed the following panels (Table 3.1):

Platelet identification marker: CD41a

Platelet activation markers: CD62P, LAP, GARP, TGF-β.

Table 3.1. **Antibodies with the fluorochromes employed in the study.**

Platelet markers of identification and activation	
Antibody	Fluorochrome
CD41a	APC
LAP	PE
GARP	BV421
CD62P	BV510
TGF-β	Alexa Fluor ® 488

Gating strategy employed

Platelet marker, CD41a, was used as a gating marker since it is only expressed by platelets and megakaryocytes (Gobbi *et al*, 2003). To identify platelets, the following gating protocol was followed (Figure 3.3). Forward scatter-area versus time to identify the cells present. Forward scatter-height versus forward scatter-area to exclude clumped cells present in the sample. Lastly, SSC-A versus Comp-APC-A CD41a APC-A (where Comp-APC-A was in log format) to identify the CD41a+ cells. All other parameters were determined from the resulting singlet cells (Figure 3.3), with CD41a on the x-axis and other antigens presented on the y-axis, in log format. FMOs were performed to ensure the gating strategy employed would accurately differentiate true positive and negative populations for the regions of interest.

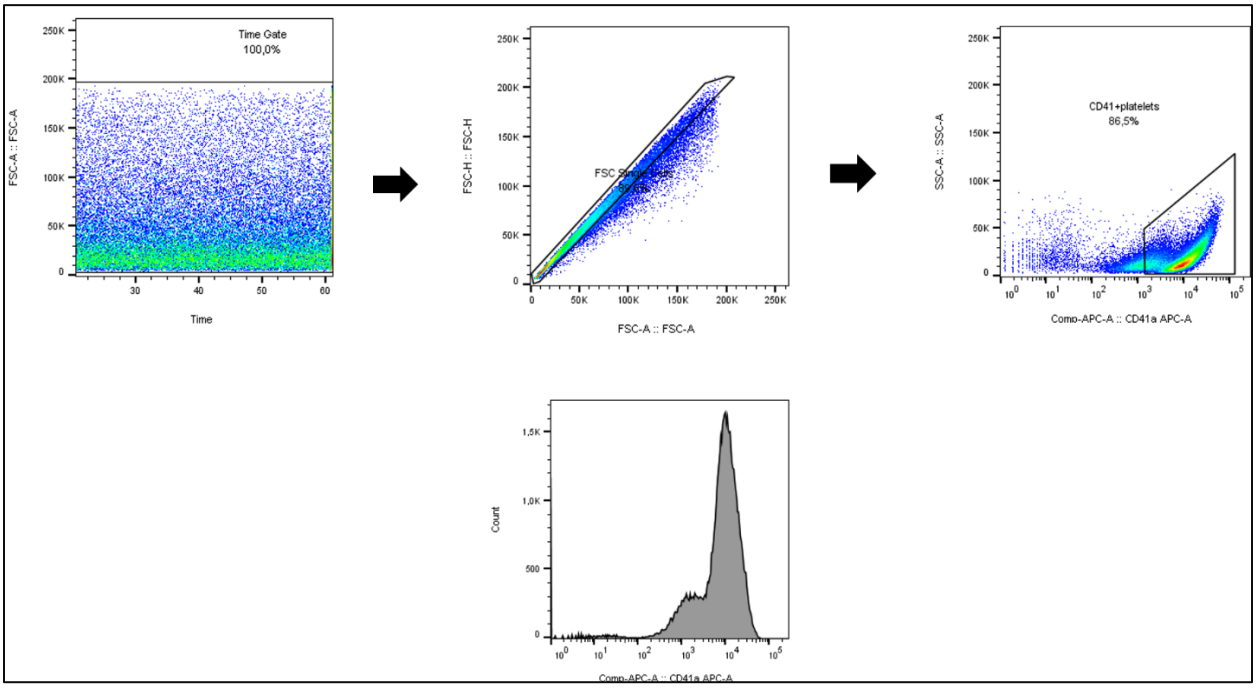

Figure 3.3: Flow cytometry scatter plots and histogram illustrating the platelet gating strategy based on CD41a expression. FSC: forward scatter, SSC: side scatter

3.3.2. Comparing protocols (fixed vs fresh) for optimal platelet flow cytometry

In order to determine which staining protocol would yield the optimal fluorescence, two platelet staining methods were tested using blood samples obtained from a healthy person. Both samples were drawn on the same day.

3.3.2.1. Fresh protocol

Two sodium citrate vacutainers were used to draw blood from a healthy donor. The first sample was discarded to avoid hyperactivated platelets that would be present. The second sample was thereafter used and centrifuged at 0.1 g for 15 minutes to obtain platelet rich plasma (PRP) (as per protocol detailed below). In parallel, six microfuge tubes were prepared and labeled according to the antibody that would be added, in addition to an unstained sample for the control. Subsequently,

100 µL PRP was added to a labeled microfuge tube followed by the addition of 5 µL calcium ionophore A23187 (concentration of 1 nmol/mL) and left for 5 minutes. This was done in order to activate the platelets and to ensure that platelet activation markers would be adequately expressed for flow cytometric analyses. Antibodies for extracellular markers (Table 3.1) were added to the appropriately labeled tubes according to the manufacturers' instructions, and this was left to incubate for 30 minutes in the dark. Here the samples were wrapped in foil and put in a dark cupboard to ensure no light would influence the light sensitive antibodies. Thereafter 500 µL 1X FACS lysing solution (BD Biosciences, San Jose, CA) was added to each sample and left for 10 minutes. This was to lyse and fix cells as FACS lysing solution also contains <15% formaldehyde, a fixative. Subsequently, the samples were centrifuged for 10 minutes at 1.2g. The supernatant was decanted, leaving behind a pellet to which 500 µL 1X Perm/wash solution (BD Biosciences, San Jose, CA) was added and left for 10 minutes at 4°C. The 1X Perm/wash solution is used when there is intracellular staining as it permeabilizes cells and acts as an antibody diluent and wash buffer. Thereafter 500 µL staining buffer was added to each sample and centrifuged at 1.2 g for 10 minutes in order to obtain a pellet after decanting the supernatant. Hereafter, 5 µL of the intracellular TGF-β antibody was added to the appropriately labeled tube and left to incubate for 30 minutes at room temperature. After this 1000 µL staining buffer was added to each tube for a final washing step, and the sample again centrifuged at 1.2 g for 10 minutes. The supernatant was decanted and 500 µL staining buffer added. Samples were then stored at 4°C until ready for analyses using the BD FACS Aria (BD FACS Aria I, BD Biosciences, San Jose, CA).

3.3.2.2. Fixed protocol

Two sodium citrate vacutainers were used to draw blood from a healthy donor. The second sample was used and centrifuged at 0.1 g for 15 minutes to obtain PRP (as per protocol detailed below).

Six microfuge tubes were prepared and labeled according to the antibody that would be added in addition to an unstained sample for the control. Subsequently, 100 µL PRP was added to a labeled microfuge tube and 5 µL of 1 nmol/mL calcium ionophore added and left for 5 minutes in order to activate the platelets and to ensure that the platelet activation markers (Table 3.1) would be adequately expressed for flow cytometric analyses. Thereafter 500 µL 1x FACS lysing solution (BD Biosciences, San Jose, CA) was added to samples to fix and permeabilize the cells, and left to incubate for 10 minutes at room temperature. The samples were subsequently centrifuged at 1.2 g for 10 minutes and the supernatant decanted to leave behind the pellet. We then added 500 µL of 1x Perm/wash solution to each sample that was left to incubate for 10 minutes at 4°C. Thereafter, 500 µL staining buffer was added and centrifuged at 1.2 g for 10 minutes, with the supernatant then decanted to leave behind a pellet. Extra- and intracellular antibodies were then added to the pellet and then left incubate for 30 minutes at room temperature. After incubation, 1000 µL staining buffer was added to the samples and centrifuged at 1.2 g for 10 minutes as a final washing step. Thereafter the supernatant was decanted and 500 µL SB added, and the pellet then resuspended. The samples were stored at 4°C in foil to preserve the light-sensitive antibodies, until ready to analyze using the BD FACS Aria (BD FACS Aria I, BD Biosciences, San Jose, CA).

3.3.3. Antibody titrations

To optimize antibody concentrations for platelet flow cytometric analyses, antibody titrations were performed on isolated platelets (from a healthy donor) and activated with 1 nmol/mL calcium ionophore. This was done to ensure that all markers of interest would be expressed (Table 3.1). Titrations were done to determine the optimal volumes that generate a signal strong enough to allow for precise measurement of antibody fluorescence. Furthermore, this also minimizes non-specific binding and ensures that the most cost-effective approach is employed.

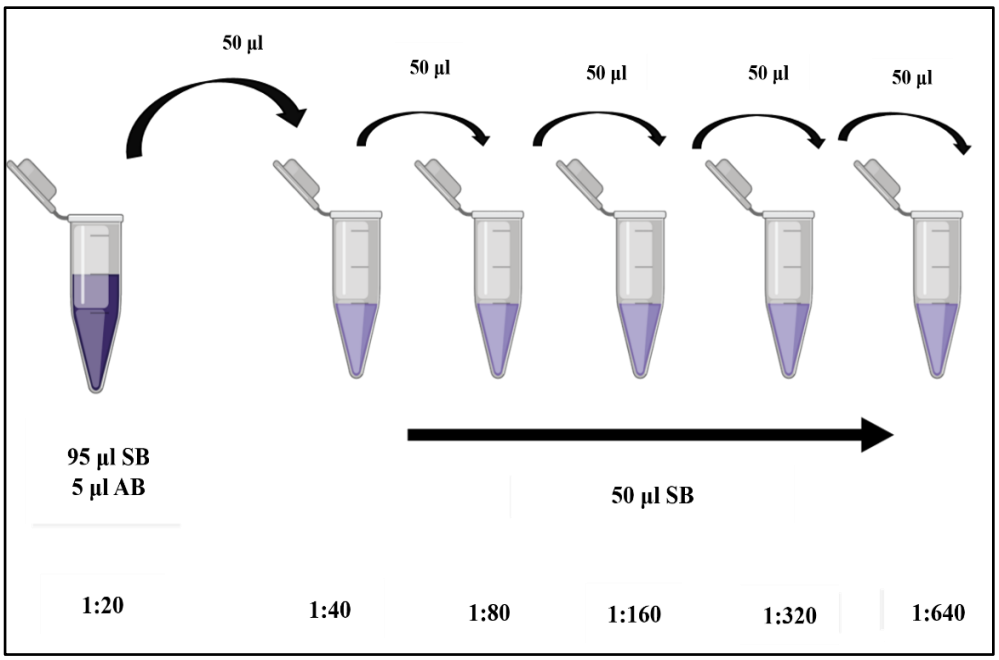

Figure 3.4. Antibody titrations. Titrations were employed to ensure optimal volumes were used in order to generate a signal strong enough to allow for precise measurement of antibody fluorescence and to minimize non-specific binding.

Each antibody underwent the same series of dilutions in order to determine which ones would be optimal. Titrations were performed in doubling dilution steps (Figure 3.4). The fresh protocol (as explained in section 3.3.2.1) was employed when doing the dilutions. Two samples were drawn into sodium citrate vacutainers (inverted 8 times) from a healthy donor and the second sample used to minimize platelet activation. The sample was centrifuged at 0.1 g for 15 minutes to obtain the PRP. The PRP was aliquoted into appropriately labeled microfuge tubes. 1 nmol/mL calcium ionophore A23187 was then added to activated the platelets in order for all the activation markers to be expressed. For each marker (CD41a, CD62P, LAP, GARP, TGF-β), six tubes were labeled with different dilution factors (Figure 3.4). The appropriate volumes of staining buffer and antibody were added to the first tube, after which samples were titrated downwards. 50 µL of activated

sample was then added to each tube and incubated for 30 minutes at room temperature in the dark. This is to preserve the antibodies that are light sensitive. Important to note, TGF-β is an intracellular marker. Therefore, its' sample preparation and titration consisted of first treating samples with FACS lysing solution (BD Biosciences, San Jose CA) for 10 minutes. This also serves as a fixative before adding the intracellular antibody and incubating it in the dark for 30 minutes. The rest of the steps were the same (Figure 3.4). After incubation, 500 µL FACS lysing solution (BD Biosciences, San Jose, CA) was added to fix the samples - 10 minutes in the dark at room temperature. After fixation, samples were centrifuged at 1.2 g in a microcentrifuge for 10 minutes. The supernatant was decanted and left behind a pellet. 500 µL of 1 Perm/wash solution (BD Biosciences, San Jose, CA) was then added to each sample and this was left to incubate for 10 minutes at 4°C. Thereafter 500 µL PBS was added to the samples, followed by centrifugation at 1.2 g for 10 minutes as a washing step. The samples were subsequently decanted to leave behind only a pellet to which 1000 µL PBS was added as a final washing step. The sample was then centrifuged at 1.2 for 10 minutes. Thereafter this was decanted to leave behind a pellet, to which 500 µL PBS was added. The samples were then covered with foil and stored at 4°C until used for analyses on the BD FACS Aria (BD FACS Aria I, BD Biosciences, San Jose, CA).

After data acquisition using the BD FACS Aria (BD Biosciences, San Jose CA), analyses were performed on the data using FlowJo™ software. Both negative and positive populations were identified for each antibody. The staining index (SI) value for each titration was calculated using the equation below:

The equation used to calculate the staining index and to generate the titration curve. MFI= mean fluorescence intensity, SD= standard deviation.

$$SI = \left(\frac{MFI_{pos} - MFI_{neg}}{2(rSD_{neg})}\right)$$

Table 3.2. Titrated antibody volumes, manufacturer information and isotypes employed.

Antibodies	Manufacturer	Volume (µL)	Optimal dilution	Isotype
CD41a APC	BD Biosciences, San Jose CA	1.25	1:80	MsIgG1,κ (HIP8)
CD62P BV510	BD Biosciences, San Jose CA	0.625	1:160	
LAP PE	BD Biosciences, San Jose CA	2.5	1:40	Ms(BALB/c)IgG1, κ (TW4-2F8)
GARP BV421	BD Biosciences, San Jose CA	1.25	1:80	MsIgG2b, κ (7B11)
TGF-β1 AF488	BD Biosciences, San Jose CA	2.5	1:40	Ms(BALB/c)IgG1, κ (TW4-9E7)

3.3.4. Color compensation

Multi-colored flow cytometry has the advantage of identifying and characterizing very low cell frequencies. A significant issue with this method is the overlap between fluorescent dyes. Therefore, where emission spectra of fluorochromes may overlap, compensation must be made to prevent or limit spill-over of fluorescent signals from one detector channel (filter) to another. Some fluorochromes can also be excited by more than one laser and this can therefore contribute to spill over. The main goal of compensation is to eliminate spill over, especially considering the technical limitation of tandem dyes that tend to be more susceptible to damage upon exposure to light or

high temperatures (Baumgarth & Roederer, 2000; Bagwell & Adams, 1993). Compensation beads therefore capture species-specific antibodies conjugated to fluorophores and other types of reagents, with its main purpose being to set voltages and gating parameters in order to obtain accurate fluorescence signal (Baumgarth & Roederer, 2000).

Procedure

Positive and negative BD compensation beads (BD Biosciences, San Jose CA) were used and vortexed before using to ensure the proper mixing with less sedimentation. Microfuge tubes were labeled appropriately for the respective fluorochrome antibody. The antibodies were added at optimal concentrations to staining buffer in order to maintain the optimal titrations (Table 3.3). One drop of both positive and negative compensation beads was added in the appropriate microfuge tubes. This was then vortexed and incubated for 30 minutes in the dark at room temperature. Subsequently, 1, 000 µL staining buffer was added to each microfuge tube and centrifuged at 1.2 g for 10 minutes, whereafter the supernatant was discarded and the pellet resuspended in 500 µL SB. This was placed at 4°C until analyses on the BD FACS Aria (BD FACS Aria I, BD Biosciences, San Jose, CA). Compensation beads were also run as another control measure – in addition to the prevention of spill over - to ensure the instrument is functioning optimally. This was therefore essential in setting up the panel on the instrument and also before every sample run. This was done along with the 8 peak beads (detailed later on).

Table 3.3. The antibodies were added at the optimal antibody to staining buffer concentration in order to maintain the optimal titrations.

Antibody	Volume antibody (µL)	Volume staining buffer (µL)	Optimal dilution
CD41 APC	1.25	48.75	1:80
CD62P BV510	0.625	49.375	1:160
LAP PE	2.5	47.5	1:40
GARP BV421	1.25	48.75	1:80
TGF-β1 AF488	2.5	47.5	1:40

3.3.5. Fluorescent minus one (FMO)

To ensure that the gating strategy employed was able to accurately differentiate true positive and negative populations, FMO controls were performed for regions of interest. This is an additional and essential parameter required that involves the addition of all fluorescent reagents to a cell sample, except for the marker of interest. It is also important especially where positive signals are expressed at low levels, for example GARP.

Procedure

Five microfuge tubes were prepared, i.e. each one with all antibodies except for the antibody it was labeled with. After the appropriate antibodies were added to the tubes, 50 µL PRP from a normal control (preparation of PRP detailed below) were added to the tubes and mixed, and thereafter incubated at room temperature in the dark for 30 minutes. The staining protocol employed during the antibody titration experiments (Table 3.3) was also used in this instance and followed accordingly for cellular and intracellular markers.

3.4. Routine cell separation, preparation and staining

This was the method we employed to prepare platelets on a weekly basis as new samples were obtained. The following staining procedure was employed (Table 3.4).

Table 3.4. **Composition of flow cytometric panels**. APC: allophycoyanin, PE:Phyroerythrin, BV: Brilliant violet, AF: Alexa Fluor

Platelet identification marker	**Platelet activation marker**	**Intracellular marker**
CD41a APC	CD62P BV510	TGF-β AF-488
	LAP PE	
	GARP BV421	

3.4.1. Preparation of antibody staining mixture

Antibody panels consisted of both cellular surface and intracellular markers. The antibody mixtures were made up as indicated (Table 3.5) and appropriate volumes used to maintain the optimal antibody titrations (after addition of 50 µL sample).

Table 3.5. The antibody mixture that was made up using the volumes indicated.

Antibodies	µL/test
CD41a	1.25
DC62P	0.625
GARP	1.25
LAP	2.5
TGF-β	2.5
Staining buffer	41.875
Total	50

3.4.2. Cell preparation for flow cytometric analyses

Samples collected in sodium citrate vacutainers were centrifuged at 0.1 g for 15 minutes to separate cellular components and produce the PRP. It was also centrifuged at this speed to avoid platelet activation by using too much force. For each patient 50 µL of PRP was added to the antibody mixture as described above (Table 3.5).

Staining

500 µL of 1x FACS lysing solution (BD Biosciences, San Jose, CA) was added to microfuge tubes containing 50 µL of the patient's PRP for the simultaneous fixing and lysing of cells (for appropriate intracellular staining). After incubating for 10 minutes, it was centrifuged at 1.2 g for 10 minutes to isolate a platelet pellet. The supernatant was thereafter decanted leaving behind approximately 50 µL (the pellet), which was resuspended in 500 µL of 1x Perm/wash solution.

This was left to incubate at 4°C for 10 minutes. Thereafter 500 µL of staining buffer was added and this was then centrifuged at 1.2 rcfs for 10 minutes. The supernatant was decanted leaving behind a pellet suspended in remaining staining buffer of approximately 50 µL. The antibody cocktail mentioned above (Table 3.5) was added to the pellet and left to incubate in the dark at room temperature for 30 minutes. Samples were also covered in foil as an extra measure to ensure that light sensitive antibodies were not degraded. After incubating cells with the fluorescent antibodies, 1000 µL staining buffer was added to each sample which was then centrifuged at 1.2 g for 10 minutes as a final wash step. Thereafter, the supernatant was decanted leaving behind a pellet that was resuspended in 500 µL staining buffer and stored at 4°C wrapped in foil, to preserve light sensitive antibodies, until flow cytometric analyses within 24 hours.

3.4.3. Control measures

There were several control measures put in place with each run to ensure that sample runs produced optimal results. These included analyzing unstained patient samples in addition to the stained samples, and running positive and negative compensation beads along with the 8 peak beads. The purpose of running an unstained sample on each occasion is to identify the location of the negative population. This will also allow for the determination of the level of background fluorescence (or autofluorescence) to set suitable voltages and negative gates. Here 8-peak beads contain eight different populations that differ only in the amount of fluorophore contained within them. These beads are designed to check the sensitivity of fluorescence, resolution and linearity in flow cytometers and cell sorters. This is achieved through measuring the position of the unlabeled peak and separation between all peaks, respectively. The 8-peak beads remain the gold standard for estimating how a flow cytometer is performing in its ability to measure subtle differences in fluorescence levels.

3.4.4. Sample acquisition and data analyses

The samples were acquired using the BD FACS Aria flow cytometer (BD FACS Aria I, BD Biosciences, San Jose, CA) and analyzed using FlowJo™ software. The appropriate number events were collected per sample tube and expression of each marker was determined based on the total gated events. Data were exported from FlowJo™ software to an Excel spreadsheet to allow for further analyses.

3.5. Statistical analysis

GraphPad Prism 8 (GraphPad Software Inc, San Diego) was used for all statistical analyses. All data were tested for normality using the Shapiro-Wilk normality test. For normally distributed data, ordinary one-way ANOVAs were performed to compare different sub-study groups (HIV-negative, CD4<500 and CD4>500). Data was sorted according to the patients' HIV status (positive or negative) and according to CD4 count; low (CD4<500) and high (CD4>500). This was to indicate the patients' immunity and overall disease progression. For non-parametric data, Kruskal-Wallis tests were performed. Unpaired t-tests were utilized to compare data between two specific groups (HIV-negative versus HIV-positive). Here, Mann-Whitney tests were performed for on-parametric data. Additionally, Spearman's correlation test was performed to determine correlations and presented with linear regression XY data, together with significant co-efficient r-and p-values. Differences were considered significant if the p-value was <0.05. All data presented as the mean percentage ± standard error of the mean (SEM) unless otherwise stated.

Chapter 4– Results

All data presented in this chapter show the mean percentage ± standard error of mean (SEM) unless otherwise stated. One way-ANOVAs were performed to compare different sub-study groups: HIV-negative, CD4 <500 and CD4 >500 (extra analyses includes CD4 <350 and CD4> 350 sub-groups). Student t-test analyses were performed to compare data between two specific groups. A Spearman's correlation test was utilized to determine correlations presented with linear regression XY data, together with significant co-efficient r- and p-values.

4.1.Patient demographics

The targeted population for this study were HIV-positive and HIV-negative males and females that attended the People's Healthcare Clinic (Worcester, SA) - to ensure they shared similar CVD risk and socio-economic factors (Table 4.1). A total of 36 patients were recruited for this study. This included both a control group of 13 HIV-negative individuals and also 23 HIV-positive individuals. The age range of participants varied from 18-55 years, with a median age of ~43 years. Here 42% were males (n= 15) while females comprised the rest. Data collected from patient histories revealed that 22% of the HIV-positive participants (n=5) exhibited a family history of CVD, 39% were smokers (n=9) and 30% physically inactive (n=7). (Table 4.1).

Table 4.1. **Basic participant characteristics at blood sampling.**

Variable	HIV-positive (n=23)	Control (n=13)
Age (years)	42.4 ± 9.1	43.4 ± 10.2
BMI	26.18 ± 5.5	27.5 ± 5.2
Diastolic blood pressure (DBP) (mmHg)	78.2 ± 13.6	89, 7 ± 11.8
Systolic blood pressure (SBP) (mmHg)	83.4 ± 8.6	124, 7 ± 15.0
HR (bt/min)	78.2 ± 13.6	70.6 ± 10.2
HIV/DX (years)	9.1 ± 5.5	N/A
cART use		
Current	21	N/A
Duration (weeks)	326.2 ± 170.4	N/A
CD4 cell count (cells/mm^3)	542.9 ± 215.9	N/A

N/A: not applicable, DBP: diastolic blood pressure, SBP: systolic blood pressure, HR: heart rate, HIV/DX: time of HIV diagnosis, cART: combined antiretroviral therapy, BMI: body mass index (defined as the weight in kilograms divided by the height in meters squared).

4.2.Markers of disease progression

CD4 counts and VL were measured to assess disease progression and the degree of immunity. HIV-positive participants displayed CD4 counts ranging from 100- 900 cells/mm^3. Moreover, 31% of the HIV-positive patients displayed a CD4 count <500 c/mm^3 whereas 61% exhibited a CD4 count >500 c/mm^3. Deeper analyses indicated that 26% of these patients had a CD4 count <350 c/mm^3. The mean VL of the HIV-positive group was 4, 127 ± 3, 020 cells/mm^3 (median = 50 cells/mm^3). For the two sub-groups, i.e. CD4 count <500 and CD4 count >500, a Mann-Whitney test revealed no significant difference in VL between HIV-positive persons (Figure 4.2.A). However, the mean VL between the two groups is higher, i.e. 10, 412 ± 22, 446 cells/mm^3 (median =196) versus 87.36 ± 13.3 cells/mm^3 (median= 50), respectively. Moreover, a Spearman correlation performed

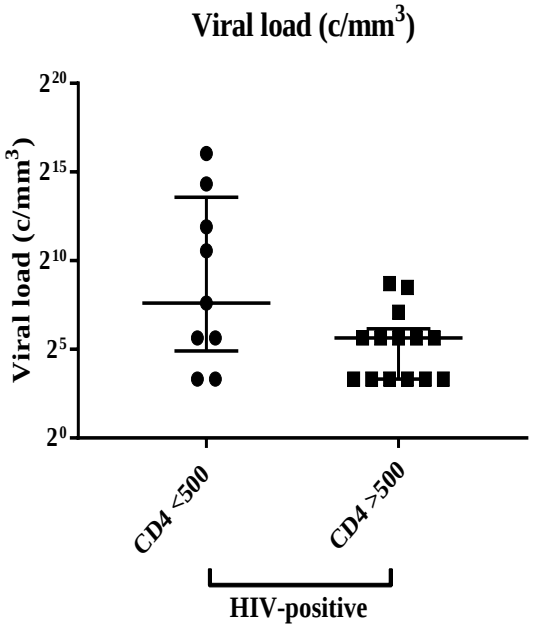

Figure 4.2.A. Viral load in HIV-positive persons based on CD4 <500 vs CD4>500). Data displayed as median ± IQR; statistical analyses: Mann- Whitney test, n= 23

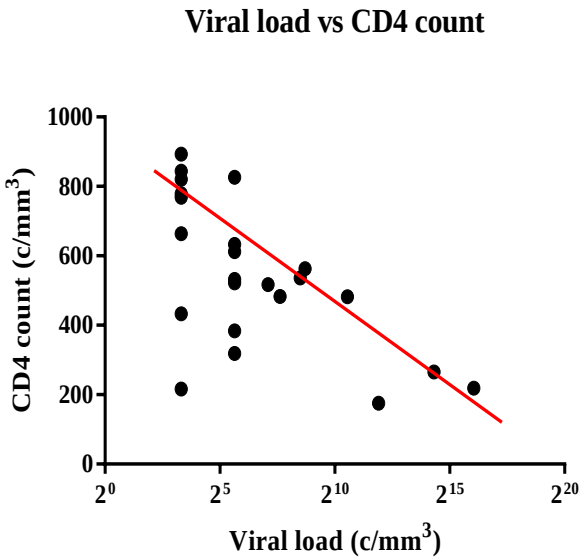

Figure 4.2.B. The relationship between viral load and CD4 count. Data presented in log format; statistical analyses: Spearman correlation, n=23 (r= -0.57; p= 0.005).

between VL and CD4 count showed a moderate, negative relationship (r= -0.57; p= 0.005) (Figure 4.2.B).

4.3.Blood pressure

Analyses were performed to determine the variations in BP between HIV-negative and HIV-positive persons (Figure 4.3). An unpaired t-test revealed a significant difference in SBP between the two groups ($p<0.0001$). The average SBP for the HIV-negative group was 124.7 ± 4.147 mmHg versus 83.44 ± 1.785 mmHg for the HIV-positive group. The mean DBP for the HIV-negative group was 89.7 ± 3.3 versus 78.2 ± 2.9 mmHg for the HIV-positive group. Here an unpaired t-test revealed a significant difference in DBP between these two ($p<0.05$).

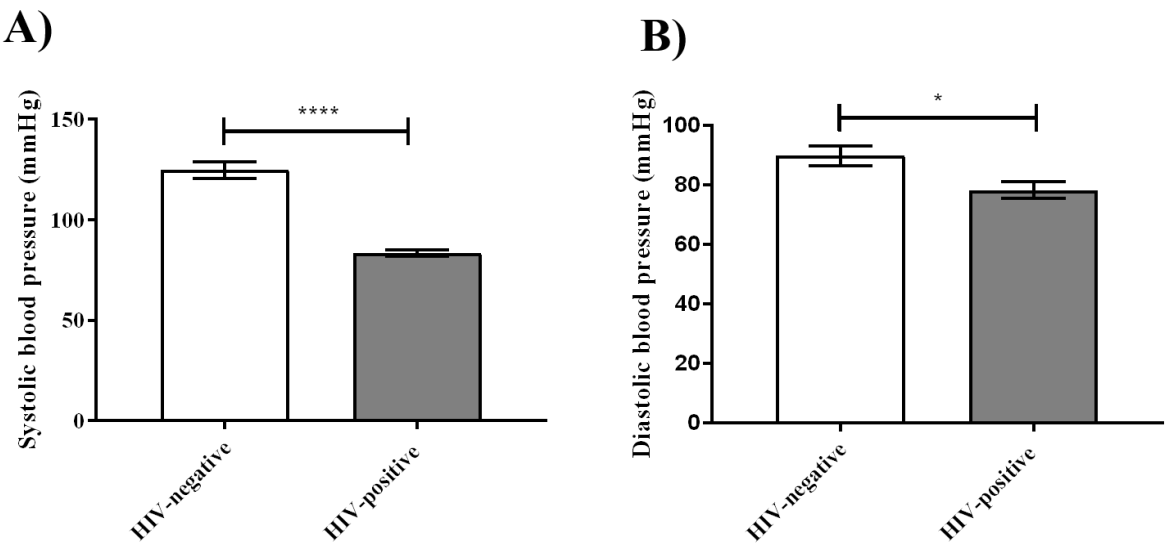

Figure 4.3. **Blood pressure in HIV-positive patients vs HIV-negative patients**. Lower SBP (A) and DBP (B) in HIV-positives patients. Data presented as mean ± SEM; statistical analyses: unpaired t-test; ****$p<0.0001$, *$p<0.05$, n=36.

4.4. Heart rate

Analyses were performed to determine the variations in heart rate between HIV-negative and HIV-positive persons. An ordinary one-way ANOVA revealed a significant difference in heart rate between the three groups ($p<0.05$) (Figure 4.4). The average heart rate for the HIV-negative group was 70.64 ± 2.83 (bt/min) versus 71.99 ± 2.40 (bt/min) and 82.14 ± 4.10 (bt/min) for HIV-positive CD4 <500 and CD4 >500, respectively.

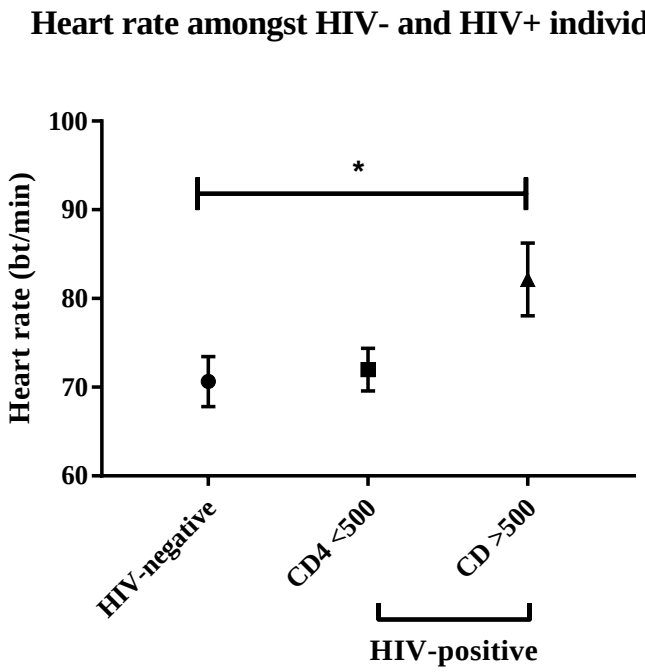

Figure 4.4. The variation in heart rate amongst HIV-positive and HIV-negative patients. A higher heart rate in HIV-positives patients was observed. Data presented as mean ± SEM; statistical analyses: ordinary one-way ANOVA; *$p<0.05$, n=36.

4.5.Endothelial function

Endothelial function was measured via FMD analyses, with values <6% considered abnormal as previously employed by our group (Teer *et al*., 2017). 49% of the HIV-positive group presented with an FMD of <6%, with a moderate decrease in average FMD (%) in patients with a lower CD4 count (<350 cells/mm^3). When divided into sub-groups i.e. HIV-negative, CD4 <500 and CD4 >500 cells/mm^3 we found no significant differences between the groups (p= 0.16). The mean FMD (%) across groups were 5.90 ± 2.33, 5.02 ± 2.64 and 8.01 ± 4.17 % for HIV-negative, CD4 <500 and CD4> 500, respectively. For deeper analyses the groups were sub-divided into HIV-negative, CD4 <350 and CD4>350 and here the FMD (%) revealed a significant difference (p<0.05). An unpaired t-test between HIV-positive individuals with CD4<350 and CD4>350 revealed a very significant difference for the FMD data (p<0.005) (figure 4.5).

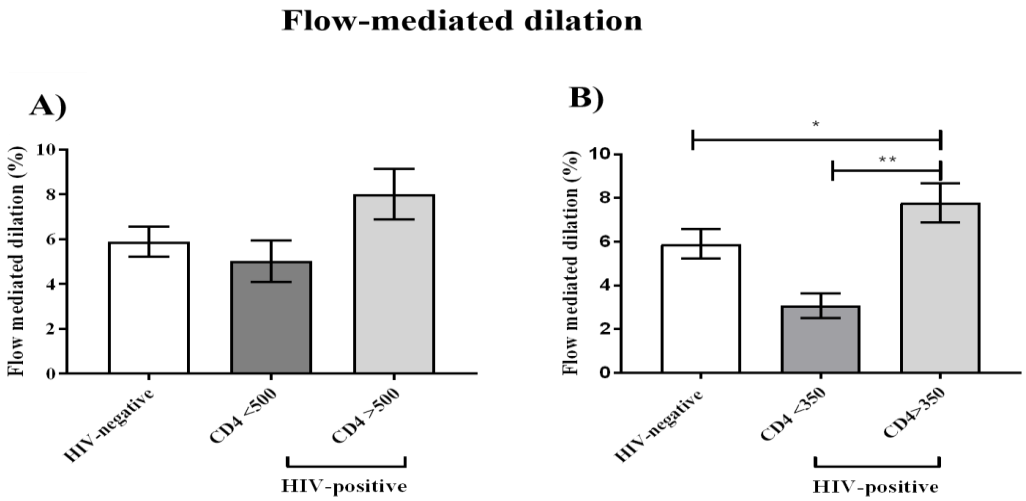

Figure 4.5. Flow-mediated dilation amongst HIV-positive vs HIV-negative patients. Results reveal a significant difference between HIV-negative and HIV-positive persons with a lower FMD in patients with a CD4 count <350 cells/mm3, (B). Data presented as mean ± SEM; statistical analyses: Kruskal- Wallis test, unpaired t-test; *p<0.05, **p= 0.005 n=36.

4.6.Platelet evaluations

4.6.1. Platelet count

Platelet counts were also performed to assess the effect of HIV infection on thrombocytes. When divided into sub-groups (HIV-negative, CD4 <500 and CD4 >500) an ordinary one-way ANOVA revealed no significant difference between groups (p= 0.4721). The mean platelet counts for HIV-negative, positive CD4<500 and CD4 >500 was 311 ± 18.95, 298.20 ± 31.07 and 277.40 ± 14.97 cells/mm^3, respectively. Further analyses revealed a significant difference across the patient sub-groups (p=<0.05). The mean platelet counts for HIV-negative, positive CD4<350 and CD4 >350 was 311 ± 19.00; 361.40 ± 33.40 and 264.50 ± 13.2 cells/mm^3, respectively. An unpaired t-test between HIV-positive persons with a CD4<350 and CD4>350 revealed a significant difference (p<0.005) whereas an unpaired t-test between the HIV-negative group and CD4>350 groups also showed significance (p<0.05) (Figure 4.6).

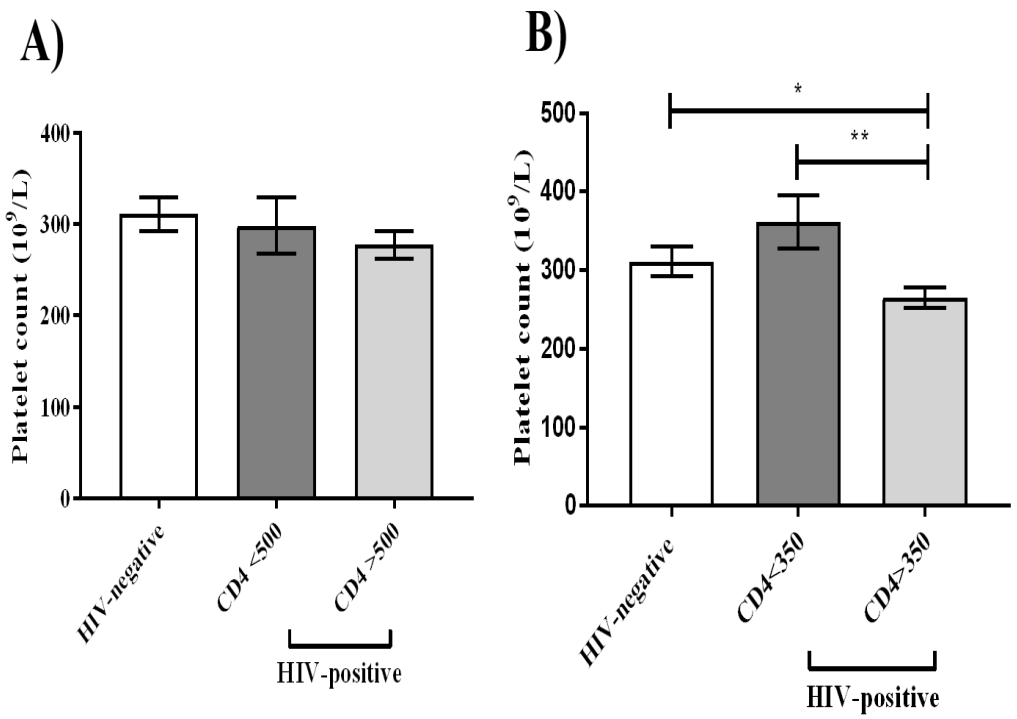

Figure 4.6. Platelet count in HIV-positive vs HIV-negative patients. Results revealed a significant difference between HIV-negative and HIV-patient patients (B) with a higher platelet count in HIV-positive patients with a CD4 <350 cells/mm^3.Data presented as mean ± SEM; statistical analyses: One-way ANOVA, unpaired t-test; *p=<0.05, **p<0.005.

4.6.2. Platelet activation

To assess platelet activation in HIV-positive persons, expression of CD62P, LAP and GARP was evaluated.

4.6.2.1.Platelet CD62P expression (%)

No significant difference was observed when comparing expression of CD62 between HIV-negative and HIV-positive when sub-dividing according to the CD4 <500 and CD4> 500 subclass (Figure 4.7.A). However, further analyses revealed a significant difference in CD62 expression

between HIV-negative individuals and HIV-positive individuals with CD4 <350 (p=0.03) (Figure 4.7.D). The mean ± SEM for the HIV-negative group and HIV-positive CD4<350 and CD4>350 group was 95.66 ± 1.59, 90.96 ± 2.47 and 93.74 ± 1.84, respectively. A one-way ANOVA revealed no significant differences (p= 0.11).

4.6.2.2.Platelet LAP expression (%)

No significant difference was observed when comparing LAP expression between HIV-negative and HIV-positive groups (Figure 4.7.B). Unpaired t-tests also revealed no significant differences between the various sub-groups. The average LAP expression ± SEM for HIV-negative, HIV-positive CD4 <350 and CD4>350 individuals was 82.2 ± 8.10, 94.34 ± 3.51 and 77.51 ± 7.58, respectively (Figure 4.7.E).

4.6.2.3.Platelet GARP expression (%)

A one-way ANOVA revealed no significant difference between groups (Figure 4.7.C). Here the mean ± SEM was 37.44 ± 6.93, 57.08 ± 11.67 and 39.57 ± 8.70, respectively. The mean ± SEM indicates a higher average in patients with a CD4 count <500 c/mm^3. However, an unpaired student t-test revealed a significant difference between HIV-negative individuals and HIV-positive individuals with CD4 <350 (p=0.05) (Figure 4.7.F). The average ± SEM for HIV-negative, CD4 <350 and CD4 >350 was 37.44 ± 24.97; 71.38 ± 25.82 and 39.49 ± 33.08, respectively.

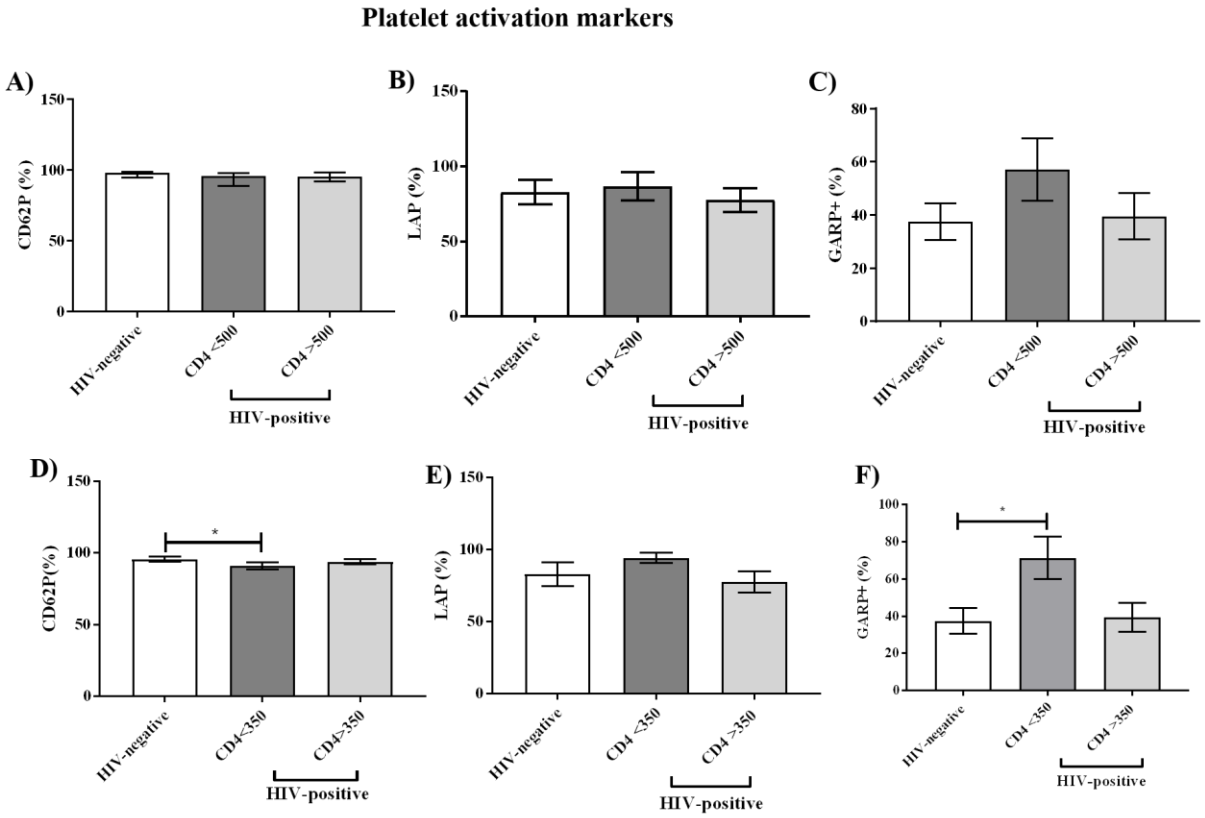

Figure 4.7. Expression of platelet activation markers in HIV-positive persons; CD4<500 – CD4>500 cells/mm^3(A-C) and CD4<350 – CD4>350 cells/mm^3 (D-F). Data displayed as mean ± SEM; statistical analyses: one-way ANOVA, Kruskal-Wallis test, unpaired t-test; *p<0.05, n=36.

4.6.3. Platelet expression of TGF -β

No significant difference was observed across the groups here compared. However, the mean ± SEM data indicate that the group with CD4 <500 displayed a higher average TGF-β expression. Further analyses showed a significantly higher TGF-β expression when analyzing data according to sub-groups (CD4< 350 and CD4 >350). An unpaired t-test between HIV-positive individuals with CD4<350 and CD>350 revealed a significant difference ($p<0.05$), whereas a one-way ANOVA between all three groups also revealed significance ($p=0.05$) (Figure 4.8).

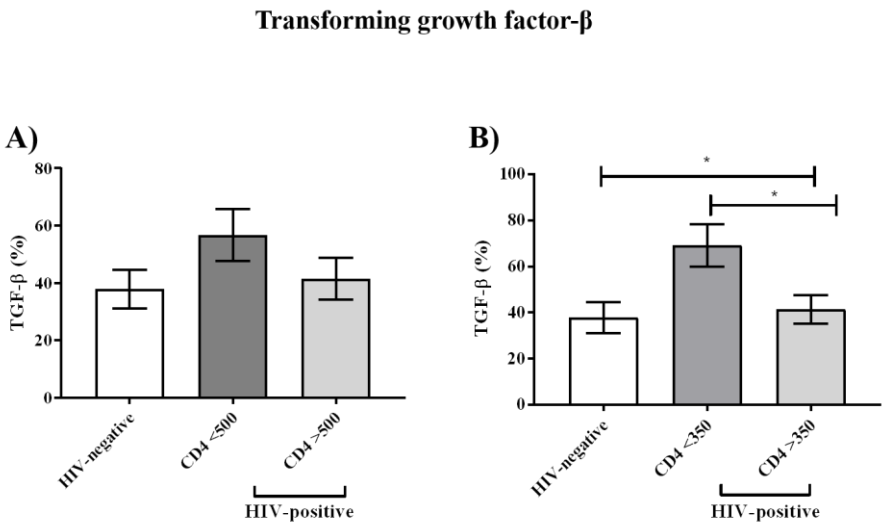

Figure 4.8. Transforming growth factor-β expression in HIV-positive and HIV-negative patients. Data presented as mean ± SEM; statistical analyses: one-way ANOVA, unpaired t-test; *$p<0.05$, *$p=0.05$, n=36.

4.7.The relationship between platelet activation and blood pressure

Correlative tests were performed to assess the relationship between platelet activation and BP readings. A weak negative correlation exists between DBP and CD62P expression, r=-0.38; p<0.05 (Figure 4.9.A). In addition, a weak negative correlation was also observed between SBP and CD62P platelet expression, r=- 0.39; p<0.05 (Figure 4.9.B). LAP platelet expression and correlative studies revealed a negative relationship between SBP and LAP, r=- 0.41; p<0.05 (Figure 4.9.D). Although LAP showed no significant correlation with DBP a negative trend was observed (r= -0.27) (Figure 4.9.C)

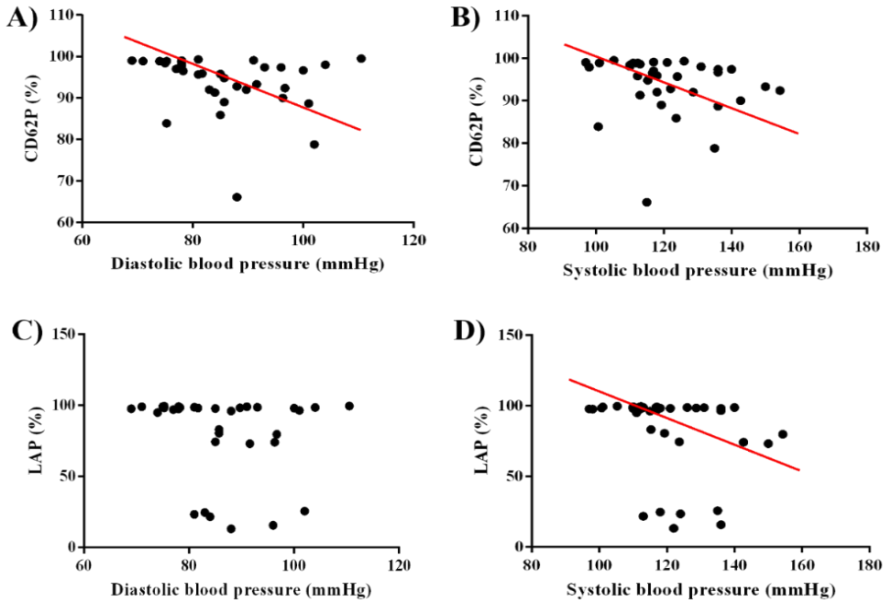

Figure 4.9. The relationship between platelet activation and BP. Results revealed weak, significant negative correlations between CD62P (%) and both DBP, SBP (mmHg) (A-B). LAP also had a significant, negative correlation with SBP (mmHg)(D). Data presented linear regression XY data; statistical analyses: Spearman's correlation test; *p<0.05, n=36.

4.8.The relationship between GARP, disease progression and fibrosis

Correlations were performed and a moderate positive relationship was identified between DBP and GARP (r= 0.335; p<0.05) (Figure 4.10.A). A moderate positive relationship was observed between GARP and VL, a well-known marker of disease progression, r=0.4351; p<0.05 (Figure 4.10.B). Additionally, a very strong relationship was observed when correlating GARP with TGF-β (r=0.8467; p<0.0001) (Figure 4.10.C).

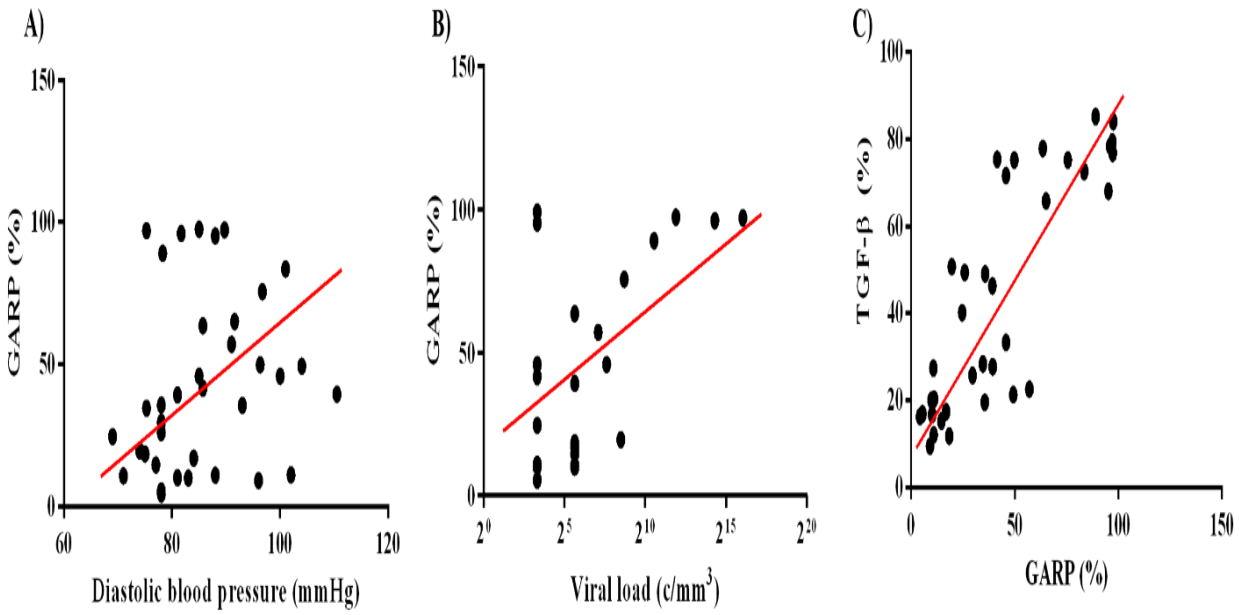

Figure 4.10. The relationship between GARP, disease progression and fibrosis. Analysis reveal a moderate, significant positive relationship between GARP and DBP (A,), VL (B) and TGF-β (C). Data presented as linear regression with XY data; statistical analyses: Spearman's correlation, *p<0.05, ****p<0.0001, n=36.

4.9.QT interval in HIV-positive persons

While only 14% of the HIV-positive persons presented with an irregular sinus rhythm, a significant difference was observed in the QT interval between HIV-negative and HIV-positive patients, $p<0.05$. HIV-positive persons displayed a shorter average QT interval – although still within the normal range. 61% of the HIV-positive patients presented with a QT interval <400 ms, while only 13% of these HIV-positive patients had a CD4 count <500 c/mm^3. Further analyses revealed a significant difference between HIV-negative, CD4 <500 and CD4 >500 ($p<0.05$) (Figure 4.11.A). Deeper analyses also showed a greater significance between the HIV-negative group and persons with CD4 >500 ($p<0.05$) (Figure 4.11.A). When evaluating the average QT interval data, we found that HIV-positive persons displayed a shorter QT interval, with mean values of 404.40 ± 8.70, 394.10 ± 6.71 and 375.20 ± 7.24 for HIV-negative, CD4< 500 and CD4> 500, respectively.

However, QRS complex analyses showed no significant differences between HIV-positive and negative persons. Further analysis of the sub-groups also revealed no significance. However, HIV-positive persons did exhibit a delayed QRS complex with the average for the sub-groups being 93.90 ± 2.52; 91.41 ± 2.28 and 87.86 ± 1.96 for HIV-negative, CD4 <500 and CD4 >500, respectively (Figure 4.11. B).

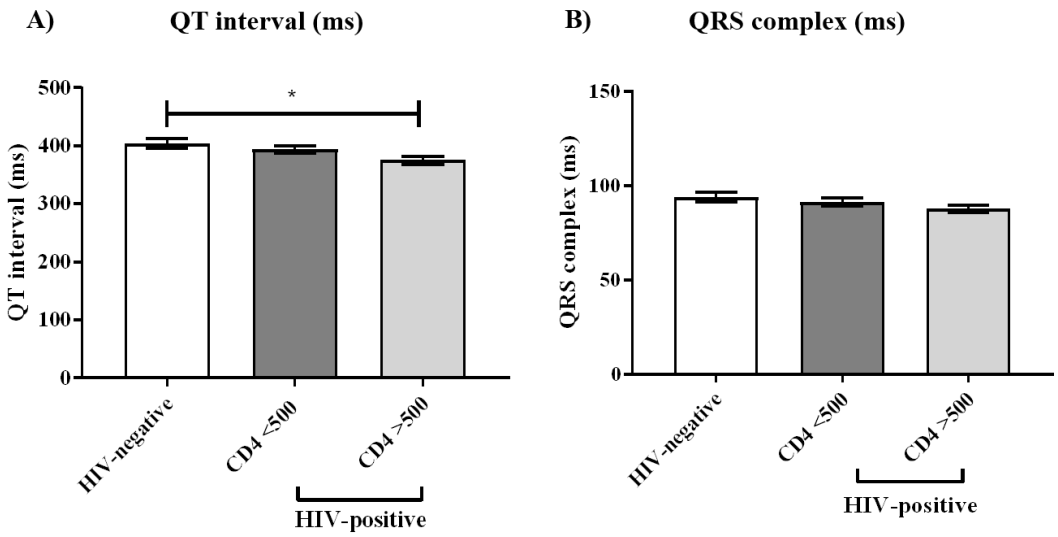

Figure 4.11. The repolarization and depolarization of the heart in HIV-positive and HIV-negative patients. HIV-positive patients display shorter QT intervals (A), with no significant difference in QRS interval (B). Data presented as mean ± SEM; statistical analyses: one-way ANOVA, unpaired t test, $p<0.05$, n=36.

Chapter 5– Discussion

The hypothesis of this study was that chronic HIV-infection induces immune dysregulation that can trigger persistent platelet activation and the release of mediators that contribute to an increased CVD risk. Markers of platelet activation were investigated in South African participants that shared similar CVD risk factors and socio-economic risk factors. These parameters were then correlated with clinical tests of cardiac function (BP, ECG and FMD), markers of disease progression (CD4 and VL) and a pro-fibrotic marker (TGF-β). The major findings of this study for HIV-positive individuals are: a) the observation of lower BP (systolic and diastolic), b) GARP upregulation and its strong correlation with disease progression (CD4 and VL) and fibrosis (TGF-β) markers, c) the identification of a negative association between platelet activation markers and BP, d) lower FMD and e) shorter QT intervals. Together we propose that such factors likely contribute to an increased risk of HF/CVD in HIV-positive persons (Figure 5.1)

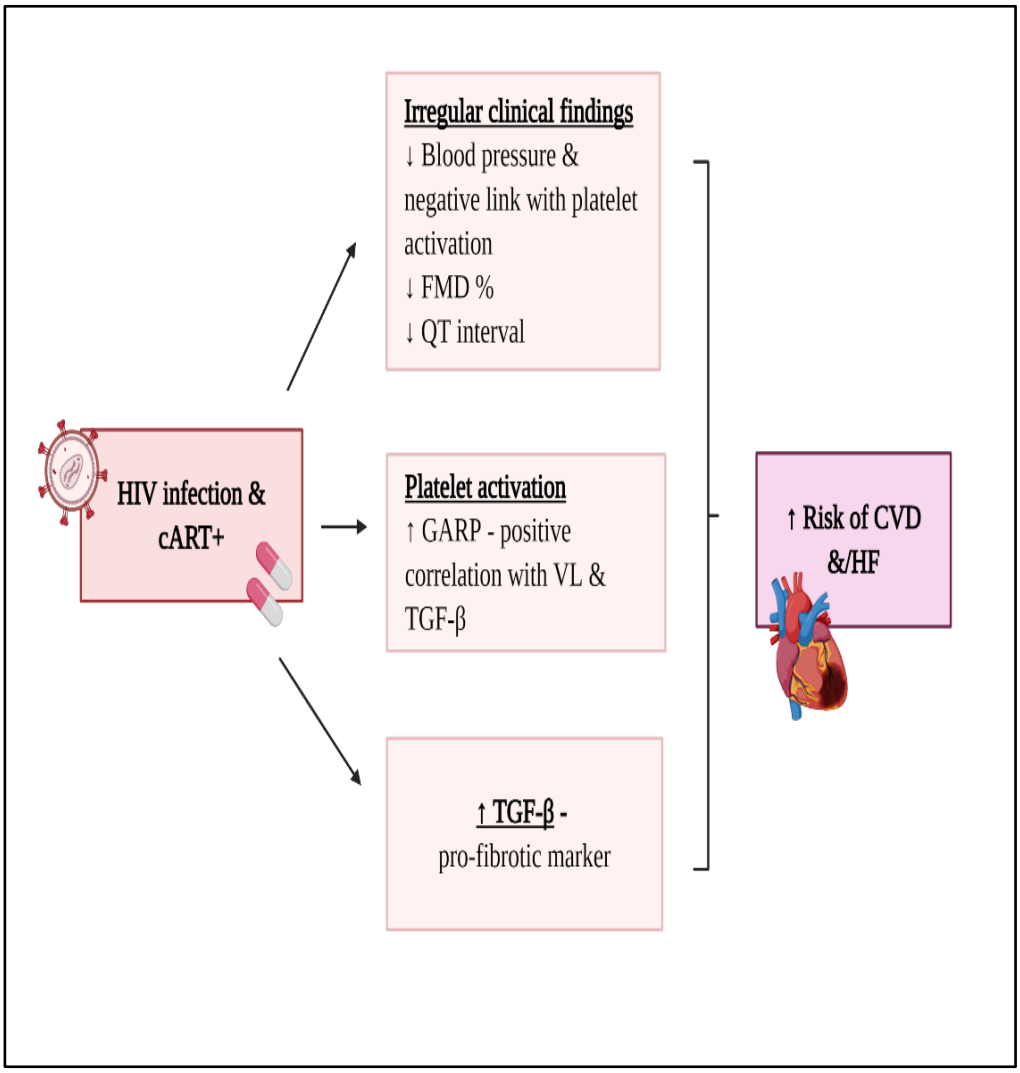

Figure 5.1. Summary of major findings generated by this study. Our data reveal enhanced expression of a platelet activation marker (GARP) that correlated with disease progression markers, higher VL with lower CD4 counts. In addition, we found lower BP and FMD% together with a shorter QT interval. We propose that such effects together with cART side-effects may contribute to the onset and progression of CVD/HF in HIV-infected individuals. cART: combined antiretroviral treatment, CVD: cardiovascular disease, FMD: flow-mediated dilation, GARP: glycoprotein A repetitions predominant, HIV: human immunodeficiency virus, HF: heart failure, TGF-β: transforming growth factor-β, VL: viral load.

5.1. Abnormal clinical findings

5.1.1. Lower BP in HIV-positive patients

Since an abnormal BP reading is one of the clinical presentations of left ventricular failure (LVF) and DD we evaluated this parameter in our study. Here the data revealed lower BP readings, both systolic and diastolic, in HIV-positive persons. Since the vast majority of studies evaluating BP in HIV-positive patients observe hypertension (Palacios et al., 2006; Chow et al., 2015; Fiseha et al., 2019; Wilson et al., 2009), these findings were contradictory. However, there are some studies that also reported lower BP measurements in this population (Table 5.1).

Table 5.1. Studies assessing the BP in HIV-positive persons.

Study #	Study design	Outcome	Reference
1	Prospective, observational study of 95 HIV+ patients (78 men) starting cART, and maintaining the same regimen for 48 weeks. Baseline and >48-week BP measurements were recorded.	SBP, DBP and pulse pressure were increased (121.8 vs 116.6 mmHg, p=0.0001; 76.3 vs 69.7 mmHg, p=0.004, 46.9 vs 43.8 mmHg, respectively)	Palacios et al., 2006
2	Retrospective study examining the effects of ARVs on SBP and DBP of 286 patients (ARV vs ARV-naïve).	An increase in SBP and DBP was observed by 4.71 mmHg/year, p=0.005 and	Chow et al., 2015

	This included 4 visits and BP measurements every 6 months.	2.26 mmHg/year, p=0.76 in patients on cART. Patients on PI regimens – 4.75 mmHg increase in SBP (p=0.002) and 1.96 mmHg in DBP (p=0.042). Patients on non-nucleoside reverse transcriptase inhibitors (NNRTI) - containing regimens – 3.21 mmHg increase in SBP (p=0.011) and 2.62 increase in DBP (p=0.050).	
3	Cross-sectional study of 408 HIV+ patients (69% females) on treatment for at least 12 months.	29% of patients were hypertensive	Fiseha et al., 2019
4	Cross-sectional survey of 612 HIV+ patients	NNRTI-treated HIV+ patients displayed a higher BP (+4.6/4.2 mmHg) than HIV+ patients without treatment.	Wilson et al., 2009

5	Cross-sectional study of 1182 HIV+ patients (71% males).	29.3% patients exhibited hypertension. No correlation with PIs or NNRTIs.	De Socio et al., 2012
6	Population based survey of 2, 687 HIV+ patients.	50% patients exhibited hypertension (stage I and II). SBP was lower by 3.5 mmHg in HIV+ patients versus HIV-negative persons, p=0.001.	Barnighausen et al., 2008
7	Retrospective analyses of medical records of an HIV treatment program in Kenya (2005 to 2010).	Low BP was associated with highest mortality incident rate (IR) (systolic <100 mmHg IR 5.2, diastolic <60 mmHg IR 9.2). However, an increased rate of mortality also occurred in men with increased BP, who were not in an advanced diseased state.	Bloomfield et al., 2014
8	Meta-analysis of 63, 554 participants from published studies (2011-2016).	35% HIV+ displayed hypertension	Xu et al., 2017
9	Cross-sectional study of 258 participants; 100 with AIDS,	Lower SBP and DBP amongst HIV-positive patients.	Okeahialam et al., 2006

	78 asymptomatic HIV-positive and 80 HIV-negative		

AIDS: acquired immunodeficiency syndrome, BP: blood pressure, cART: combined antiretroviral treatment, DBP: diastolic blood pressure, HIV: human immunodeficiency virus, IR: incident rate, NNRTI: non-nucleoside reverse transcriptase inhibitor, PI: protease inhibitor, SBP: systolic blood pressure.

For example, Bloomfield et al., (2014) found both increased and decreased BP in HIV-positive patients, with low BP associated with higher mortality rates (Bloomfield et al., 2014). In agreement, others established that low BP in this population is associated with a more advanced diseased state (Okeahialam et al., 2006). There is also a link between low BP and HF. For example, 15-25% patients admitted for HF are burdened with low BP, although sometimes asymptomatic in nature (Gheorghiade et al., 2013). The lower BP and an elevated HR as displayed by our HIV-positive participants (Figure 4.3 and 4.4) could occur due to decreased cardiac output. In agreement, HF patients with a lower BP exhibited decreased left ventricular ejection fractions (Gheorghiade et al., 2006). Thus, it should be further investigated whether the decreased BP and increased HR may be indicative of early LVF.

However, the individuals in our study were relatively healthy with only 31% presenting with a CD4 count <500 cells/mm^3 and 17% with a VL > 1000 cells/mm^3. The impact of ARVs in this context should also be considered. For example, patients with a longer duration of nucleoside reverse transcriptase inhibitor (NRTI) usage and exposure experienced DD that suggests that this ARV class may potentially mediate this complication (Hsue et al., 2010). Additionally, NRTIs were previously implicated in terms of mitochondrial damage and cardiomyopathy (Frerichs et al., 2002). Of note, most of our HIV-positive cohort were on first-line cART which contains an NRTI.

There are multiple reasons that may help explain the lower BP observed in the HIV-positive participants. These may include adrenal insufficiency, anemia, autonomic dysfunction, concomitant infections and microbial translocation (Meya at al., 2007; Marchetti et al., 2008; Compostella et al., 2008). In contrast, some literature supports the association between increased SBP and mortality in HIV-positive persons. However, most of these studies were done in developed countries and it needs to be established whether this is also the case for developing nations. For example, relatively limited attention has been paid to the incidence of low/high BP in HIV-positive persons and HIV-related CVD onset and progression in the SSA region (Bloomfield et al., 2014). Moreover, a large systematic review and meta-analysis (including 38 studies from SSA) that investigated mortality in adults on cART in low- and middle-income countries, did not identify BP as a covariate of interest (Gupta et al., 2011). Another, similar systematic review and meta-analysis focusing on SSA indicated that HIV-positive persons on average displayed lower BP than their HIV-negative counterparts (Dillon et al., 2013).

Although untreated HIV is typically associated with lower BP, our HIV-positive participants were relatively healthy with higher CD4 counts. Moreover, there are inconsistent findings whether individuals on cART exhibit a higher prevalence of increased or decreased BP compared to HIV-negative persons (Xu et al., 2017; Peck et al., 2014; Feinstein et al., 2016; Gelpi et al., 2018; Armah et al., 2014; Fahme et al., 2018). Collectively these findings indicate that both high and low BP can elicit effects on HIV-positive patients and that a multitude of factors may be responsible for such differences. Thus, we suggest a) increased awareness to monitor BP fluctuations in HIV-positive patients in SSA and b) further studies with larger SSA HIV-positive cohorts to better assess whether lower or higher BP actually predominates and also its downstream effects on cardiovascular health and overall well-being.

5.2. Higher GARP expression in HIV-positive patients and strong correlation with markers of disease progression and fibrosis

Findings from the current study revealed an upregulation of the anti-inflammatory activation marker, GARP, on platelets in our HIV-positive participants. There are limited studies investigating the expression of GARP on platelets, even less so in the context of HIV. However, the studies that are available all allude to an increase in GARP expression in HIV (Teer et al., 2019; Wang et al., 2009). A study by Vermeersch et al., (2017) revealed a 2.2-fold increased GARP expression after platelets were activated (Vermeersch et al., 2017). Due to its anti-inflammatory nature and immunosuppressive function, the upregulation of GARP may inhibit HIV-specific immune responses which would allow viral replication to persist, promoting disease progression (Gianesin et al., 2011; Appay & Sauce, 2008). This is in agreement with our results which show a moderate correlation between GARP and VL. Additionally, there was a greater upregulation of GARP in patients with a low CD4 count. It is clear that the upregulation of GARP is related to disease progression. This is further supported by previous findings from our group that found an upregulation of GARP on regulatory T cells during chronic HIV infection in patients with a low CD4 count (Teer et al., 2019). One of the mechanisms whereby GARP can contribute to disease progression, is through the depletion of CD4+ T cells, by converting CD4+CD25- T helper cells to regulatory T cells, possibly through an activated GARP-TGF-β complex (Miller et al., 2014). The expansion of Tregs would aid in the inhibition of the immune response, in addition to the decrease in CD4 count. GARP has been identified as a docking receptor for TGF-β enhancing the secretion and activation of latent TGF-β (Wang et al., 2012; Tran et al., 2009; Cuende et al., 2015). On platelets, the co-expression of GARP and LAP which associates with TGF-β, was reported (Wang et al., 2012; Tran et al., 2006). This supports the findings in our study which found a very strong

correlation between GARP and TGF-β. A study by Rashidi et al., (2017) showed that platelet-intrinsic GARP plays an essential role in the activation of TGF-β (Rashidi et al., 2017). Further analyses revealed higher TGF-β expression in our HIV-positive patients. Of note, immunosuppressed patients (CD4 count <350 c/mm^3) showed greater expression of TGF-β on platelets as opposed to HIV-positive patients with a higher CD4 count, potentially linking TGF-β to disease progression. TGF-β is a pro-fibrotic growth factor, and the increased expression thereof may lead to elevated fibrotic signaling, collagen production and deposition in the heart (Barrientos et al., 2008; Yun et al., 2019; Ma et al., 2018).

Disease progression, occurs as CD4 count is depleted, with or without viral suppression. This is linked to a pro-inflammatory and hypercoagulable state, which increases the risk of CVD (Deeks, 2013).

5.3. The expression of CD62P, LAP and links to BP

No significant increase in any of these markers were found in the current study. This contradicts findings of previous studies that indicate an increase in CD62P (Mayne et al., 2012). For example, some found increased CD62P expression on platelets in HIV-positive patients compared to HIV-negative controls (Nkambule et al., 2013). There are various reasons that may help explain this finding. Here CD62P expression on platelets was determined after exposing it to an agonist (adenosine diphosphate) whereas in our study baseline CD62P expression was measured without exposing it to such agents. The nature of ARV treatment may also help explain such discordant findings. Here platelet activation was mainly linked to PIs (Van der heijden et al., 2017; Loelius et al., 2017; Ahamed et al., 2016; Mayne et al., 2012; Laurence et al., 2017; Satchell et al., 2011; Kort et al., 2011) while most of the HIV-positive patients in our cohort were on first-line cART without PIs.

While no differences were observed in CD62P or LAP expression, a significant negative relationship was observed between these activation markers and BP (systolic and diastolic). Bloomfield et al., (2014) also observed that low BP correlated with the highest risk of mortality in HIV-positive patients and thus it is likely that BP decreases due to the downstream effects of immune dysregulation and subsequent platelet activation.

5.4. Lower FMD in HIV-positive patients with low CD4 count

Our data revealed that 49% of the HIV-positive group presented with an FMD of <6%, with a moderate decrease in average FMD % in patients with a lower CD4 count (<350 and <500 c/mm3). These findings are supported by several studies that observed endothelial dysfunction with disease progression (Islam et al., 2012; Kline & Sutliff, 2008; Lambert et al., 2016; Zhang, 2008; Steyers & Miller, 2014). Moreover, perinatally HIV-infected youths displayed a higher degree of endothelial dysfunction and immune activation than their HIV-positive counterparts who were infected later in life (Dirajlal-Fargo et al., 2017). This could be due to a longer, cumulative duration and exposure to the virus and also cART (Kline & Sutliff, 2008; Lambert et al., 2016). The downstream effects of pro-inflammatory cytokines promote the activation of endothelial cells which increases the expression of adhesion molecules and subsequent monocyte adherence, increased permeability and elevated production of reactive oxygen species. This all contributes to the development of endothelial dysfunction (Zhang, 2008; Steyers & Miller, 2014) and can helps to explain why individuals with a relatively lower CD4 count can exhibit a lower FMD.

Of note, our results show that patients with a CD4 > 500 cells/mm^3 displayed a higher FMD than both HIV-positive patients with a lower CD4 count (<500 cells/mm^3) and their seronegative counterparts. This is consistent with the notion that a higher CD4 count together with decreased VL signifies an enhanced immunological status and endothelial function. The patients here

included exhibited greater immunity as only 31% displayed a CD4 count <500 cells/mm^3 whereas only 17% showed a VL > 1000 cells/mm^3. This may be a possible explanation for the higher FMD values and preserved endothelial function we found for the HIV-positive CD4 > 500 cells/mm^3 group.

While our results differ from most studies (reduced FMD in HIV-positive persons), such studies usually include patients that are on PI that is harmful to the vasculature and endothelial lining . Thus, we propose that another reason for the largely preserved FMD observed in our study may be due to most of our patients receiving first-line treatment. In contrast, second-line treatment usually includes PI that can exert more harmful effects on the endothelium and vascular structure (Wang et al., 2009; Hurlimann et al., 2005; Bruder-Nascimento et al., 2020). Our findings are also consistent with the larger EndoAfrica cohort that found preserved FMD in HIV-positive patients with CD4 >500 c/mm^3 .

5.5. Shorter average QT interval in HIV-positive cohort

The QT interval indicates ventricular repolarization while the QRS measures ventricular depolarization. Our data show a significant difference in the QT interval between HIV-negative and HIV-positive patients, with a shorter average QT interval for HIV-positive persons but that is still within the normal range. This contrasts the findings of other studies that observed a prolonged QT interval in HIV-positive patients (Sani et al., 2005; Liu et al., 2020; Vallejo et al., 2002). A prolonged QT interval occurs due to an irregularity of the electrical activity of the heart which increases the patients risk of ventricular arrhythmias.

Most studies that reported on prolonged QT intervals in HIV-positive patients related it to the specific drug regimens used by such patients (Vallejo et al., 2002). Here drug-induced QT

prolongation may be caused by inhibition of of cardiac potassium channels. For example, certain PIs (lopanavir, nelfinavir, ritonavir and saquinavir) can cause a dose-dependent inhibition of such channels and subsequently result in prolonged QT intervals (Villa et al., 1995; Liu et al., 2020). Such effects may also occur by alternate mechanisms, e.g. these drugs can inhibit the hepatic CYP3A metabolic pathway and thereby lead to clinically significant drug-drug interactions with increased drug exposure and toxicity (Fantoni et al., 2001; Liu et al., 2020). However, some first-line drugs such as Efavirenz (an NNRTI), were also implicated in the prolongation of QT intervals and severe ventricular arrhythmia (Villa et al., 1995; Liu et al., 2020). Only one patient in our study was on second-line treatment (containing a PI) whilst the rest received first-line treatment that contained Efavirenz. Despite NNRTIs not being as detrimental as PIs, a possible explanation for our patients preserved QT interval could likely be that they are relatively healthy (higher CD4 counts) with suppressed viremia. In agreement, several studies associated lower CD4 counts (<200 c/mm^3) with a prolonged QT interval (Sani et al., 2005; Shaaban et al., 2010; Wongcharoen et al., 2014; Gili et al., 2017; Ayyadurai et al., 2016).

Although our ECG data revealed shorter QT intervals still within the normal limit this may indicate a potential risk. While most research focuses on the effects of prolonged QT intervals, a shorter QT interval may also cause an elevated risk of arrhythmias such as atrial and ventricular fibrillations and thereby increase overall SCD risk (Liu et al., 2020). However, there is limited literature available in this regard and thus further studies are required to evaluate the functional effects of a shorter QT interval in HIV-positive patients. It is therefore clear that both a short and prolonged QT interval is an independent predictor of cardiovascular mortality and is associated with an increased risk for atrial and ventricular arrhythmias (Schouten et al., 1991; Liu et al., 2020).

As HIV-positive patients exhibit an increased risk of developing both short and prolonged QT intervals, we suggest increased ECG monitoring in this population, if logistically feasible.

5.6. Limitations

There were a few limitations related to the methodology, such as using PRP instead of whole blood to perform the flow cytometry. While using PRP is an established method for platelet flow cytometry, it may result in further platelet activation due to the centrifugation and pipetting steps. This could skew results. Another limiting factor was the fixative used. The samples were fixed with <15% formaldehyde, prior to storing in a 4°C fridge, before analyses. The purpose of fixing the samples was to preserve their function and antibodies. However, upon further research it was revealed that formaldehyde can stimulate platelet activation. For future studies, it is recommended that whole blood be used instead of PRP. This will produce background artefacts when performing the actual flow cytometry but the sample will not be hyperactivated. Another recommendation is to use a different fixative to preserve the cells or to analyze the cells directly after incubating with fluorescent antibodies. Additionally, a relatively small sample size was investigated due to the constraints imposed by the national lockdown due to the SARS-CoV2 pandemic. This small sample size certainly impacted on the robustness of our statistical analyses. However, since this is an ongoing study and also part of a larger study samples from additional patients can be collected and further investigated to help strengthen the current findings. Another limiting factor is the relatively limited number of immune activation parameters investigated. While previous findings from our group indicated increased immune activation in the larger EndoAfrica cohort, investigating additional markers of immune dysregulation in addition to coagulation (including mean platelet volume) and lipid markers may strengthen the current findings and also contribute to an improved

understanding of the interplay between HIV, immune dysregulation and platelet activation and CVD risk.

Chapter 6– Conclusion

The global burden of HIV-associated CVD has increased 3-fold during the past two decades; the greatest impact in SSA and Asia-Pacific regions. More than two-thirds of PLHIV die from HF – presenting with no known risk factors. This can be due to HIV-associated systolic and diastolic dysfunction. Persistent immune activation and immune dysregulation is associated with HIV-infection. Activated platelets have been implicated as a key role-player in inflammatory conditions and CVD. Studies investigating the role of platelet activation in the context of HIV are scarce. The aim of this study was to evaluate platelet activation in HIV-positive patients and contribution to increased risk of HF/CVD. The most notable findings of the current study were decreased BP in HIV-positive patients with a significant link to platelet activation (Figure 5.1). Platelet activation in this context may occur due to immune dysregulation and/or cART, contributing to increased HF/CVD risk. The decreased BP, reduced FMD and shorter QT interval can be an early sign of HF in PLHIV, a common cause of mortality in SSA. Platelet activation marker, GARP, correlated with disease progression markers. This signifies a relationship between platelet activation and disease progression in HIV. Furthermore, this study highlights the role of platelet activation, in HF/CVD. Additionally, it introduces the need for novel screening techniques such as the ones utilized in this study (BP, FMD, ECG) as screening tools for risk of HF/CVD in PLHIV since current techniques are invasive and expensive. However, further studies are needed to elucidate the exact role of platelet activation in this pathology, especially in SSA.

Chapter 7– References

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