HIV, Immune activation and Endothelial Damage in Malawian Adults

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by

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Declaration

I declare that this thesis was composed by me, and that the work contained therein is my own, except where explicitly stated otherwise. The work within this thesis has not been submitted for any other degree or professional qualification.

Chapter 2 “DISCORDANT IMMUNE RESPONSE WITH ANTIRETROVIRAL THERAPY IN HIV-1: A SYSTEMATIC REVIEW OF CLINICAL OUTCOMES” was prepared for publication with the input of co-authors including Katherine Gaskell who helped with screening papers for inclusion. Peter MacPherson who provided senior technical support on manuscript composition and Paul Garner who provided advice on systematic review methodology. Work for Chapter 6 “CHARACTERISATION OF ENDOTHELIAL MICROPARTICLES”, was greatly aided by assistance of Rijan Gurung, PhD student at UCL, who provided onsite training in microparticle quantification and helped with the analysis.

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Abstract

Mortality from cardiovascular disease (CVD) is predicted to surpass that of infectious disease in sub-Saharan Africa (SSA) by 2030. HIV doubles the risk of CVD in high resource settings, but the contribution of HIV and immune activation to the risk of CVD in SSA is unknown.

HIV-1-infected adults with CD4<100 cells/ul were recruited 2 weeks following initiation of anti-retroviral therapy (ART) within the REALITY trial (NCT01825031), along with healthy HIV-uninfected adults and followed for 44-weeks. Acute infections (malaria, TB, cryptococcal meningitis, pneumonia, gastroenteritis) were recorded. Pulse wave velocity (PWV) was assessed using the Vicorder system. Flow cytometry identified T-cell activation (HLA-DR/CD38+), exhaustion(PD1+) and senescence(CD57+) in all participants and circulating microparticles(CMPs) in 72 participants. Independent predictors of PWV were identified using linear regression. Backwards elimination was performed with an exit of p>0.1 Variables with univariable p<0.2 were included (spearman-rho or Wilcoxon ranksum).

279 HIV-infected adults had similar median(IQR) age [36(31-43) vs 35(3-41) years, p=0.4], but lower systolic BP [120(108-128) vs 128(114-134) mmHg, p<0.01], BMI [20(18-21) vs 22(20-25) kg/m^2, p<0.01] and proportion of women [122(44%) vs 66(60%), p<0.01] than 110 HIV uninfected adults. Following adjustment for confounders, HIV infection was associated with a 12%-increase in PWV (p<0.01) at baseline, which remained at week 10 (14%-increase, p=0.02) but resolved by week 24. %CD4-PD1 and %CD8-PD1 were independently associated with PWV at baseline (fold change 2% and 3% per 10%increase, p=0.06 and 0.05 respectively). A decrease in %CD4-PD1 was associated with improvement in PWV by week 44 (rho 0.20, p=0.02). At baseline, median (IQR) CMPs were increased in HIV infection [5.1(2.0-18.0) x10^6 versus 0.4(0.2-6.0) x10^6, p<0.00001) and in high versus low immune activation [4.0(2.3-5.6) x10^6 versus 0.3(0.1-0.5) x10^6, p<0.0001)]; and were strongly related to PWV (rho 0.42, p<0.001). An acute infection during the study carried a 51% adjusted increase in %CD8 activated T cells at week 44 (p=0.02) and an increase in PWV at week 44 of 0.80m/s [versus -0.10m/s (p=0.01)] for HIV uninfected participants.

These results strongly implicate HIV and immune activation in increased endothelial damage during the first 12 weeks of ART therapy. Improvement in PWV on ART and cotrimoxazole is associated with decreases in immune activation. HIV and co-infections may present modifiable CVD risk factors in low resource SSA setting.

List of abbreviations

ACE	Angiotension converting Enzyme
AETC	Accident, Emergency and Treatment Centre
AIDS	Acquired Immune Deficiency Syndrome
AnV	Annexin V
aOR	adjusted Odds Ratio
ApoE	Apolipoprotein E
ART	Antiretroviral Therapy
BMI	Body Mass Index
BNP	Brain Natriuretic Peptide
BP	Blood Pressure
CAC	Coronary Artery Calcium
CB	Carotid Bulb
CCA	Common Carotid Artery
CD	Cluster of Differentiation
CHD	Coronary Heart Disease
CI	Confidence Interval
cIMT	Carotid Intima Media Thickness
CKD	Chronic Kidney Disease
CMV	Cytomegalovirus
COMREC	College of Medicine Research and Ethics Committee
CRP	C Reactive Protein
CV	Cardiovascular

CVA	Cerebrovascular Accident
CVD	Cardiovascular Disease
DAG	Directed Acyclic Graph
DDI	Didanosine
DIR	Discordant Immune Response
DNA	Deoxyribose Nucleic Acid
EBV	Epstein Barr Virus
ECA	External Carotid Artery
EMPs	Endothelial Microparticles
EPCs	Endothelial Progenitor Cells
ESC	European Society of Cardiology
ESH	European Society of Hypertension
FMO	Flow Minus One
FRS	Framingham Risk Score
HAART	Highly Active Anti-Retroviral Therapy
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HDL	High Density Lipoprotein
HIV	Human Immunodeficiency Virus
HLA	Human Leucocyte Antigen
HR	Hazard Ratio
HSV	Herpes Simplex Virus
HTN	Hypertension

ICA	Internal Carotid Artery
ICAM	Intracellular Adhesion Molecule
ICH	Institute of Child Health
IQR	Interquartile Range
IR	Immune Response
LDL	Low Density Lipoprotein
LFA	Lymphocyte function-associated antigen
LMIC	Low and Middle Income Countries
LPS	Lipopolysaccharide
LTRs	Long terminal repeats
MCP	Monocyte Chemoattractant Protein
MFI	Mean Fluorescence Intensity
MI	Myocardial Infarction
MLW	Malawi-Liverpool-Wellcome
MMPs	Matrix Metalloproteinases
MPs	Microparticles
NCDs	Non-Communicable Diseases
NK	Natural Killer
NNRTI	Non-Nucleoside Reverse Transcriptase Inhibitors
NRTI	Nucleoside Reverse Transcriptase Inhibitors
OI	Opportunistic Infection
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate-buffered Saline

PCA	Principal Components Analysis
PECAM	Platelet Endothelial Cell Adhesion Molecule
PI	Principal Investigator
PPP	Platelet Poor Plasma
PWV	Pulse Wave Velocity
QECH	Queen Elizabeth Central Hospital
RCT	Randomised Controlled Trial
REALITY	Reduction in Early Mortality Trial
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute Medium
RUSF	Ready to Use Supplementary Food
RUTF	Ready to Use Therapeutic Food
SHIELD	Study into HIV, Immune Activation and Endothelial Damage
SMCs	Smooth muscle cells
SOP	Standardised Operating Procedure
SSA	Sub Saharan Africa
TB	Tuberculosis
TDF	Tenofovir
TF	Tissue Factor
TIA	Transient Ischaemic Attack
TNF	Tumour Necrosis Factor
UCL	University College London
UNAIDS	Joint United Nations Program on HIV/AIDS

VCAM	Vascular Cell Adhesion Molecule
VCT	Voluntary Counselling and Testing
VL	Viral Load
WHO	World Health Organisation

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1 CHAPTER 1: INTRODUCTION

1.1 Overview of HIV

1.1.1 HIV virus

Human immunodeficiency virus (HIV) was originally a zoonotic cross-species infection which belongs to the family *Retroviridae* and genus *Lentivirus* [1]. It exists in two separate species: HIV-1 and HIV-2. HIV-1 infection is distributed globally compared to HIV-2 which is confined to West Africa. HIV-1 infection progresses rapidly to acquired immune deficiency syndrome (AIDS). The HIV-1 species is further divided into groups (M, O and non-MO). More than 90% of HIV-1 infections are group M, and this group is further divided into nine main clades A-D, F-H, J and K. Many recombinant forms also exit. The distribution of HIV-1 subtypes is shown in Figure 1-1 [2].

Figure 1-1 Global distribution of HIV-1 subtypes[1]

HIV is made up of a core and an envelope. The envelope contains viral glycoproteins (gp120 and 41). It is gp120 that binds with the CD4 receptor and one of two accessory receptors (CCR5 or CXCR4) to effectuate a conformational change allowing viral entry to the cell. The core contains two single sense strands of RNA containing 3 structural genes (gag, pol and env) and six accessory genes (tat, rev, nef, vif, vpr and vpu). The pol gene is responsible for the production of HIV viral enzymes including reverse transcriptase which allows

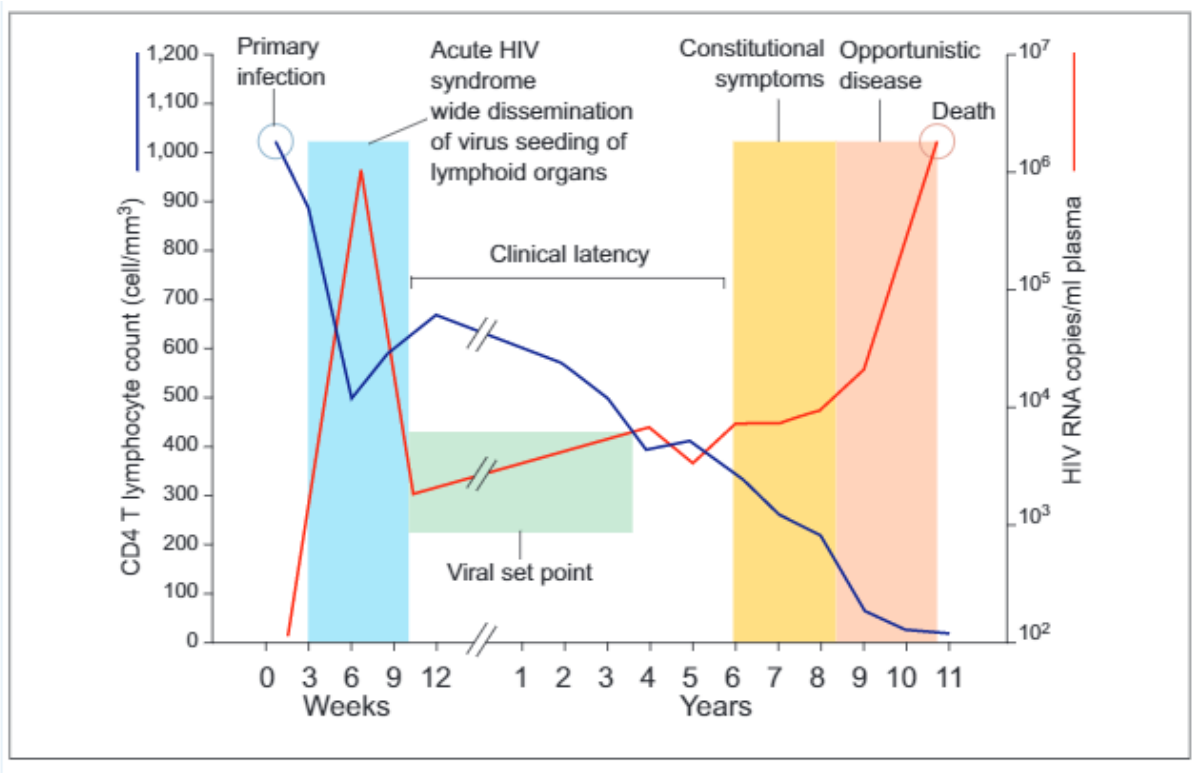
[1] From 2. Perrin, L., L. Kaiser, and S. Yerly, *Travel and the spread of HIV-1 genetic variants.* The Lancet Infectious Diseases. **3**(1): p. 22-27.

the viral RNA to be copied into DNA within the host cell. Integrase then facilitates the integration of DNA into the host cell genome.

1.1.2 Natural history of HIV infection

Sexual transmission is the most common mode of infection for HIV and occurs across a mucosal surface [3]. The first cells to be infected are either CD4 T cells within the epithelium, or Langerhans cells (a type of dendritic cell)[4]. CD4 T cells are found closer to the surface of the epithelium during activation in response to a current infection [5]. During the first week of infection, there is local replication of the HIV virus within neighbouring CD4 T cells [6]. T cells then migrate to local draining lymph nodes before spreading out to secondary lymphoid organs during the second week of infection[7]. It is at this point that viral reservoirs are established in lymphoid tissue a well as in organs such as brain, lungs and liver [8, 9]. Simultaneously, activation of the immune system in response to the HIV virus leads to a large pool of CD4 T cells that are highly permissive to HIV infection and destruction of CD4 T cells begins [10]. A compensatory increase in CD8 T cells results, with the development of HIV specific cytotoxic T cells [11]. It is at this point that maximal HIV replication occurs and peripheral blood viral loads peak and patients can often experience an 'acute retroviral syndrome', manifesting as 'flu-like' symptoms including fever and general malaise. The process of immune activation leads to systemic inflammation and 'bystander death' which contributes to the depletion of the CD4 T cell pool [12]. SIV sootey mangabey models and comparisons with HIV-2 infections demonstrate that the quantity of CD4 T cells is preserved despite a high viral load because of an absence of significant activation of the immune system. This supports the hypothesis that CD4 T cell death in HIV infection is an immunopathological process [13]. Continued antigenic stimulation in the context of impaired T cell renewal capacity due to thymic fibrosis together are likely responsible for T cell count depletion in HIV infection [14]. T regulatory responses limit some of this heightened response, and predict the extent of immune activation [15]. However, the ability of HIV specific cytotoxic CD8 T cells to evade this suppression may be important in viral control [16]. Following this period of rapid replication, the HIV virus then enters a latent phase and the level of plasma viral load is referred to as the viral set point. The length of time spent in this latent phase varies considerably from person to person but, without treatment, generally leads to the development of AIDS. It was recognised early in the epidemic that HIV virus continues to replicate during this latent stage within lymphoid tissue [17] and that such sanctuary sites and latently infected cells would be a major obstacle to the eradication of HIV [18]. A summary of CD4 and viral load (VL) dynamics over the course of infection is given in Figure 1-2 [19].

Figure 1-2 Immunological, virological and clinical stages of untreated HIV infection[2]



1.1.3 History of the HIV epidemic

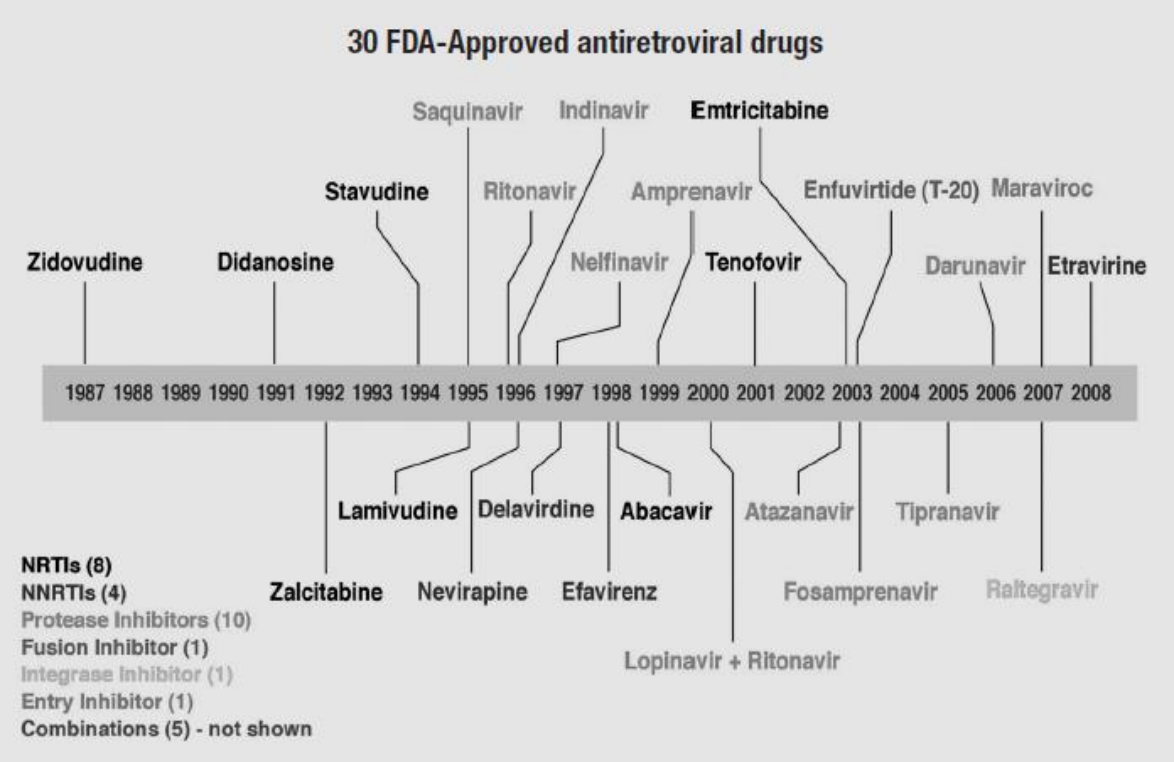
The acquired immunodeficiency syndrome (AIDS) was first reported in a case series of patients with *pneumocystis carinii* pneumonia published by the Centres for Disease Control and Prevention in 1981 [20]. In 1983 The Pasteur Institute in France first identified the HIV virus, which they referred to as Lymphadenopathy associated virus (LAV) [21]. Soon after, Robert Gallo and colleagues also identified what they referred to as HTLV-III [22]. Four years later, reports of 'slim disease' emerged from clinics in sub-Saharan Africa [23]. In 1985, the first serological test for detection of HIV specific antibodies was designed [24] and over 10 years later, quantification of HIV viral load in the plasma was made possible using PCR methods [25]. This not only supported efforts to diagnose HIV infection, but importantly served as an outcome marker to assess therapeutic interventions [26].

Zidovudine, from the nucleoside analogue reverse transcriptase (NRTI) class, was the first anti-retroviral agent used to treat HIV and became popular towards the end of the 1980s [27]. Although Zidovudine increased AIDS free survival, this was only a short term effect and mortality remained unchanged; it was soon discovered that this was due to the development of resistant mutations [28]. Dual therapy was attempted with two agents from the NRTI class [29, 30], but again, the mortality benefit was short-lived.

[2] From 19. Rodger, A.J., M.A. Johnson, and T.W. Mahungu, *HIV/AIDS : An Atlas of Investigation and Management*. Atlas: HIV2011, Oxford: Clinical Publishing.

Nevirapine was the first of the non-nucleoside analogue reverse transcriptase inhibitors (NNRTI) to be used to treat HIV infection. Shortly after this, a landmark paper from the INCAS group provided the first evidence of the benefits of triple therapy, or highly active anti-retroviral therapy (HAART), using two different drug classes [31]. Around the same time drugs from the protease inhibitor class became available and guidelines were soon released recommending a protease inhibitor as the 'backbone' of HAART along with two NRTIs [32]. Although HAART was first officially used in Uganda and Cote d'Ivoire in 1998 [33], roll-out in sub-Saharan Africa began in earnest between 2000 and 2004 following the first ever WHO guidelines for use of HAART in resource poor settings: 'Scaling up anti-retroviral therapy in resource limited settings' [34].
The introduction of HAART between 1995 and 1997 revolutionised the treatment of patients with HIV [35]. The first confirmation of this came from a report of the HIV outpatient study in 1998 which demonstrated that HAART decreased AIDS related mortality from 29.4 per 100 person years to 8.8 per 100 person years over only 2 years [36]. The timeline for the introduction of some of the most commonly used antiretrovirals (ARVs) today is given in Figure 1-3 [37]. In the years following the introduction of HAART, it became clear that some patients experienced failure of these first line drugs [38].The next HAART milestone came with introduction of the concept of second line regimes [39]. More recent work has shown that even short, planned interruptions of HAART lead to viral rebound and increased AIDS related events and mortality [40].

Figure 1-3 Timeline of introduction of anti-retroviral drugs[3]



During just over 3 decades of the epidemic, a total of 78 million people have been infected with HIV and 39 million have died [41]. Antiretroviral Therapy (ART) has globally reduced progression to AIDs and HIV related mortality [42]. WHO first published guidelines on eligibility for ART initiation in 2002 and since then the CD4 count threshold has steadily increased. In 2013, WHO recommended initiation of ART for anyone with a CD4 count equal to or less than 500 cells/uL and in September 2015, this guidance was updated to recommend universal treatment regardless of CD4 count [43]. 15.8 million people were accessing ART at the end of June 2015, representing 41% of those eligible [44]. 7.8 million deaths are estimated to have been averted thanks to ART between 2000 and 2014 [45].

The vast majority of the global burden of HIV disease is found in sub-Saharan Africa (SSA) and efforts to roll-out ART have focussed on the region. As of the end of 2014, 25.6 million (70%) of the 36.9 million people infected with HIV were living in sub-Saharan Africa [44]. Nearly 5% of adults in SSA are living with HIV. SSA saw 5000 new adult infections a day in 2014, which is 66% of new infections globally [46]. Although more people are being initiated on ART, evidence suggests that globally CD4 counts at ART initiation are not increasing significantly. In other words, a significant proportion of people still present with advanced immunosuppression. A recently published meta-analysis found that between 2002 and 2013, there has not

[3] From 37. Palmisano, L. and S. Vella, *A brief history of antiretroviral therapy of HIV infection: success and challenges.* Ann Ist Super Sanita, 2011. **47**(1): p. 44-8.

been a significant increase in CD4 count either at presentation or at ART initiation [47]. Although a recent report from Rwanda found that between 2007 and 2008 CD4 count at ART initiation increased 110 cells/uL, there were still clear disparities for some groups including male patients [48].

Because of this disproportionate burden of disease, the number of people receiving ART has increased rapidly in SSA compared to other regions (see Figure 1-4 [46]). A stark illustration of the impact of this roll-out of ART in SSA is demonstrated by looking at the impact in life expectancy in South East Africa, where the highest prevalence of HIV is found (Figure 1-5 [49]).

Figure 1-4 Estimated global ART coverage 2000 – 2015, by WHO region[4]

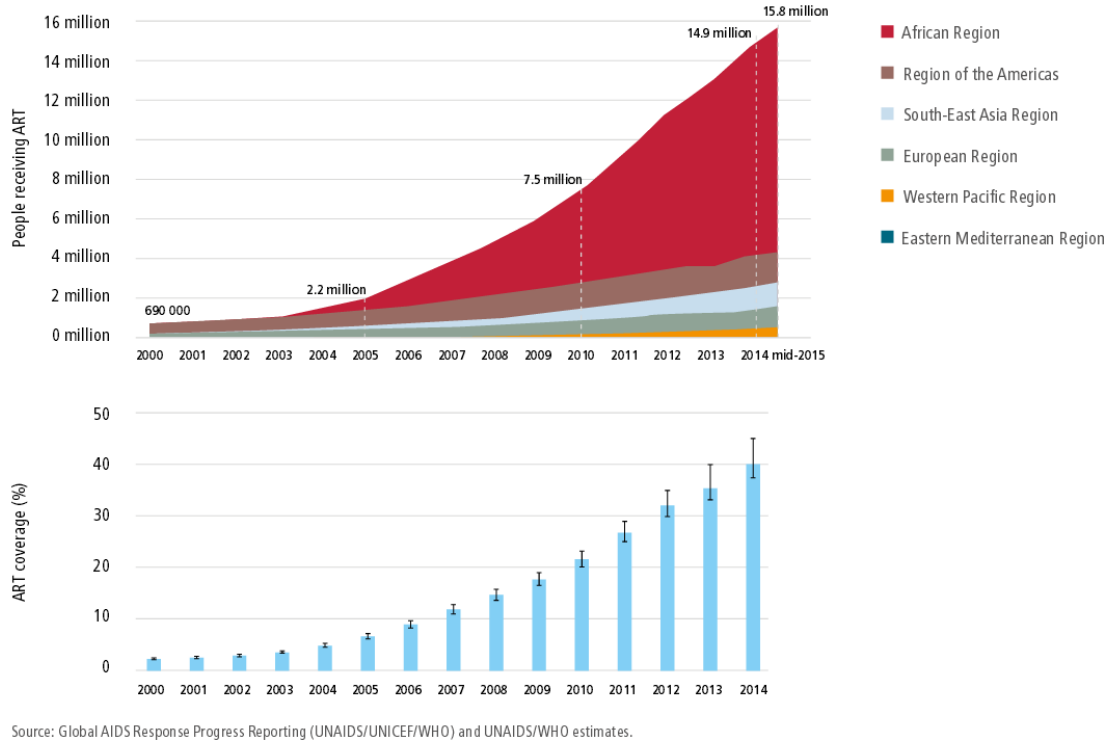
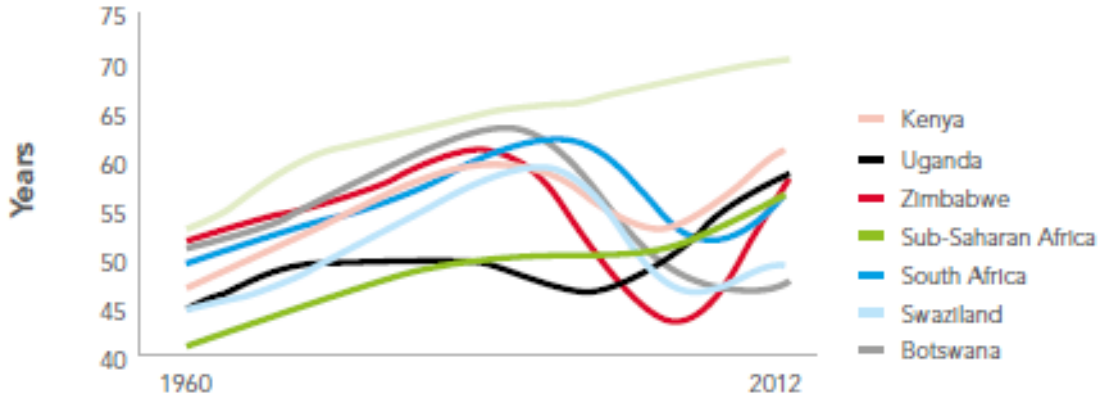


Figure 1-5 Impact of HIV response on life expectancy in SSA[5]



Despite this progress, recent estimates from WHO and UNAIDS project that failing to widen global ART coverage could lead to a rise in HIV incidence to 2 million new annual infections by 2030 [49]. UNAIDS 2016-2021 Fast track targets have been published, aiming for 90% of HIV infected people to know their status,

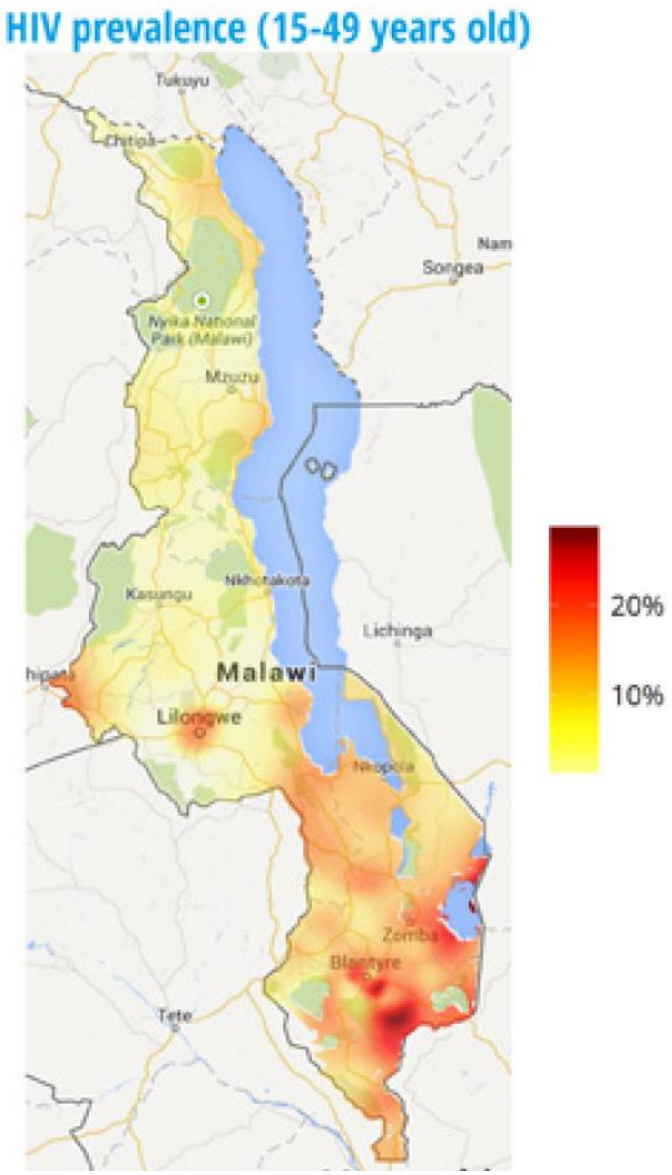
[4] From 46. UNAIDS, *Core Epidemiology Slides*, 2015.
[5] From 49. UNAIDS, *HIV TREATMENT IN AFRICA: A looming crisis*, in *ISSUES BRIEF*2015.

90% of positive people to be on ART and 90% of people on ART to be virally suppressed by 2020 [50]. This strategy underpins new Sustainable Development Goals which aim for zero new infections and zero AIDS related deaths by 2030 [50].

1.1.4 Epidemiology of HIV in Malawi

In Malawi, 12% of the population – just over 1 million people - are living with HIV [51]. This accounts for 4% of all people living with HIV in SSA. The epidemic is concentrated in the Southern region; Blantyre has a HIV prevalence of 17.8% (110,000 adults) [52]. Although the prevalence is high, the incidence of new HIV infections is falling: there were around 34,000 new infections in Malawi in 2014 , which has declined from 98,000 in 2005 (see Figure 1-6) [52].

Figure 1-6 Distribution of HIV in Malawi in 2014[6]



Around 500,000 Malawians are currently alive on ART which represents 69% of the 745,000 people ever initiated on ART in Malawi [51]. In all, 67% of all those currently eligible for ART are receiving it [53]. Just over 110,000 people were initiated on ART in 2014. ART roll out has been largely successful in Malawi with 48,000 AIDS related deaths in 2014 representing a 51% decrease since the roll out of ART [52]. Although the death rate of ART patients has fallen steadily from 2005, the default rate has remained more or less static at around 1 – 2%.

[6] From 52. AVERT. *HIV AND AIDS IN MALAWI*. Gap report 2014 2014 [cited 2016 31st March]; Available from: http://www.avert.org/professionals/hiv-around-world/sub-saharan-africa/malawi.

1.2 Chronic immune activation is a limitation to the success of ART

1.2.1 Overview of chronic immune activation in HIV

The focus in HIV care is shifting to managing HIV as a chronic disease [54]. Despite the unequivocal success of ART in reducing mortality related to HIV and AIDS, ART does not restore patients to full health. Under normal physiological conditions, immune cells are usually at rest. Activation of immune cells in response to most pathogens is short-lived whilst the pathogen is cleared from the host system. However, in the case of chronic infections such as HIV, the immune system remains activated at low levels, even in patients who are virologically suppressed on ART. Patients on ART have long since been recognised to have markedly elevated levels of activated immune cells compared to HIV negative patients [55, 56]. Activation of CD4 and CD8 T cells leads to complications such as immune exhaustion, poor immune reconstitution on ART and AIDS related illnesses [57-59]. Research conducted at the Malawi Liverpool Wellcome Clinical Research Program (MLW) suggests that many factors specific to resource limited settings contribute to immune dysregulation and that the consequences may also be unique in sub-Saharan Africa [60-63]. For example, both T cell and B cell functions have been shown to be predictive of pneumococcal infection even without HIV infection and differences have been demonstrated in mucosal immunity in children from this low resource SSA setting.

1.2.2 Pathogenesis of immune activation, exhaustion and senescence

Stimulation with HIV antigens promotes T cell differentiation and proliferation. During HIV infection, failure to replenish the CD4 T cell pool results from both a decreased production in cells as well as a decreased half-life. ART allows the production of cells to increase but the half-life is still shortened [64]. Marked expansion of the CD8 T cell pool occurs with 80-90% of cells exhibiting activation with CD38 expression during acute HIV infection (CD4 T cells show much less CD38 expression) [65]. There is both direct activation through recognition of specific antigens that individual T cells are primed to recognise (such as cytomegalovirus (CMV), Epstein Barr Virus (EBV) and HIV itself), as well as indirect, non-specific activation [65]. Immune activation in HIV infected patients has been demonstrated for many immune cells including NK cells, B cells, neutrophils and plasma DCs [66-69]. However, most of the attention has been focussed on T cells and monocytes which seem to represent two quite distinct inflammatory axes.

T cell activation has been strongly associated with acute HIV pathogenesis, disease progression, AIDS related events and non-AIDS related events. CD38 was one of the first markers used to identify T cell activation and at the time was shown to independently predict HIV disease progression [70-72]. The expression of HLA-DR is also regarded as an activation marker on T cells and its expression has been shown to be increased in cells that have undergone multiple rounds of replication [73]. In addition to activation markers, HIV infection has been associated with the expression of inhibitory markers on the cell surface which prevent the cell from

being able to respond to any new stimulus. Two main processes of cell inhibition can occur: immune senescence and immune exhaustion.

T cell senescence occurs when a cell has undergone many rounds of replication (which can be measured by telomerase length). Telomerase shortening leads to induction of the DNA damage response within the cell (DDR). DDR leads to growth arrest and if this goes unchecked by internal repair mechanisms ultimately leads to permanent growth arrest which is then irreversible. This process is likely to represent an anti-cancer control mechanism and would also be useful for the control of latent infections. The cells do not die, however, but instead remain in a state of limbo where they are unable to replicate or function.

Upregulation of CD57 and downregulation of CD28 expression on T cells characterises highly differentiated cells and therefore is highly expressed on senescent cells [65, 74, 75]. Around 40% of CD8 T cells in patients with AIDS also express CD57, a senescence marker which heralds the inability of the cell to further divide [75]. Lee et al demonstrated that expression of CD57 on T cells could be reversed by early ART [76]. It should be noted that other chronic viral infections promote the expansion of the senescent T cell phenotype, with CMV being the most notable example [77]. Furthermore, CMV infection has been associated with HIV progression [78], non-AIDS diseases [79] and CD4 T cell recovery on ART [80] .

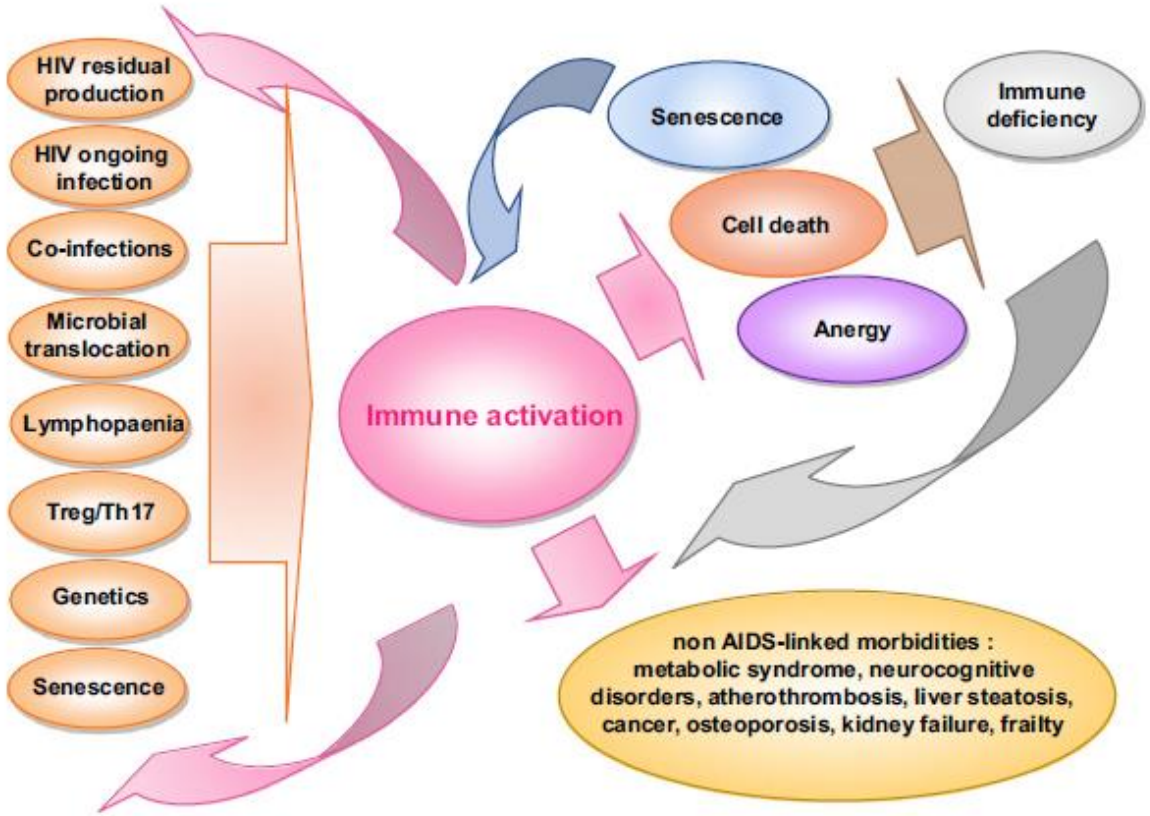
In contrast, T cell exhaustion occurs in response to a high antigenic load. Usually either viral or tumour antigen leads to differentiation and upregulation of inhibitory cell surface receptors such as PD-1. A signalling cascade is initiated which results in growth arrest, decreased function and cell death. However, T cell exhaustion can be reversed as demonstrated by studies using PD-1 blockade [81]. Cockerham *et al* found that PD-1 expression on CD8 T cells was closely related to CD8 T cell activation and viral load, whereas expression on CD4 T cells was related to CD4 T cell activation and low CD4 counts [82].

Monocytes expressing CD16 were identified as a distinct subpopulation in 1989 [83]. Three monocyte subsets are now recognised: classical (CD14++CD16-), intermediate (CD14++CD16+), and nonclassical (CD14+CD16+). HIV infected subjects have an expanded population of intermediate monocytes, which produce pro-inflammatory cytokines including TNF alpha and IL-1 [84]. It is hypothesised that TNFα production by monocytes drives expansion of nonclassical monocytes which in turn causes an upregulation of TLR4 expression on CD16+ monocytes [85]. The nonclassical monocyte subset is associated with HIV disease progression [86] and is more permissive to HIV infection [87]. Interestingly, expansion of the nonclassical monocyte population was protective against the development of tuberculosis (TB) immune reconstitution inflammatory syndrome (IRIS) in a South African HIV population [88]. In mice models, CD16+ monocytes express proatherogenic chemokine profiles and exist in higher numbers in atherosclerosis lesions [89]. In particular, CCR2 - the main receptor for the pro-inflammatory cytokine MCP-1 involved in the initiation of atherosclerosis - is upregulated in CD16+ monocytes.

1.2.3 Drivers of chronic immune activation in HIV

The exact mechanisms driving chronic immune activation in HIV infection are the subject of intense research and have not been fully elucidated. Although several main pathways are under investigation, it is likely that several, or even all, of these potential mechanisms can exist in any one individual with chronic immune activation. Figure 1-7 gives a simplified overview of the relationship between potential drivers and the cellular consequences of immune activation [90]. The three most important drivers are the effects of HIV itself, chronic coinfections and microbial translocation. All of the main factors contributing to chronic immune activation in HIV infection are likely to be more pronounced in the context of advanced immunosuppression. Using Ki-67 as a marker of active replication, the percentage of dividing T cells in untreated HIV infection in those with a CD4 count <100 is 10 fold greater than those with a CD4 count above 100 [12]. T cell activation and senescence is closely associated with low nadir CD4 counts [91]. T cell activation has been associated with CD4 T cell counts in a Ugandan cohort [92].

Figure 1-7 Overview of the drivers and cellular consequences of chronic immune activation[7]



[7] From 90. Younas, M., et al., *Immune activation in the course of HIV-1 infection: Causes, phenotypes and persistence under therapy.* HIV Med, 2016. **17**(2): p. 89-105.

1.2.3.1 HIV-1 infection per se

The most obvious cause for activation of the immune system during HIV infection would be the effect of HIV itself. There are two main ways in which the HIV virus might stimulate the immune system, even under virological control with ART [93]. The first theory is that viral replication continues at very low levels, below lower limits of detection of current assays. Viral replication can be measured by quantifying long terminal repeats (LTRs) within cells as a proxy marker for intracellular HIV replication [94]. The second is that HIV proteins may stimulate the immune system even when replication is not present. HIV-1 RNA may stimulate TLR pathways [95] and the presence of intracellular HIV DNA may activate caspase-1 pathways, even without the production of a competent virus [96]. However, the effects of HIV replication are not necessary to cause immune activation during HIV disease, because low CD4 counts are independently predictive of immune activation and are more strongly predictive than viral load [72]. Furthermore, the effects of the HIV virus itself are not sufficient to explain the pathogenesis of immune activation. Activation of T cells is not confined to those cells infected by HIV or that are specific for HIV antigen and cells of the innate immune system such as NK cells, pDCs and monocytes also show high levels of activation [97, 98]. Viraemia in several SIV models (such as sooty mangabeys and macaques) exists without evidence of immune activation or CD4 depletion [99]. Lastly, active viral replication with high viral loads are seen in long term non-progressors who do not exhibit the same degree of immune activation [100].

1.2.3.2 Chronic coinfection

Several chronic infections have been associated with increased levels of immune activation in people with HIV infection. These include hepatitis C virus (HCV) [101], hepatitis B virus (HBV) [102], CMV [103], herpes simplex virus (HSV) [104] and EBV [105]. Infection with TB is also an important contributor to immune activation in HIV infection [106]. Sullivan and colleagues found elevated levels of T cell activation in patients with latent TB in a South African cohort, but there was no TB uninfected control group [107]. A vicious circle exists in that inflammation in HIV is also associated with an increased risk of TB infection [108]. Evidence suggests that helminth infection can exacerbate immune activation in HIV and that intervening with anti-helminths improves HIV related outcomes [109].

1.2.3.3 Microbial translocation

The gut is now regarded as a major site in the immunopathogenesis of HIV disease. During acute SIV infection, there is a massive loss of CD4 memory cells in peripheral blood mononuclear cells (PBMCs) and in tissues, including the gut [110] and this has been confirmed with biopsies in untreated HIV-1 infected adults [111]. In SIV models, the loss of CD4 T cells in the gut seems to occur earlier than even loss in peripheral

lymph nodes and as such is a major site of immune depletion [112]. This has been confirmed in biopsies from HIV-1 infected adults which show that the CD4 T cell depletion in the gut is much more pronounced than in lymph nodes or blood samples [113]. As a result of this damage to the gut's immune mechanisms, the gut mucosa becomes compromised and inflamed, and bacterial products can cross into the blood stream. LPS is an endotoxin which can originate from gut bacteria and can be detected in blood [114]. Markers of gut epithelial barrier disruption and innate immune system activation have been associated with mortality in cohorts where there was only a minor association found with T cell activation [115]. But other microbial products may directly activate T cells [116]. In a South African cohort, CD16 positive monocytes were associated with HIV viraemia and normalised on ART; however, LPS remained elevated despite effective ART [117].

1.3 Immune activation is a risk factor for cardiovascular disease in people living with HIV

1.3.1 Pathophysiology of endothelial damage

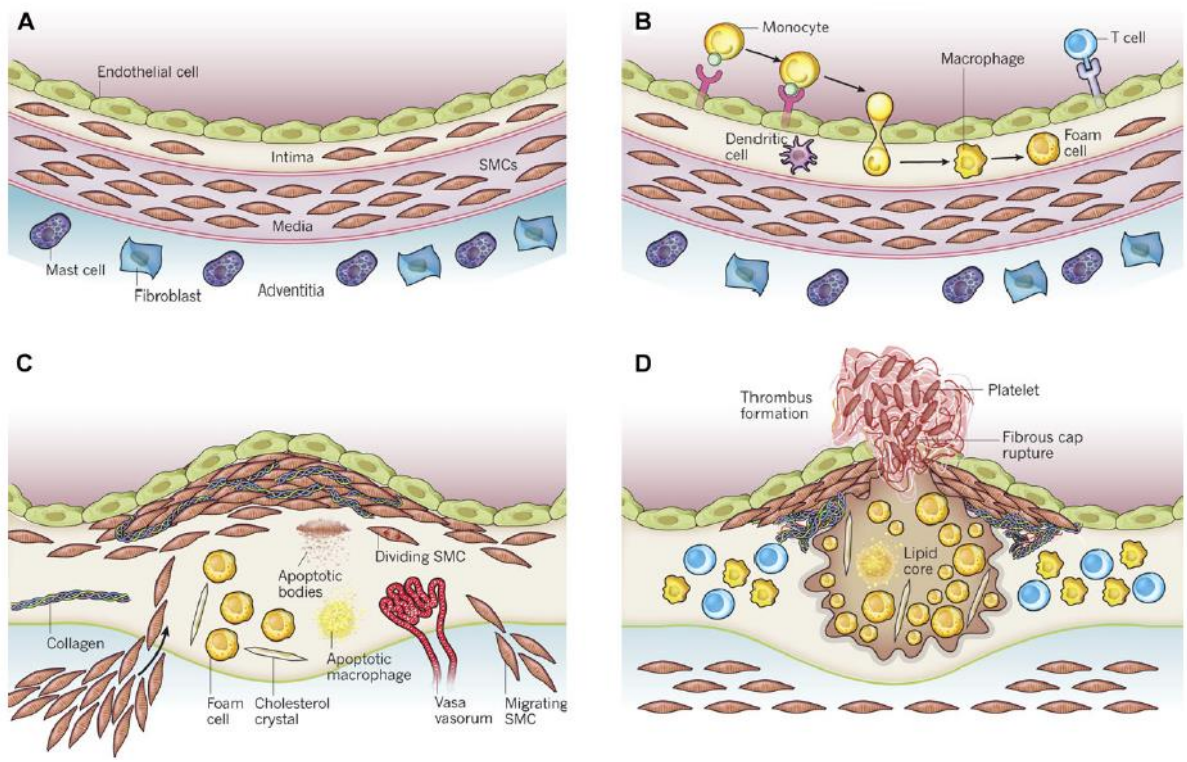
The arterial endothelium plays a critical role in the inflammatory response to a pathogenic stimulus [118]. Atherosclerosis can be viewed as a pathogenic consequence of the inflammatory response, occurring when the immune system responds to an aggravating stimulus, usually in the form of modified LDL (oxidised, glycated, aggregated or opsonised) [119]. However, activated monocytes have been shown to be atherogenic even in the absence of LDL stimulus and other stimuli which can initiate atherosclerosis include free radicals, hypertension, high plasma glucose, and even chronic infections such as HSV. Endothelial cells are activated either directly by adherence of monocytes and T cells to receptors on the endothelial surface, or indirectly through the release of chemokines such as MCP-1. As a result, adherence markers such as VCAM-1, ICAM-1, selectins and integrins are upregulated and both endothelial cells and recruited leucocytes signal to attract additional T cells and monocytes [120, 121].

Following endothelial activation, the next component of endothelial inflammation is 'rolling' where leucocytes, mainly monocytes, form low affinity bonds with endothelial markers causing them to roll along the endothelium from one marker to the next. VCAM-1 and selectins are the most important receptors involved in rolling and are essential in the early stages of atherosclerosis. Nonclassical monocytes preferentially express PSGL-1, a ligand for VCAM-1 and are more adept at rolling on activated endothelium than monocytes not expressing CD16 in mouse models [122].

When the leucocytes encounter a high affinity bond with endothelial molecules, they become 'stuck'. ICAM-1 and integrins are responsible for firm tethering, providing stronger connections with molecules such as LFA-1 and CR3 on leucocytes. Interestingly, LFA can be induced by gp120 and is also a key determinant of CD4 T cell infection [123]. This leads to the process of transcytosis where monocytes cross the endothelial barrier to enter the subendothelial intimal layers, becoming resident macrophages. Macrophages then

engulf lipid molecules creating foam cells that grow in size forming a fatty streak - the earliest stage of atherosclerosis. Leucocyte recruitment is much more important at this early stage of atherosclerosis than later on in the process. Th-1 subsets, in particular, have been identified as being proatherogenic through the production of IFNgamma [124]. Signalling between macrophages and T cells promotes the release of MMPs which lead to degradation of the surrounding extracellular matrix. Eventually accumulation of foam cells in the intima leads to the development of plaque and vascular smooth muscle cells in the media can migrate into the intima and proliferate there to form a fibrous cap [125]. It is this process that leads to thickening of the intima-media wall that can be seen on ultrasound of the carotid artery. Many approaches now exist to measure endothelial damage non-invasively and include carotid intima media thickness (cIMT) which provides information on the arterial wall thickening and atherosclerosis, and pulse wave velocity (PWV) which gives a measure of arterial stiffness. These will be discussed in more detailed in the methods chapter. Figure 1-8 gives an overview of the four stages of atherosclerosis.

Figure 1-8 Representation of the four stages of atherosclerosis[8]



A. endothelial activation B. leucocyte rolling and transcytosis C. formation of fatty streak D. plaque formation

[8] From 126. Lo, J. and J. Plutzky, *The biology of atherosclerosis: general paradigms and distinct pathogenic mechanisms among HIV-infected patients.* J Infect Dis, 2012. **205 Suppl 3**: p. S368-74.

1.3.2 HIV and cardiovascular disease

HIV infected participants demonstrate a profile of age related comorbidities similar to HIV uninfected adults who are 10 years older [127]. The risk of cardiovascular disease in people with HIV in high income settings is thought to be increased by about 2 fold compared to matched HIV uninfected adults [128]. In the VACS study, risk of myocardial infarction (MI) in prehypertensive HIV infected patients was 1.6 compared to healthy controls [129]. Having HIV infection doubles the risk of developing heart failure 12 months following an MI [130] and some reports suggest that diastolic dysfunction, in particular, is more common in HIV infection [131]. The number of people presenting with stroke who had HIV infection increased by 60% in the USA between 1997 and 2006 [132].

More evidence for the relationship between HIV infection and cardiovascular risk comes from the use of physiological markers such as carotid intima media thickness and arterial stiffness measurements. Several studies have assessed patients without traditional cardiovascular risk factors and found that PWV was increased in HIV infected compared to uninfected adults, and also closely correlated with features of heart failure [133, 134]. An interesting study from Lekakis and colleagues compared 56 HIV normotensive, 28 HIV uninfected patients with hypertension and 28 HIV uninfected participants without hypertension and found average PWV values of 8.1, 9.0 and 6.7 m/s respectively [135], indicating that PWV values for normotensive patients with HIV approached those for patients with diagnosed hypertension. Furthermore, when comparing HIV infected patients on ART to those who were ART naïve, those on ART had higher PWV values (8.4 m/s compared to 7.5). Ugandans aged over 40 and on ART had nearly double the risk of an ankle/brachial index >1.2 when compared to healthy HIV uninfected controls [136]. A South African cohort showed higher cIMT and lower arterial distensibility in a small HIV infected group on ART compared to uninfected controls [137].

The main drivers for the increased risk of cardiovascular disease in HIV fall into three main groups: direct effects of the HIV virus, side effects of ART drugs, and immune activation. Although this thesis focusses on the contribution of immune activation, these effects likely overlap and it is often difficult to tease apart whether any increased risk is mediated by immune activation as opposed to other processes, such as metabolic syndrome, because HIV untreated immunosuppressed patients are not available for comparison [138].

1.3.2.1 Direct effects of HIV virus

HIV is able to infect endothelial cells in vitro, but leads to an abortive infection unless rescued by mononuclar cells or CD4 T lymphocytes [139, 140]. Entry may occur via coreceptors such as CCR5 independently of CD4 [141]. Proteins such as gp120 and tat may also activate the endothelium [142], meaning that endothelial cells can be activated without direct infection and, even, in the absence of actively replicating virus. HIV has also

been shown to have a direct effect on the atherosclerosis process through effects on cholesterol metabolism: it can block cholesterol efflux from macrophages within atherosclerotic lesions and can also block egression of macrophages from the intima back into the artery lumen, potentiating the formation of atherosclerotic plaques [143].

The SMART study found an increased risk of cardiovascular disease in patients who were randomised to receive scheduled treatment interruptions on ART; this correlated with higher viral loads as well as inflammatory markers (C reactive protein (CRP), Ddimer and IL-6) and was highly predictive of cardiovascular disease (CVD) related morbidity [144]. The VACS cohort found an adjusted hazard ratio of 1.81 for incident heart failure in adults with HIV, which increased to 2.28 when only including those with a VL of over 500 copies/ml [145]. Further evidence of the link between HIV disease and cardiovascular events comes from the observations that earlier initiation of ART prevents cardiovascular events in several cohorts [146]. A study of 47 patients with HIV and pulmonary arterial hypertension found that ART led to an improvement in artery pressure as well as mortality due to pulmonary arterial hypertension [147]. Markers of endothelial damage have also been shown to be related to HIV viral load [148]. However, the direct effects of the virus are not the only explanatory factor for the increased risk of cardiovascular disease in HIV; an analysis of the VACS cohort found that the risk of MI in HIV infected people remained significant in those with a suppressed viral load (aHR 1.38 in virologically suppressed patients versus 1.48 in all HIV infected patients) [149].

1.3.2.2 Effect of ART

It may be possible that the increased risk of non-AIDS events in HIV is prevented if ART is started early enough [150], thus reducing the length of time exposed to viral replication and preventing advanced immunosuppression. However, this needs to be balanced with the cardiovascular risk posed by ART drugs themselves. ART has been recognised as a risk factor for cardiovascular disease in high income cohorts for many years, with the risk of myocardial infarction increasing by 26% for every year a patient is exposed to ART [151]. The DAD study reported a 3-fold increase in the risk of myocardial infarction in patients on a regimen containing a protease inhibitor compared to those taking an NNRTI [152]. Arterial stiffness has been shown to be significantly increased in people on a protease inhibitor compared to those not (PWV 9.0 +/- 1.4 compared to 8.1+/-1.3 m/s) and history of protease inhibitor treatment increased the positive correlation between PWV and age [153]. However, Efavirenz use has also been implicated as increasing cardiovascular risk as compared to other NNRTIs [154]. When looking at NRTIs, Abacavir has been strongly associated with both cardiovascular events and endothelial dysfunction [155].This effect is potentially mediated by many factors including the action of various ART classes on lipid profiles, metabolic syndromes, oxidative stress and direct endothelial damage [156]. However, in a small group of men who switched from

Abacavir to Tenofovir, augmentation index (a measure of arterial stiffness) decreased significantly at 24 weeks and a significant decrease in cholesterol was observed [157].

Overall evidence from high income settings suggests that patients established on ART have less evidence of endothelial damage than those not yet on ART [158]. This discrepancy is probably explained by the fact the damage from ART is acquired over the longer term [159]. PWV data for patients on ART in the sub-Saharan Africa setting is limited and not as conclusive as high income data. In Cameroon, blood pressure (BP) was higher in patients on ART than those not on ART, but PWV was similar (7.2 +/-1.5 in treated and 7.46 +/- 2.2 in untreated) [160]. In Rwandan women, PWV was not higher in HIV infected patients with short exposure to ART [161]. Most evidence points to the trend that coronary heart disease risk is increased in HIV, and that this is further increased on those on ART for longer durations of time [162].

1.3.2.3 The relationship between chronic immune activation and cardiovascular disease in HIV

Seminal work on the relationship between T cell activation and cardiovascular disease in HIV was produced by Kaplan and colleagues, who showed that in HIV infected women, carotid artery stiffness as measured by distensibility was closely related to T cell activation and that this was independent of effects of CD4 count and HIV viral load [163]. This association was found to persist even after 6.5 years of ART [164]. Activated CD8 T cells, in particular have been associated with cIMT [165]. Critics of this association believe that the increased risk of cardiovascular disease seen in people with HIV results from a higher burden of traditional risk factors in this group and a small study from Goulenok and colleagues found that cIMT was not associated with T cell activation nor HIV in patients who had never smoked [166]. It should be highlighted here that CMV infection is very relevant to the discussion of the association between immune compromise, immune activation and endothelial dysfunction. CMV infection is increased in advanced HIV infection and has been independently linked with endothelial damage and cardiovascular disease [79, 167].

Particular interest has been focussed recently on the activation of cells of the monocyte lineage and their close associations with soluble markers of inflammation and coagulation; these markers are strongly related to an increased risk in cardiovascular disease and mortality [168-171]. However, the role of monocyte subsets in the development of cardiovascular remains unclear. Although patients with coronary artery disease have high levels of inflammatory monocytes (CD14 +CD16+) in some studies [172], classical monocytes (CD14++CD16-) have also been associated with acute myocardial infarction [173]. In addition, there is lack of clarity around whether it is the microbial translocation or the resultant monocyte activation that increases cardiovascular risk [174, 175].

Whatever the cellular mechanism, the resultant inflammation has been closely linked with clinical outcomes. In post hoc analysis of the SMART study, each 2 log increase in IL6 and D-dimer as a composite

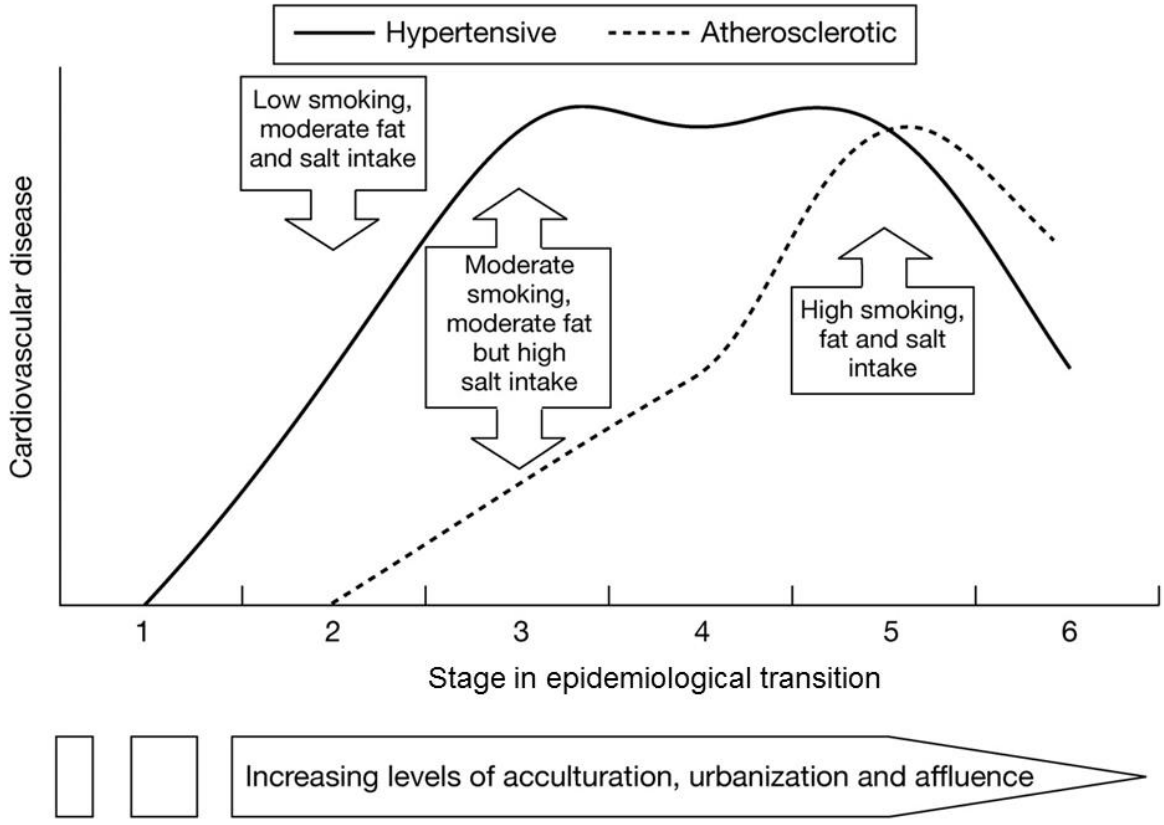
marker increased the risk of a serious cardiovascular event by 60% [176]. Markers of inflammation such as CRP and fibrinogen were independently related to death in the FRAM cohort [177] and have been shown to be of similar importance in a South African cohort [178]. Persistently raised inflammatory biomarkers correlated with subclinical atherosclerosis in a Ugandan cohort of HIV infected patients [179].
Patients with lower CD4 counts at ART initiation also have higher inflammatory biomarkers and evidence of increased monocyte activation [180]. Advanced immunosuppression at the time of ART initiation is also an important predictor for non-AIDS co-morbidities in HIV infection [181-183] and a long term cohort of ART patients in Brazil showed that those with CD4<200 had higher PWV and that it also correlated with age, gender and BP [184]. In an Italian study, HIV was associated with ambulatory arterial stiffness index and baseline CD4 count [185]. High CD4 count, or more specifically, CD4 count more than 500 may be the important factor in protection against CVD in HIV, as opposed to early initiation of ART [186]. A CD4 count less than 500 cells/uL carried an attributable risk of around 20% towards the development of cardiovascular disease in the HIV outpatient study, which was greater than the contribution calculated for cigarette smoking [187]. The association between low CD4 count and cardiovascular disease has not been replicated in some cohorts [188], but higher CD4 count reconstitution on ART is associated with lower risk of cardiovascular disease [189]. Supporting the importance of absolute T cell counts as biomarkers of cardiovascular disease in HIV, a CD4/CD8 ratio of <0.8 has emerged as a predictor for cardiovascular disease in HIV and was significantly predictive in a cohort of patients with CD4 count >350 cells/mm^3 [190]. Both low CD4 count and high CD8 T cell count were independent predictors of MI in the French HIV Database cohort [191].

1.4 A clash of two epidemics: shifting epidemiological trends in cardiovascular risk factors in SSA

1.4.1 Epidemiological transition of cardiovascular disease in sub-Saharan Africa

The global burden of disease survey showed an increasing incidence of cardiovascular disease in low and middle income countries and a trend towards increasing contribution of non-communicable diseases in the sub-Saharan Africa region to mortality [192]. As urbanisation increases, lifestyle changes result in alterations in the relative contribution of important cardiovascular risk factors such as hypertension, atherogenic diets and smoking [193]. This concept of an 'epidemiological transition' occurs as countries transition through the stages of economic development and the prevalence of subclinical atherosclerosis increases compared to cardiovascular disease caused by hypertension (see Figure 1-9). A global task force was launched in 2016 to address the research gap around HIV and non-communicable diseases in LMICs [194].

Figure 1-9 Gillum's stages of cardiovascular disease in the epidemiological evolution of patterns among people of sub-Saharan Africa[9]



1.4.2 Cardiovascular disease and HIV infection in sub-Saharan Africa

Data on the risk of cardiovascular disease in HIV infection in sub-Saharan Africa are limited. A cohort study of cerebrovascular events in two regions of Tanzania showed an age-standardised stroke incidence of 315 per 100,000 in an urban setting [195]. The same study reported a 15% prevalence of myocardial infarction on ECG amongst non-stroke patients [196]. A comparison of a cohort of patients with HIV infection in Botswana to a cohort in Nashville found that, when standardised for age, the incidence of cardiovascular disease was 8.4 compared to 5.4 per 1000 person years respectively [197]. Attempts to characterise subclinical atherosclerosis in SSA have revealed higher than expected levels of carotid intima-media thickening [198, 199]. A study in Botswana found HIV to be twice as frequent in patients with cardiomyopathy than in the general population [200] and a separate study in Rwanda diagnosed dilated cardiomyopathy in over 17% of HIV infected adults not established on ART [201].

[9] From 193. Cappuccio, F.P., *Commentary: epidemiological transition, migration, and cardiovascular disease.* Int J Epidemiol, 2004. **33**(2): p. 387-8.

The risk factors for cardiovascular disease in sub-Saharan Africa are likely to be different from those in high-resource countries [202] and management of cardiovascular disease will be particularly challenging in low income countries, where health systems are often fragmented and centred around single attendance episodes or infectious disease prevention [203]. Hypertension is an important risk factor for strokes in SSA, but the resources for secondary prevention are currently not available [204].

1.4.3 Cardiovascular disease and HIV in Malawi

Within Malawi, a cohort of stroke patients had a high prevalence of hypertension (55%), diabetes (21%), HIV (33%) and high cholesterol (17%)[205]. In addition, the majority of patients from a recent SSA multi-centre study on stroke had evidence of metabolic syndrome (78%)[206]. Looking instead at patients on ART, a cross-sectional study in Malawi showed a high prevalence of cardiovascular risk factors: insufficient fruit and vegetable diet (67.6%), raised blood pressure (45.9%), increased waist-hip ratio (45.4%), raised total cholesterol levels (31.0%) and low physical activity level (27.0%)[207]. A prospective study in Malawi found that HIV infection was more common in young adults with ischaemic strokes, who did not have many traditional risk factors [205]. A case control study by Benjamin et al found that patients with stroke were more likely to have HIV infection and that the highest risks were in those with untreated HIV (aOR 4.48) and those within the first 6 months of therapy (aOR 15.6). This was likely related to advanced immunosuppression within this cohort and demonstrates the need for further investigation into the aetiology of cardiovascular disease in patients in a setting like Malawi.

However, when examining the existing data from SSA, there is a clear disparity on an epidemiological level in that the reported prevalence of levels of traditional risk factors is not able to fully account for the event rate of cardiovascular disease. Together, the increased life expectancy and increased risk of diseases associated with aging in HIV pose a significant threat to health systems in low-income countries [194]. The current study hypothesises that HIV infection and immune activation are risk factors for endothelial damage as measured by carotid intima medial thickness (cIMT) and pulse wave velocity (PWV) in adult Malawians.

1.5 Assessment of cardiovascular risk

1.5.1 Cardiovascular risk prediction scores

The Framingham heart study pioneered the field of cardiovascular risk scoring and produced a risk estimation calculator which is still the most widely used scoring system [208]. It incorporates age, gender, total cholesterol, HDL, smoking status, diabetes and systolic blood pressure. Other risk calculators have been developed subsequently including the European Society of Cardiology SCORE calculator, which does not

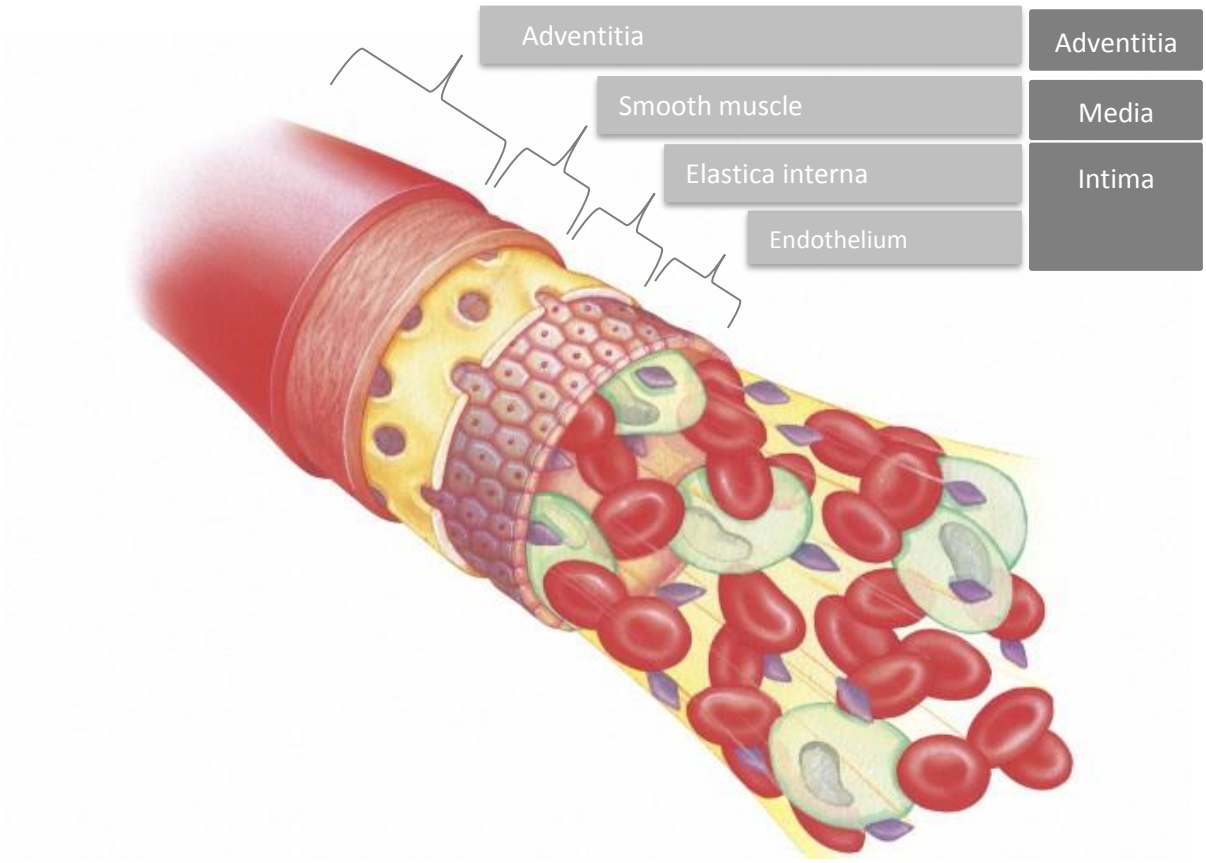
include HDL or diabetes [209] and the American Society of Cardiology ASCVD calculator which does include HDL and diabetes but also makes a limited assessment of the contribution of race [209]. These scores have all been developed in general populations and do not cover risk factors specifically related to HIV such as immunosuppression, HIV viral load and chronic immune activation. The DAD study group produced a prediction score using a population of patients with HIV infection which incorporates CD4 count, but this has not been shown to be of increased predictive value [210]. Cardiovascular risk scores for patients with HIV infection remain inadequate compared to their use in populations with HIV infection [211]. A recent study in Tanzania identified a high lifetime CVD risk in HIV infected compared to uninfected participants (34.7% versus 17.0%) [212]. However, the application of cardiovascular risk scores in low income sub Saharan Africa is also limited by the absence of population specific data as well as pragmatic issues around availability of blood tests such as cholesterol. WHO in conjunction with the International Society of Hypertension have produced risk estimation charts specific for countries in sub Saharan African for settings with and without access to cholesterol measurements. Additionally, they take into account local mortality rates and are classified according to mortality stratum [209]. Malawi falls into stratum E with high child mortality and very high adult mortality.

1.5.2 Physiological markers of cardiovascular risk

1.5.2.1 Pathophysiology of arterial stiffness

The artery wall is comprised of three layers: intima, media and adventitia (Figure 1-10). The main component of the intima is the endothelial cell layer which is surrounded by the media layer. The media contains lamellar units of elastin, smooth muscle and collagen. Elastin is responsible for artery distensibility and is found in high content in large arteries [213].

Figure 1-10 Layers of the arterial wall[10]



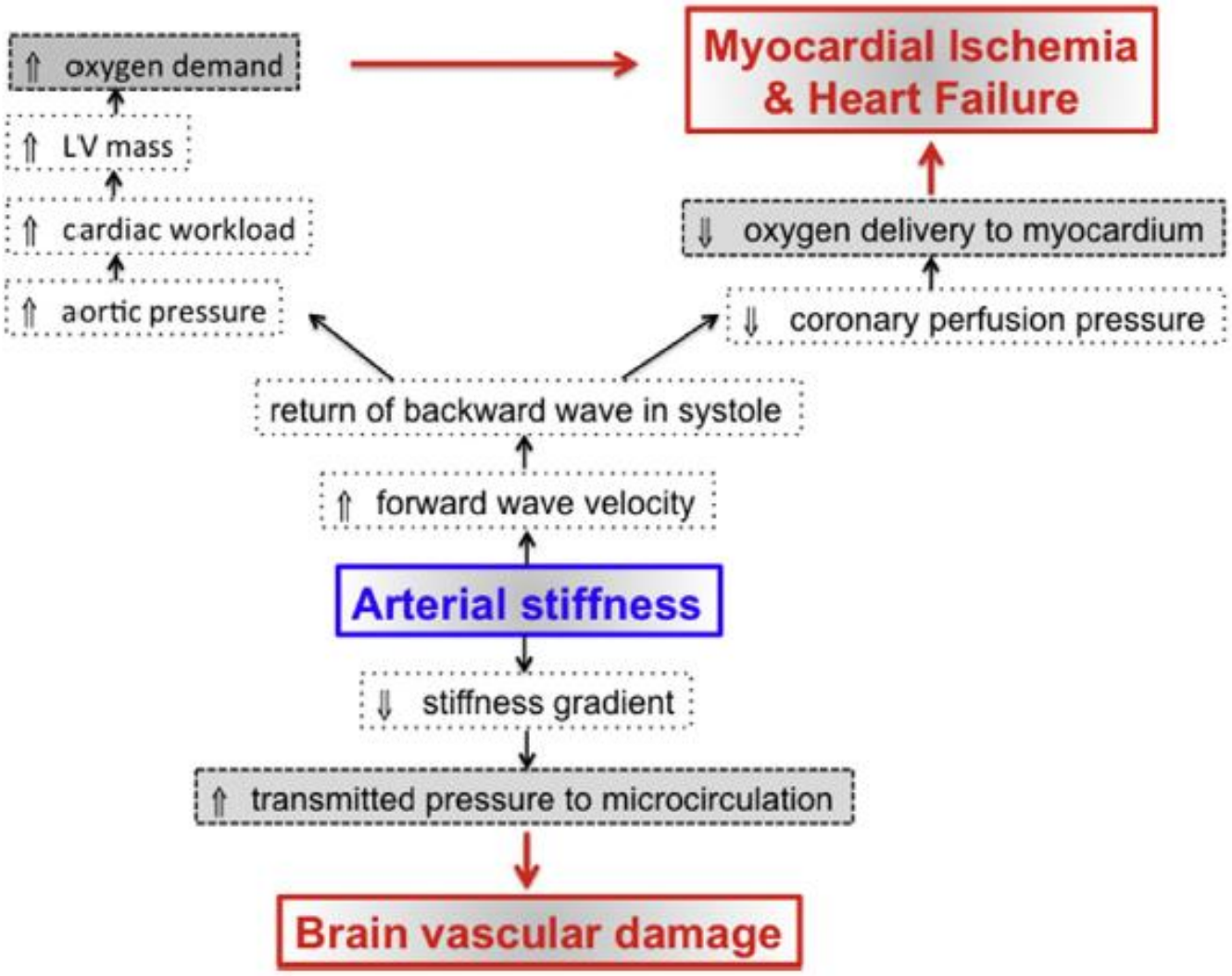
Elastin resists pressure within the artery at physiological levels and collagen functions to provide strength when the threshold of resistance for elastin is overcome. Collagen is stiffer by 100 – 1000 fold when compared to elastin and isn't distensible. Adults are unable to manufacture elastin so damage caused by enzymes such as elastin like proteases (including MMPs) is irreversible [214], whereas collagen can be produced in response to damage to the media layer. Advanced glycation end products accumulate with time and cause extensive cross-linking of both collagen and elastin, which leads to an increase in stiffness. Calcification of the medial layer of the arterial wall also occurs with aging [215] and recently, it has been proposed that smooth muscle cells may develop osteogenic phenotype under stress, which could also contribute to arterial wall calcification and stiffening [216]. Stiffness of the media layer can lead to disruption of the endothelial barrier, indicating that arterial stiffness may play a direct role in the development of atherosclerosis [217].

Aortic stiffness leads to early end systolic reflections instead of the usual diastolic reflections. One consequence of this is the increased backwards pressure on the left ventricle during systole which causes increased pressure in the left ventricle and left ventricular remodelling. A higher pulse pressure also occurs, leading to remodelling of the arterial wall and intima medial thickening [218]. Another consequence is that

[10] From Wellcome Images

there is less backwards pressure during diastole which decreases coronary perfusion pressure. Together, an increased pulse pressure and decreased diastolic pressure increase transmission pressure to the microcirculation including the brain and kidney [213] (Figure 1-11).

Figure 1-11 Effect of arterial stiffness on end organs[11]



[11] From 219. Palombo, C., et al., *Circulating endothelial progenitor cells and large artery structure and function in young subjects with uncomplicated type 1 diabetes.* Cardiovasc Diabetol, 2011. **10**: p. 88.

Stiffness also means that a higher blood pressure is needed to distend the artery and the ensuing hypertension propagates the cycle of increased stiffness through mechanical shear stresses. Studies have demonstrated that increased arterial stiffness can be picked up before the development of clinical hypertension [220] and has been shown to precede structural alterations in mice [221].

1.5.2.2 Methods of measurement of arterial stiffness

Non-invasive measures of arterial stiffness can be categorised into three groups: measuring PWV, relating change in diameter of an artery to distending pressure, and assessing arterial pressure waveforms [222]. There are two main tools for measuring arterial stiffness: Doppler ultrasound or applanation tonometry. Applanation tonometry is based on a small micromanometer flattened against an artery [222].

Aortic PWV is considered the gold standard for measurement of arterial stiffness [223] and is the method recommended by the European Network for Non-Invasive Investigation of Large Arteries [224]. The arterial stiffness analysis of the Framingham cohort compared various techniques for measurement of arterial stiffness and found that carotid-femoral PWV was by far the most predictive of cardiovascular outcomes (Table 1-1) [225]. PWV has also been chosen as a biomarker for the assessment of cardiovascular risk in clinical practice by the European society of Cardiology [226].

Table 1-1 Comparison of the ability of arterial stiffness measurements to predict clinical outcomes in the Framingham cohort[12]

Hemodynamic measure	Hazard Ratio (LCI,UCI)	*P*
Carotid-femoral (aortic) pulse wave velocity	1.48 (1.16, 1.91)	0.002
Carotid-radial (muscular artery) pulse wave velocity	1.07 (0.92, 1.25)	0.77
Augmentation index	0.91 (0.77, 1.07)	0.24
Central pulse pressure	1.00 (0.99, 1.01)	0.98
Pulse pressure amplification	0.86 (0.19, 3.82)	0.84

1.5.2.3 PWV as a tissue biomarker

In contrast with the non-tissue biomarkers of cardiovascular disease arterial stiffness provides a measurement of cumulative cardiovascular damage. It is considered an intermediate step between cardiovascular risk factors and cardiovascular disease and an elevated PWV>10m/s indicates asymptomatic end organ damage according to ESC-ESH guidelines [227]. Experts are calling for the integration of PWV into

[12] From 225. Mitchell, G.F., et al., *Arterial stiffness and cardiovascular events: the Framingham Heart Study.* Circulation, 2010. **121**(4): p. 505-11.

risk prediction tools as well as interventions to target arterial stiffness [228]. The European Society of Cardiology guidelines recommend the use of PWV as one marker of target organ damage [229] and its use is advocated as an end point in clinical trials [230].

Criteria for novel markers of cardiovascular risk were published in a consensus document by the American Heart Association in 2009 [231]. Evidence for each of these criteria will be presented in more detail.

1.5.2.3.1 PWV validation against clinical outcomes in prospective studies

There is strong evidence validating PWV as an independent predictor of cardiovascular disease. A meta-analysis of 20 prospective studies that reported the predictive value of aortic PWV for cardiovascular (CV) events or death found that for groups with high versus low PWV, the RR was 2.26 (95 % confidence intervals (CI) 1.89 – 2.24) for CV events, 2.02 (1.68 – 2.42) for CV mortality and 1.90 (1.61 – 2.24) for all-cause mortality [232]. A more recent individual participant meta-analysis found that adjusted risk ratios for high aortic PWV were 1.23 (CI 1.11 – 1.35) for coronary heart disease, 1.28 (1.16 – 1.42) for stroke and 1.30 (1.18 – 1.43) for all cardiovascular events [233]. When looking only at studies that assessed the general population, an analysis of the Framingham cohort found that for every SD increase in PWV, the adjusted HR for a CVD event was 1.48 (CI 1.16 – 1.91) [225]. In the Rotterdam study analysis, aHR for participants in the third compared to the first tertile of aPWV was 2.45 for coronary heart disease and 2.28 for stroke [234].

1.5.2.3.2 Evidence of incremental change in PWV with clinical outcomes

The association between PWV and cardiovascular events has been shown to increase incrementally [232]. The meta-analysis by Vlachapoulos et al also found an incremental adjusted association between PWV and clinical outcomes: a 1 m/s increase in PWV correlated with an increase of 14% for CV events, 15% for CV deaths and 15% for all-cause mortality. In the Rotterdam study, an incremental increase was seen when comparing the second and third tertiles of PWV to the first for both coronary heart disease (aHR 1.72 vs 2.45 respectively) and stroke (aHR 1.22 vs 2.28 respectively) [234].

1.5.2.3.3 Evidence for clinical relevance of PWV

The meta-analysis from Ben-Shlomo et al found that adding aortic PWV to the Framingham risk score (FRS) improved the net reclassification index modestly for all outcomes, but was particularly helpful for those with an intermediate risk on framingham risk score, showing a 13% improvement in classification of 10-year risk of CVD in those with intermediate risk [233]. In the Framingham risk study addition of aortic PWV to the standard risk factor model gave an upward reclassification of 6.7% of participants with CVD and upward reclassification of 1.2% with no event. For those in the intermediate risk category, there was upward reclassification of 14.3% for those with CVD event and downward reclassification of 1.4% for those who did not [225]. A subanalysis of the EDIVA cohort showed that there was a marked improvement in prediction of

CV events when PWV was added to the traditional HeartSCORE, especially for those with intermediate risk [235]. Several studies have recently reported the ability of PWV to improve the predictive capability of the FRS [236, 237]. However, another study from Holland aiming to identify new markers that may add to the predictive accuracy of the FRS found additive utility only with coronary artery calcium and to a lesser extent brain natriuretic peptide (BNP). Other markers, including PWV, were only marginally helpful [238].

1.5.2.3.4 Evidence that modification of PWV improves clinical outcomes

Trials to test the theory that reversal of arterial stiffness can also lead to improvement in clinical outcomes are currently lacking. However, several methods of reversing arterial stiffness are under study. Targeting the Renin-Angiotensin--System has shown the most promise. A meta-analysis of 5 trials using Angiotension Converting Enzyme inhibitor (ACEi) vs placebo to reduce arterial stiffness reduced PWV by an average of 1.69 m/s independently of any change in BP [239]. Perindopril significantly decreased PWV compared to controls in a small group of hypertensive South African patients [240]. The combination of ACE inhibition and angiotension II receptor (ARB) blockade may have a synergistic effect on reversal of arterial stiffness. In a trial which investigated the reduction in risk of CVD in chronic kidney disease with dual inhibition of the RAS, PWV was decreased on ACEi and ARB dual therapy compared to monotherapy independently of BP [241]. Aldosterone has been shown to directly increase collagen deposition in elastic artery walls and spironolactone or other aldosterone inhibitors may also show promise in reversal of arterial wall stiffening [242] .

Data suggest that statins exert anti-inflammatory and antiproliferative actions on vasculature beyond their lipid lowering properties. In a systematic review published in 2010, 2 of 4 studies assessing the effect of statins on PWV found a favourable decrease [243]. More recently, Kanaki and colleagues found that after 26 weeks of statin therapy in patients with mild hypertension and hypercholesterolaemia, PWV (sd) was 9+/-1.5 in the statin group and 10.9+/-2.6 in the placebo group ($p<0.001$) [244]. In an elderly population, 6 months of atorvastatin therapy led to a reduction in brachial ankle PWV as well as markers of oxidative stress [245].

In terms of the effect of other anti-inflammatories on arterial stiffness, most work so far has been in the field of rheumatological disease. Anti-TNFα antibodies have shown a beneficial effect on arterial stiffness, but it is unclear whether this is an anti-inflammatory mediated effect or a direct effect of the antibody itself [246]. A trial to investigate the Il-6 receptor blocker tocilizumab resulted in a worsening of arterial stiffness after 12 weeks [247]. Another randomised controlled trial (RCT) testing golimumab in ankylosing spondylitis also showed a significant increase in PWV compared to placebo [248].

Inhibition of the soluble receptor for advanced glycation end products is currently the only therapeutic target specifically designed to de-stiffen arteries and has recently showed some promise. In animal studies, use of "AGE-breaker" ALT-711 reversed arterial stiffness in diabetic rats [249].

1.5.2.4 Risk factors for arterial stiffness

1.5.2.4.1 Hypertension

Around 70% of the variance in arterial stiffness can be accounted for by age and BP [250, 251]. Arterial stiffening alone may provide sufficient explanation for hypertension with aging [252]. A systematic review of risk factors for arterial stiffness found that the only consistent predictors were age and hypertension [253].The question over the direction of the relationship between hypertension (HTN) and arterial stiffness is unclear, but HTN develops in people with high arterial stiffness and no HTN at baseline [254] and arterial stiffness has been shown to be a predictor for incident HTN [255]. Further, systolic BP correlates with PWV even in pre-hypertensive ranges [251], which would support the hypothesis that arterial stiffness precedes the development of hypertension through, for example, mechanical shear stress or perturbations of the renin-aldosterone-angiotensin system. The measurement of PWV has been suggested as integral to the management of arterial hypertension [256]. Several studies from South Africa have shown that PWV is a strong predictor of masked hypertension [257-259].

1.5.2.4.2 Metabolic syndrome

The main non-haemodynamic parameters closely associated with PWV are hyperglycaemia and insulin resistance, abdominal obesity and dyslipidaemia [260-262]. The hunter-gatherer lifestyle is independently associated with lower PWV at least partly due to lower body mass index (BMI), more favourable lipid profile and lower blood pressure [263]. Some evidence suggests that metabolic derangements are likely to affect PWV irrespective of BMI [264].

In 108 HIV untreated patients in Cameroon, the prevalence of metabolic syndrome was twice as high when compared to 96 HIV uninfected controls [265]. In a South African cohort of men with low BMI, BMI negatively correlated with arterial stiffness after adjustment [266]. Wang *et al* published data from a Chinese cohort and found a higher PWV was associated with higher low density lipoprotein (LDL) and lower high density lipoprotein (HDL) [267] and in a separate study correlated with increasing levels of impaired fasting glucose [268]. HDL-C is inversely and independently associated with PWV, suggesting that it may play a role in protecting from arterial stiffness [269].

1.5.2.4.3 Genetics

There are substantial heritability components to both PWV and cIMT according to the Italian twin study [270]. This was also confirmed in a genome wide scan analysis of participants in the Framingham study

offspring cohort, with one area of linkage for reflected wave amplitude on chromosomes 4 and 8 [271]. The European ancestry cohorts showed that a variation in a locus on the BCL11B gene on chromosome 14 is associated with higher pulse wave velocity which harbours one or more gene enhancers [272]. A separate twin study showed PWV related to calcified plaque and total aorta calcification but not to cIMT or non-calcified plaque [273]. A follow-up to this showed that genes involved in arterial calcification and collagen formation were associated with cross sectional PWV and changes in PWV over time [274].

1.5.2.4.4 Race

Several studies have demonstrated that black race is associated with higher PWV factors [275-278]. In a study from South Africa, PWV and BNP levels were higher in Africans than in Caucasians and the process was partly driven by a higher systolic BP [279].

1.5.3 cIMT

1.5.3.1 What is intima-media thickness?

The carotid artery is amenable to assessment via ultrasound methods because it is a relatively large artery and is anatomically superficial (Figure 1-12). Three distinct regions of the carotid artery can be scanned: common carotid artery, carotid bulb and internal carotid artery (Figure 1-13). However, repeatability for bulb and internal measurements is limited because it is more difficult to acquire a good quality image as the artery moves distally below the mandible.

Figure 1-12 Anatomy of the carotid artery[13]

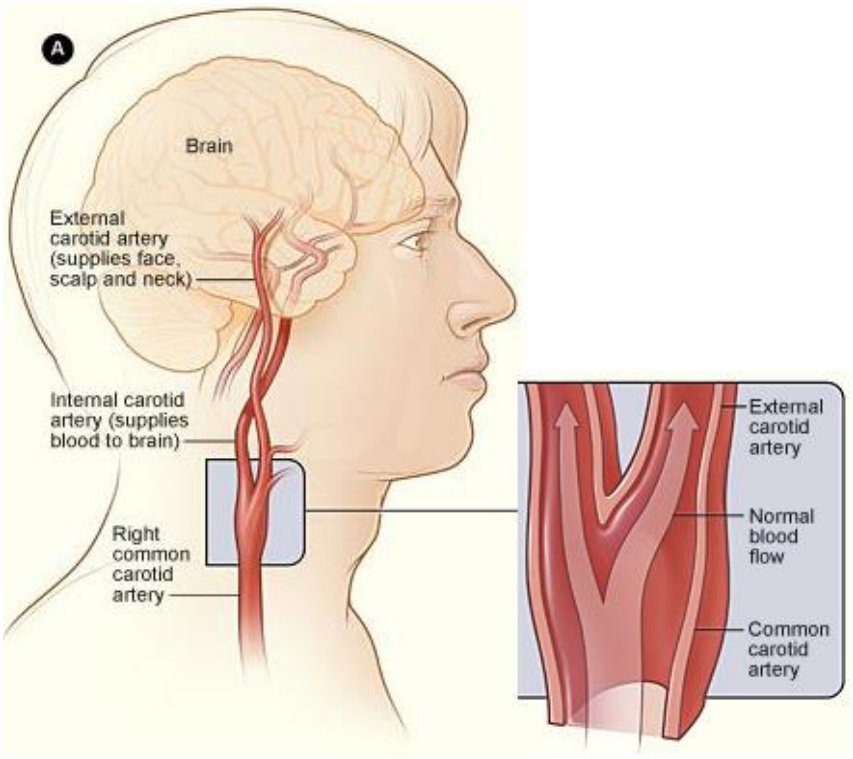
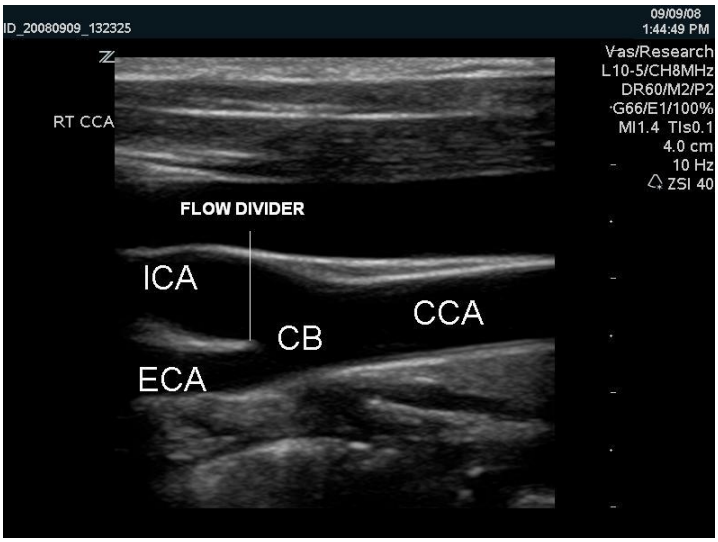


Figure 1-13 The carotid artery as seen on ultrasound



ICA: Internal carotid artery ECA: External carotid artery CB: Carotid bulb

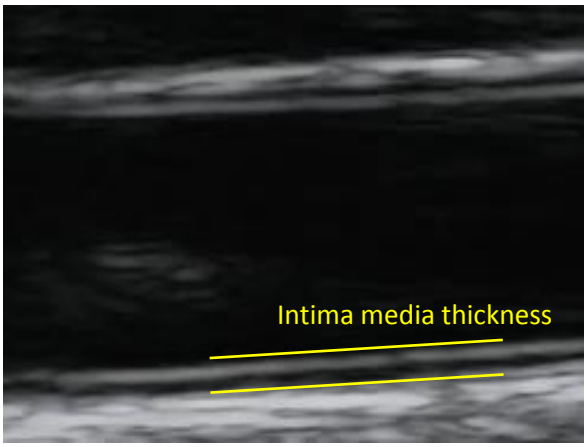
CCA: Common carotid artery

Using B-mode ultrasound, a pattern with parallel lines can be visualised at the carotid artery which provides a measurement of the intima and medial layers of the carotid wall [280]. The intima-media layer includes the vessel endothelium at the luminal aspect and extends to the far side of the media where it joins with

[13] From UCL Vascular biology unit manual

adventitia. Figure 1-14 highlights this parallel double line pattern on the far carotid wall of a still ultrasound image from a SHIELD patient.

Figure 1-14 Intima media thickness is represented by the 'double line' pattern on ultrasound



The measurement of cIMT encompasses both the intimal and medial layers of the artery wall. Uniform thickening reflects an atherosclerosis process in the endothelial layer as well as remodelling of the smooth muscle layer [280]. Early atherosclerosis involves formation of subendothelial lipid pools which lead to thickening. Macrophage infiltration then leads to the formation of a necrotic core which eventually leads to the formation of plaque [281]. Although intima-medial thickening also occurs as part of the ageing process and is not therefore necessarily related to a process of atherosclerosis, it gives a good indication of global cardiovascular risk [282]. Further, different pathologies may affect different parts of the arterial tree [283]. Atherosclerosis tends to affect areas of low shear such as the carotid bulb, whereas intima thickening in response to hypertension usually occurs in areas of high shear such as the common carotid artery near the bifurcation [284].

cIMT differs from plaque (which is a focal protrusion of the endothelial layer of the artery). cIMT is predictive of events independently from the presence of plaque [285]. An elevated cIMT is commonly cited as higher than the 75th centile of the general population value for a particular region and using a particular methodology [286].

An analysis of risk factors for higher cIMT was performed in a birth cohort in Iowa aged 33 to 42 years in 2001. This study found that the only significant risk factors for high cIMT were raised cholesterol and age [287]. The Bogalusa heart study found that systolic blood pressure explained variance in cIMT more than age and LDL cholesterol [288]. The young Finns study found that obesity, LDL and raised insulin levels predicted cIMT progression in adults with a mean age of 32 years [289]. The presence of multiple components of

metabolic syndrome present together confers a greater effect on cIMT than the sum of the effects of the individual components [290].

1.5.3.2 cIMT as a tissue biomarker

Standardised measurement of cIMT as a marker of cardiovascular risk is governed by the Mannheim Consensus, last updated in 2011 [291]. Although the American College of Cardiology Foundation and American Heart Association recommended that cIMT could be measured in asymptomatic patients graded as intermediate risk of a cardiovascular event [286], the recommendation was updated in 2013 to advise against the use of cIMT in risk assessment for primary prevention in routine clinical care [292]. Mainly, it is not clear that adding cIMT to current risk stratification measures such as Framingham risk scores add sufficient benefit to be clinically useful [293]. An analysis of the Framingham cohort found that only the internal carotid artery cIMT was useful in reclassifying cardiovascular risk, with a modest net reclassification index of 7.6% [294]. In a meta-analysis it was found that the relative reclassification index when adding cIMT for an intermediate risk group was 3.6% [293].

However, several large community studies have shown that cIMT is useful in predicting coronary events and stroke. Two large meta-analyses have been published. The first in 2007 assessed 11 studies (34335 people) and found that the HR per 1SD increase in cIMT was 1.26 (95% CI 1.21 – 1.30) for MI and 1.32 (1.27 – 1.38) for strokes [295]. A further meta-analysis published in 2012 assessed 14 studies and found that the pooled adjusted hazard ratio for risk of stroke per 0.1mm change in cIMT was 1.08 (95% CI 1.05 – 1.11) for MI and 1.12 (CI 1.10 – 1.15) for stroke [293]. The ARIC study, which was included in this meta-analysis, investigated a large cohort of community participants aged 45 – 75 and without prior history of stroke or coronary events and found an incremental association between cIMT and clinical events. The adjusted hazard ratio when comparing third to first tertile was 2.81 for women and 3.16 for men, and when comparing the second to first tertile was 1.08 for women and 1.28 for men [296]. On further assessment the relationship was non-linear, with cubic splines found to be the best fit [296]. A study of middle aged Swedish men and women found that cIMT was associated with an incremental increase in cIMT, even after adjustment for presence of carotid plaque[297]. The Tromso study included adults aged 19 to 94 with no previous MI and found that cIMT was only associated with MI when carotid bulb measurements were used [298]. The CAPS study also analysed a healthy population of 19 to 90 year olds and found that both common carotid (CCA) and bulb cIMT independently predicted myocardial infarction and stroke and that this effect was even more pronounced in the younger cohorts [299]. A large study of the general population in Germany comparing progression of cIMT with a combined cardiovascular endpoint found no association. However, when the absolute values of cIMT were assessed they were robustly associated with the combined outcome after adjustment with a HR of 1.16 (95% CI 1.10 – 1.22) [300]. Although no data exist on whether reversal of cIMT

can lead to a reduction in clinical events, several studies have demonstrated improvement in cIMT with statin therapy [301, 302].

1.6 Study aims and objectives

This study aimed to investigate the relationship between HIV, persistent immune activation and endothelial dysfunction in patients starting ART in Blantyre, Malawi. This translates into two general aims: 1) to compare subclinical carotid wall thickening in patients with and without HIV in Malawi and 2) to investigate the relationship between immune activation and endothelial dysfunction before and after initiation of ART in Malawi. This was broken down into six more specific, detailed objectives as follows:

Detailed objectives

1. Carry out a systematic review to define the clinical burden of discordant immune response to ART
   [Chapter 2]

2. Establish the range of age adjusted carotid intima medial thickness (cIMT) and arterial stiffness values in HIV negative patients and HIV positive patients with advanced HIV
   [Chapter 4]

3. Establish to what extent advanced HIV is a risk factor for increased cIMT and arterial stiffness in Malawi
   [Chapter 4]

4. Establish to what extent immune activation is a risk factor for higher cIMT and arterial stiffness
   [Chapter 5]

5. Explore the mechanisms involved in endothelial dysfunction according to different HIV related immune phenotypes
   [Chapter 6]

6. Describe the extent to which resolution of immune activation on ART alters endothelial dysfunction as measured by arterial stiffness
   [Chapter 7]

7. Investigate whether intensified initial management of HIV confers a larger decrease in endothelial dysfunction as measured by arterial stiffness, compared to standard ART
   [Chapter 7]

2 CHAPTER 2: DISCORDANT IMMUNE RESPONSE WITH ANTIRETROVIRAL THERAPY IN HIV-1: A SYSTEMATIC REVIEW OF CLINICAL OUTCOMES

2.1 Preface

Discordant immune response (DIR) to ART is a complication of chronic immune activation and has been widely reported in the literature. However, the clinical burden associated with DIR has not been systematically summarised. This systematic review was carried out to provide a summary of one of the major complications of chronic immune activation from a global view point [303].

2.2 Introduction

Antiretroviral therapy (ART) substantially reduces the incidence of acquired immunodeficiency syndrome (AIDS) and mortality, with increased CD4 cell count significantly and independently associated with improved prognosis [304-307]. Some patients do not achieve CD4 cell count reconstitution with ART, despite achieving suppression of HIV viral load in the blood [308]. This paradoxical response is referred to by various terms in the literature including DIR, poor or suboptimal immune reconstitution, incomplete immune recovery or restoration and immunological non-response. Here, we use the term discordant immune response as it was the term most frequently used by the included studies [309-313]. There is currently no agreed case definition for DIR.

Over 13 million people worldwide are on ART, with a further 22 million eligible [314]. Understanding limitations to its success will be critical in improving individual responses to treatment and regimen durability. The 2013 World Health Organization (WHO) consolidated guidelines on treatment of HIV now favour use of HIV viral load monitoring for routine identification of ART treatment failure [315], but CD4 cell counts for patients established on ART remain an important clinical and prognostic tool and are essential for identifying DIR [316, 317].

Much research has focused on CD4 reconstitution on ART, but the mechanisms promoting DIR are not well understood. Damage to CD4 T cells begins prior to ART initiation due to direct effects of the HIV virus on thymic tissue and depletion of progenitor cells[318]. Thymic output may be disproportionally affected in patients who start ART at lower CD4 counts leading to under-reconstitution of naïve CD4 T cells [319, 320]. Lymph node fibrosis is also a major feature and correlates with duration of HIV infection prior to ART initiation [321, 322]. Untreated HIV infection leads to a significant activation of the immune system [323], resulting in a cycle of systemic inflammation, persistent T cell activation, exhaustion and death [324-326]. The extent of immune activation at the time of ART initiation is associated with the development of DIR [307, 327] and predicts mortality on ART [176]. HIV induced T cell dysfunction and inflammation are closely

related to serious non-AIDS events [323, 328]. Persistent immune activation is often detected despite virologically suppressive ART [329] and can be driven by microbial translocation[330] , low level persistent HIV viral replication[331], and latent co-infections such as CMV [103, 332] and tuberculosis [333, 334]. Innate immune cells including monocytes, macrophages and NK cells also perpetuate immune activation, but this axis is more specifically driven microbial translocation, LPS antigenaemia and circulating soluble CD14 and does not necessarily correlate with T cell activation [335-338].

Non-systematic reviews have previously been carried out into aetiologies, prevalence and potential management of DIR [339-345]. However, the literature is heterogeneous and in order to better understand the burden of DIR, we sought to systematically characterise the risk of mortality, AIDS and serious non-AIDS events associated with DIR across the published literature.

2.3 Methods

The study protocol was registered with PROSPERO at the Centre for Review Dissemination, University of York (registration number CRD42014010821). The systematic review has been reported in accordance with the PRISMA guidelines [346] (See S1. Checklist: PRISMA Guidelines).

2.3.1 Eligibility criteria for study inclusion

2.3.1.1 Participants

Participants were aged 16 years or older and no restrictions were placed on language or geographical region.

Participants with DIR were defined as patients who had been taking ART for at least 6 months and who were virologically suppressed, but had a suboptimal CD4 count according to study definitions. Studies defined a suboptimal CD4 count in terms of either a failure to achieve a pre-specified rise in CD4 count or a pre-specified absolute CD4 value at a specific time point following ART initiation. Virological suppression was defined as at least one single HIV viral load measurement of below 1000 copies/ml after at least 6 months of ART. Studies that did not report on the virological status of the cohort were not included.

2.3.1.2 Outcomes

Studies were included if they estimated the risk of mortality, AIDS or serious non-AIDS events associated with DIR. Studies were included if death was verified by clinician review, tracing or verbal autopsy. AIDS was defined as any illness that met criteria for a WHO stage 4 condition [347]. Serious non-AIDS events were defined as illnesses not included in the WHO Clinical Staging System, and which were non-communicable.

These include non-communicable cardiovascular, liver, renal and bone diseases as well as non-AIDS related malignancies. Studies reporting AIDS and serious non-AIDS events were deemed to meet our inclusion criteria if the events had been verified at least by clinician review of participant records.

2.3.1.3 Study design

Studies were eligible for inclusion if they were cohort studies or randomised controlled trials (RCTs). We excluded editorials and comments, case reports and case series, qualitative studies, mathematical modelling studies, and economic analyses.

2.3.1.4 Information sources and search methods

We searched the following databases: Cochrane Central Register of Controlled Trials (CENTRAL, in the Cochrane Library issue 1, 2016); MEDLINE (PubMed; 1966 to 31st December 2015); EMBASE (OVID; 1980 to 31st December 2015). Table 2-1 shows the search strategy used in Medline (PubMed); this was modified for the other electronic databases.

Table 2-1 Search strategy

Search	
#16	Search **(#5) AND #15**
#15	Search **((((((((#6) OR #7) OR #8) OR #9) OR #10) OR #11) OR #12) OR #13) OR #14) OR #15** Field: **Title/Abstract**
#14	Search **incomplete CD4* response** Field: **Title/Abstract**
#13	Search **discordant*** Field:Title/Abstract
#12	Search **immunovirological discordance*** Field: **Title/Abstract**
#11	Search **low CD4*** Field: **Title/Abstract**
#10	Search **insufficient CD4*** Field: **Title/Abstract**
#9	Search **suboptimal CD4*** Field: **Title/Abstract**
#8	Search **low responder*** Field: **Title/Abstract**
#7	Search **suboptimal immune response*[Title/Abstract]**
#6	Search **suboptimal immune reconstitution** [**Title/Abstract]**
#5	Search **(#1) AND #4**
#4	Search **(#2) OR #3**
#3	Search **(antiretroviral[Title/Abstract]) OR ART[Title/Abstract]**
#2	Search **antiretroviral therapy [Title/Abstract]**
#1	Search **"HIV Infections"[Mesh]**

2.3.2 Study Selection and Data Collection

Titles of studies identified from the database search were independently reviewed by two authors and were excluded if the study was unrelated to the review subject. Remaining studies underwent abstract review independently by both authors and then full text review by the same two reviewers. Pre-piloted data extraction forms were independently applied to all studies that underwent full text review. Where disagreement occurred a consensus was reached by discussion or a third reviewer was consulted. Where outcome data were not reported, or if other eligibility criteria were unclear, the lead study author was contacted.

2.3.3 Risk of bias

Risk of bias assessment was based on the Cochrane Tool for Assessing Risk of Bias in Cohort Studies [348]. The Cochrane Tool for Assessing Risk of Bias in Randomised Control Trials was not used because no RCTs

targeting DIR were identified that met the inclusion criteria. Potential sources of bias were assessed in three domains: 'study design'; 'comparability'; and 'assessment of outcomes'.

The study design domain assessed whether participants were selected to be representative of adults on ART, and if there were clear selection criteria for those with and without DIR. Studies with more stringent selection criteria based on, for example, frequency of CD4 and viral load monitoring or attendance at routine clinics prior to enrolment, were deemed to be at a high risk of bias because they might exclude populations at higher risk of DIR and therefore were not representative of the entire population of patients with DIR. The comparability domain assessed if patients with and without DIR were managed according to the same standardised protocol and if outcomes were reported after appropriate adjustment for potential confounding variables. For the assessment of outcomes domain, outcomes had to be measured using clinician review, case note review, verbal autopsy or autopsy. Studies that did not report at least one of these methods were deemed high risk of bias for this category. The minimum acceptable follow-up period was one year as this is the highest risk period for adverse clinical outcomes post ART initiation [349].

An overall risk of bias assessment was made for each individual domain. A domain would be classified as high risk of bias if any one question within it failed the specified criteria. Where insufficient information had been reported in a study to make a judgement on the risk of bias, that question was recorded as unclear. 'Unclear' and 'high risk' categories were then combined for the purposes of analysis [350].

2.3.4 Summary measures and synthesis of results

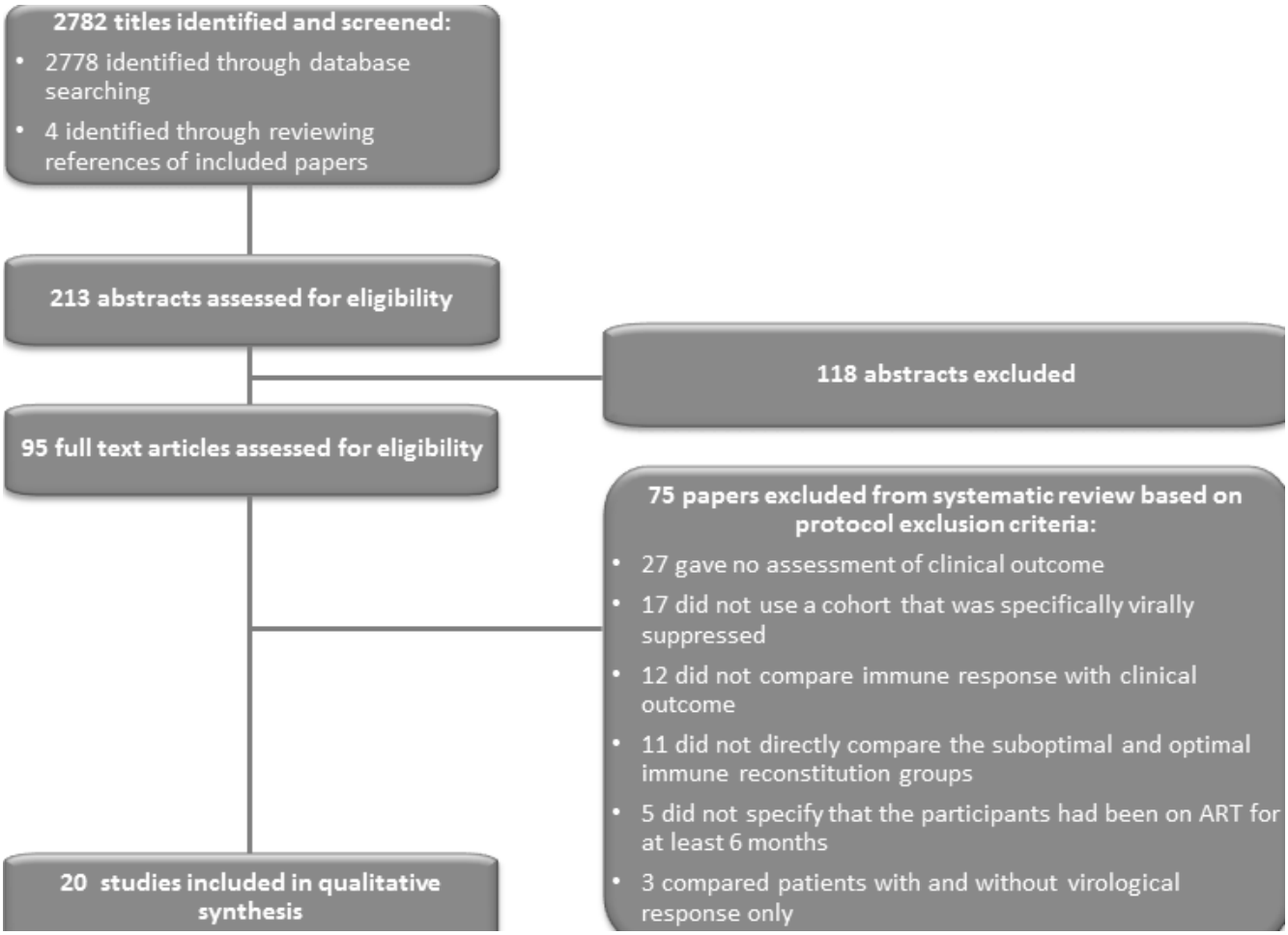
For each study, the proportion of participants with and without DIR who died, and/or experienced an AIDS-related, or serious non-AIDS-related event were estimated. Risk ratios and 95% confidence were extracted from the manuscript or calculated using Stata Version 13.1. Information was collected on variables including baseline CD4 and viral load, ART regimen, route of transmission and documentation of Hepatitis C infection. A meta-analysis with pooled effect estimates was planned including an analysis of heterogeneity using I^2 tests in Stata version 13.1. Sub analyses were planned to look for differences in effect of geographical region, year of study, exposure to ART and DIR definition. However, a meta-analysis could not be carried out due to considerable variation in both DIR definition and length of follow-up meaning that the included papers were not sufficiently comparable to produce a meaningful summary estimate.

2.4 Results

2.4.1 Study selection

2782 study titles were identified by the search. Twenty studies met inclusion criteria for full-text review (Figure 2-1). The two most common reasons for exclusion were that the study did not report a clinical outcome (36%) and the cohort was not virologically suppressed (23%). Authors from the study by Young *et al* were contacted to clarify if participants had been on ART for at least 6 months but the data were no longer available and so the study was excluded.

Figure 2-1 . Flow of paper selection from those identified following literature search through to inclusion



2.4.2 Study characteristics

Twenty studies were included [57, 307, 309-312, 351-364], all of which were cohort studies. For three studies, the cohort was established *de novo* to investigate the effects of DIR on clinical outcomes [57, 307, 352, 364]. One study analysed DIR and outcomes in the control arm of an RCT assessing ART regimes for

individuals initiating ART [351]. The remaining 14 studies conducted secondary analysis of existing datasets comprising of national or international cohorts of HIV infected patients who had data collected prospectively and systematically during routine clinical care [309-312, 353-363].

Seventeen studies recruited participants from HIV care clinics. Five studies included participants from resource-limited countries [57, 307, 312, 363, 364]: three from countries with a generalised HIV epidemic (Uganda, South Africa) [57, 307, 364]; one from Senegal [363]; and one from an international collaboration of both low-, and middle-income countries [312]. Participants were ART-naïve in 16 studies [57, 307, 309-312, 351, 352, 354, 356-360, 363, 364] whereas four studies included patients who were ART naïve or experienced [353, 355, 361, 362].

For included studies, the median proportion of male participants ranged from 31% – 100% and median age ranged from range 34 to 43 years (Table 2-2). Median CD4 cell count at ART initiation was reported for 15 studies and ranged from 80 -221 cells/mm^3. Median HIV viral load at ART initiation was reported for 10 studies and ranged from 4.5 log_{10} – 5.1 log_{10} copies/ml. The threshold for defining virological suppression ranged from <50 copies/ml to <1000 copies per ml. Participant follow-up ranged from 1 to 7 years.

Table 2-2 Description of 20 included studies

Study author	Study design	Year of publication	Median Duration of follow-up	Country	Setting	Relevant Outcomes examined	ART naïve?	%Male	Median age (years)	Median CD4 (cells/uL) at ART initiation	Median HIV VL at ART initiation (log10 copies/mL)
BAKER [351]	Control arm of ART RCT	2008	5 years	USA	Community, 80 sites	Predictors and clinical outcomes in DIR	Yes	80	39	221	5.0
BATISTA [363]	Established HIV cohort[2]	2015	7 years	Senegal	HIV care clinic	Frequency and risk factors for DIR, and incidence of OI and death	Yes	35	40	Not reported	Not reported
DRONDA [352]	Prospective cohort study[1]	2002	3 years	Spain	HIV care clinic	Immunologic and clinical outcomes in DIR	Yes	74	36	196	5.0
ENGSIG [353]	Established HIV cohort[2]	2010	4.7 years	Denmark	HIV care clinics, 8 sites	Predictors of and mortality in DIR	No	78	43[5]	Not reported	Not reported
FALSTER [354]	Established HIV cohort[2]	2008	5.4 years	Australia	HIV care clinics, sites	Prevalence of DIR, and	Yes	93[5]	Not reported	Not reported	Not reported

					not reported	clinical outcomes					
GILSON [309]	Established HIV cohort[2]	2010	3 years	UK	HIV care clinics, 10 sites	Predictors and clinical outcomes in DIR	Yes	75	37	170	5
GRABAR [355]	Established HIV cohort[2]	2000	18 months	France	HIV care clinics, 68 sites	Clinical outcomes in DIR	No	79	37	150	4.54
GUTERRIEZ [356]	Established HIV cohort[2]	2008	2.3 years	Spain	HIV care clinics, 10 sites	Predictors and clinical outcomes in DIR	Yes	75	37	160	5.0
HUNT [307]	Prospective cohort study[1]	2011	2 years	Uganda	HIV care clinic	Mortality according to CD4 account[3]	Yes	30	34	135	5.1
KAUFMANN [357]	Established HIV cohort[2]	2004	5 years	Switzerland	HIV care clinics, number of sites not reported	Predictors and clinical outcomes in DIR	Yes	74	38	180	4.9
LOUTFY [358]	Established HIV cohort[2]	2010	2.7 years	Canada	HIV care clinics, 9 sites	Clinical outcomes in DIR	Yes	83	40	180	5.0
MOORE [310]	Established HIV cohort[2]	2005	3.7 years	Canada	HIV care clinic	Predictors and	Yes	77[5]	39	199	Not reported

						mortality in DIR					
NAKANJAKO [57]	Prospective cohort study[1]	2008	1.8 years	Uganda	HIV care clinic	Prevalence of DIR and clinical outcomes	Yes	31	38	98	Not reported
NICASTRI [361]	Established HIV cohort[2]	2005	3.7 years	Italy	Hospital, 63 sites	Immunologic and clinical outcomes	No	72	35	185	4.78
PACHECO [359]	Established HIV cohort[2]	2009	6 years	Spain	Hospital, 10 sites	CD4 count recovery, predictors and mortality in DIR	Yes	32[5]	Not reported	Not reported	Not reported
TAKUVA [364]	Prospective cohort study[1]	2014	2 years	South Africa	HIV care clinic, 1 site	Mortality and AIDS in DIR	Yes	36	39	80	Not reported
TAN [311]	Established HIV cohort[2]	2008	3.2 year	USA	HIV care clinic	Clinical outcomes in DIR	Yes	76	38	213	5.4
TAIWO [362]	Established HIV cohort[2]	2009	Not reported	USA	HIV care clinics, 4 sites	Clinical outcomes in DIR	No	100	42	Not reported	Not reported
TUBOI [312]	Established HIV cohort[2]	2010	1 year	Multi-centre[4]	HIV care clinics, 31 centres	Mortality in DIR	Yes	39	34	100	Not reported

ZOUFALY [313]	Established HIV cohort[2]	2010	3.8 years	Germany	HIV care clinics, 11 sites	Predictors and clinical outcomes in DIR	Yes	77	39	80	Not reported

ART= anti-retroviral therapy, cART=combination anti-retroviral therapy, DIR=discordant immune response, VL=Viral load, PI=Protease inhibitor, NRTI=nucleoside reverse transcriptase inhibitor, DDI=didanosine, TDF=Tenofovir, LMIC-=Low and middle income countries. [1] Patients are enrolled specifically for the aims of the current study. [2] Retrospective analyses of prospectively collected data. [3]Analysis of clinical outcomes in DIR is a secondary analysis in this study. [5]Includes countries from Africa, South America and Asia. [5]Not reported for entire cohort therefore median value from optimal immune response group reported.

2.4.3 Risk of bias

Two (10%) studies had a high risk of bias in study design; 6 (30%) in comparability; and 11 (55%) in assessment of outcomes (Table 2-3).

For the study design domain, Engsig *et al* required a viral load of <50 copies /ml for more than three consecutive years before the start of the DIR observation period [353], and Kaufmann *et al* required a viral load of <1000 copies/mL during the entire 5-year observation period [357]. The frequency of visits these criteria would require may have excluded patients at higher risk of DIR. All studies detailed clear selection criteria for participants with and without DIR.

For the comparability domain, participants with and without DIR were managed according to the same treatment protocols for all studies but 6 studies did not appropriately evaluate the effects of confounders on outcomes [57, 351-354, 357].

For the assessment of outcomes domain, two studies gave no information on how deaths [357, 358] or AIDS events [358] were ascertained. Eleven studies did not describe how missing data were handled [57, 309, 351, 353, 354, 357-360, 362, 363].

Table 2-3 Risk of bias assessment for 20 included studies

Study	Study design			Comparability			Assessment of outcomes					Overall risk of bias
	Were participants selected to be representative of the wider population?	Were there clear selection criteria for those with and without DIR?	**Risk of bias**	Are patients with and without DIR managed to standardised protocol?	Are outcomes reported after adjustment for important confounding variables?	**Risk of bias**	Were procedures for measuring outcome sufficient?	Was follow-up long enough for outcome detection?	Were incomplete outcome data adequately assessed?	Are outcomes reported in full and not selectively reported?	**Risk of bias**	
BAKER [351]	Yes	Yes	**Low**	Yes	Unclear	**High**	Yes	Yes	Unclear	Yes	**High**	**High**
BATISTE [363]	Yes	Yes	**Low**	Yes	Yes	**Low**	Yes	Yes	No	Yes	**High**	**High**
DRONDA [352]	Yes	Yes	**Low**	Yes	No	**High**	Yes	Yes	Yes	Yes	**Low**	**High**
ENGSIG [353]	No	Yes	**High**	Yes	No	**High**	Yes	Yes	Unclear	Yes	**High**	**High**
FALSTER [354]	Yes	Yes	**Low**	Yes	No	**High**	Yes	Yes	Unclear	Yes	**High**	**High**
GILSON [309]	Yes	Yes	**Low**	Yes	Yes	**Low**	Yes	Yes	Unclear	Yes	**High**	**High**
GRABAR [355]	Yes	Yes	**Low**	Yes	Yes	**Low**	Yes	Yes	Unclear	Yes	**High**	**High**
GUTERRIEZ [356]	Yes	Yes	**Low**	Yes	Yes	**Low**	Yes	Yes	Yes	Yes	**Low**	**Low**
HUNT [307]	Yes	Yes	**Low**	Yes	Yes	**Low**	Yes	Yes	Yes	Yes	**Low**	**Low**
KAUFMANN [357]	No	Yes	**High**	Yes	No	**High**	Unclear	Yes	No	Yes	**High**	**High**

LOUTFY [358]	Yes	Yes	**Low**	Yes	Yes	**Low**	Unclear	Yes	No	Yes	**High**	**High**
MOORE [310]	Yes	Yes	**Low**	Yes	Yes	**Low**	Yes	Yes	Yes	Yes	**Low**	**Low**
NAKANJAKO [57]	Yes	Yes	**Low**	Yes	No	**High**	Yes	Yes	No	Yes	**High**	**High**
NICASTRI [361]	Yes	Yes	**Low**	Yes	Yes	**Low**	Yes	Yes	Yes	Yes	**Low**	**Low**
PACHECO [359]	Yes	Yes	**Low**	Yes	Yes	**Low**	Yes	Yes	No	Yes	**High**	**High**
TAKUVA [364]	Yes	Yes	**Low**	Yes	Yes	**Low**	Yes	Yes	Yes	Yes	**Low**	**Low**
TAN [311]	Yes	Yes	**Low**	Yes	Yes	**Low**	Yes	Yes	Yes	Yes	**Low**	**Low**
TAIWO [362]	Yes	Yes	**Low**	Yes	Yes	**Low**	Yes	Unclear	Unclear	Yes	**High**	**High**
TUBOI [312]	Yes	Yes	**Low**	Yes	Yes	**Low**	Yes	Yes	Yes	Yes	**Low**	**Low**
ZOUFALY [313]	Yes	Yes	**Low**	Yes	Yes	**Low**	Yes	Yes	Unclear	Yes	**High**	**High**

2.4.4 Definition of DIR

Definitions of DIR varied significantly and were classified into two categories: a failure to achieve a prespecified absolute CD4 count at a predefined time point; or a failure to achieve a prespecified rise in CD4 count from baseline at a predefined time point (Table 2-4). Five studies explored several potential definitions of DIR [57, 309, 313, 358, 364].

Eleven studies defined DIR based on a failure to achieve a rise in CD4 from ART initiation [57, 309-312, 351, 352, 355, 356, 361, 363]. Five used CD4 count thresholds of a failure to achieve a rise of at least 50 cells/ mm^3 at 6 months [309-312, 363]; two used at least 100 cells/ mm^3 at 12 months [57, 352]; 2 used at least 50 cells/ mm^3 at 12 months [355, 356]; one used at least 50 cells/ mm^3 at 8 months [351]; and one used at least 100 cells/ mm^3 at 8 months [309].

Nine studies defined DIR based on a failure to achieve an absolute CD4 count at a predefined time point [307, 313, 353, 354, 357-359, 362, 364] and used the following CD4 count thresholds: 200 cells/ mm^3 at 6 months[364] , 200 cells/ mm^3 at 12 months [313, 358]; of 350 cells/ mm^3 at 9 months [354]; of 250 cells/ mm^3 at 22 months [359]; 200 cells/ mm^3 at 36 months [353]; and 500cells/ mm^3 at 60 months [357]. Hunt and colleagues described mortality according to tertiles of CD4 counts in patients with viral suppression rather than use a single definition for DIR [307] so we compared mortality in the highest tertile (>177 cells/mm^3) to the lowest tertile (<95 cells/mm^3), using the lowest tertile of CD4 counts as the 'DIR group'.

HIV VL cut offs used by studies to define virological suppression were as follows: 50 copies/ml (seven studies) [309, 313, 352, 353, 358, 362, 363]; 400 copies/ml (four studies) [57, 351, 354, 364]; 500 copies/ml (four studies) [310, 312, 356, 361]; and 1000 copies/ml (four studies) [307, 355, 357, 359]. One study only reported using an 'undetectable' VL [311]. The time period for the definition of virological suppression was as follows: a one off cut off point between 6 months to a year post ART (eight studies) [310-312, 351, 355, 361, 363, 364]; two measurements over one year (two studies) [309, 354]; quarterly measurements for 2 years (two studies) [57, 352, 359], and quarterly measurements throughout the period of follow-up (seven studies) [307, 313, 356-358, 362, 365].

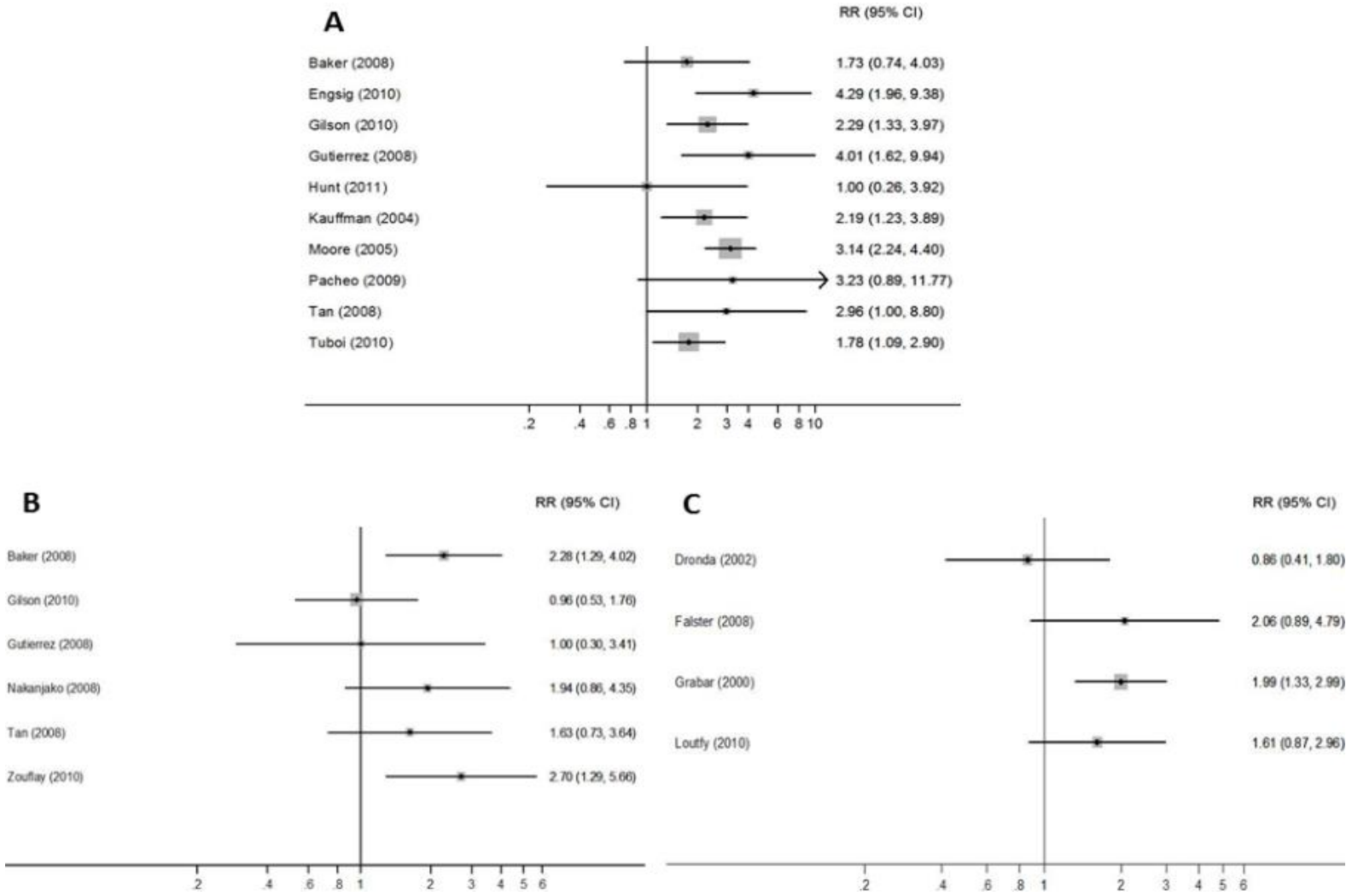
2.4.5 Effect of DIR on risk of mortality

Of the 20 studies including, 10 reported on the risk of mortality in DIR and 10 reported on the incidence or mortality in patients with DIR.

The risk of mortality ranged between 3% to 23% for patients with DIR and 1% to 7% for patients without DIR, over a median follow-up time of 2 years and 3.7 years respectively. Ten studies estimated the effect of DIR on mortality [307, 309-312, 351, 353, 356, 357, 359] (Table 2-4). Risk ratios ranged between 1.00 (95% CI 0.26 – 3.92) and 4.29 (95% CI 1.96 – 9.38). Six of ten studies showed a significantly higher risk of mortality in participants with DIR compared to participants without DIR [309-312, 356, 357] (Fig 2A). Two of these studies reported on the absolute risk of mortality in participants with DIR in resource-limited settings [307, 312], with Tuboi *et al* finding DIR to be significantly associated with an increased risk of death.

Ten studies reported the incidence of mortality in participants with DIR (Table 2-5). Six found the incidence of mortality to be significantly higher in participants with DIR compared to participants without DIR [309-312, 356, 364]. Incidence rate ratios ranged from 1.78 (95% CI 1.09 – 2.90) to 4.01 (95% CI 1.62 – 9.94). One study from a sub-Saharan Africa setting (South Africa) reported rates of mortality and found an IRR of 1.78 (1.09 – 2.90). Various different factors were assessed to investigate whether they influenced the effect of DIR on mortality. No differences in risk of mortality conferred by DIR were found according to DIR definition type, CD4 count cut off or time period post ART initiation.

Figure 2-2 Forest plot showing risk of clinical outcomes for patients with DIR across those studies reporting each outcome



A) Mortality B) AIDS events C) Combined mortality and AIDS events

Table 2-4 . Effect of DIR on rate of clinical outcomes, according to DIR definitions, for 20 studies reporting clinical outcomes

Definition of discordant immune response	First Author	HIV viral load cut off	Number virologic-ally suppress-ed	Number virologic-ally suppress-ed with DIR	Effect of DIR on risk of Mortality			Effect of DIR on risk of AIDS			Effect of DIR on risk of AIDS or mortality		
					DIR number of participants (%)	IR number of participants (%)	Risk ratio (min CI – max CI)	DIR number of participants (%)	IR number of participants (%)	Risk ratio (min CI – max CI)	DIR number of participants (%)	IR number of participants (%)	Risk ratio (min CI – max CI)
Failure to achieve rise in CD4 count of >=50 cells/mm³ at 6 months after ART initiation	MOORE [310]	<500 at 6 months	1084	235	53 (22.6)	61 (7.2)	3.14 (2.24 – 4.40)	NR	NR	NR	NR	NR	NR
	TAN [311]	Undetected at 6 months	320	35	4 (11.4)	11 (3.9)	2.96 (1.00 – 8.80)	6 (17.1)	30 (10.5)	1.63 (0.73 – 3.54)	NR	NR	NR
	TUBOI [312]	<500 at 6 months	6234	1260	23 (4.5)	51 (1.0)	1.78 (1.09 – 2.90)	NR	NR	NR	NR	NR	NR
Failure to achieve rise in CD4 count of >=50 cells/mm³ at 8 months after ART initiation	BAKER [351]	<400 at 8 months	850	149	7 (4.7)	19 (2.7)	1.73 (0.74 – 4.03)	16 (10.7)	33 (4.7)	2.28 (1.29 – 4.02)	NR	NR	NR

Failure to achieve rise in CD4 count of >=100 cells/mm³ at 8 months after ART initiation	GILSON [309]	<50 twice over one year	2584	571	26 (4.6)	24 (2.0)	2.29 (1.33 – 3.97)	15 (2.6)	33 (2.8)	0.96 (0.53 – 1.76)	NR	NR	NR
Failure to achieve rise in CD4 count of >=50 cells/mm³ at 12 months after ART initiation	GRABAR [355]	<1000 at 6 months	1486	387	NR	NR	NR	NR	NR	NR	37 (9.6)	51 (4.8)	1.99 (1.33 – 2.99)
	GUTERRIEZ [356]	<500 throughout follow-up	650	108	8 (7.4)	10 (1.8)	4.01 (1.62 – 9.94)	3 (2.8)	15 (2.8)	1.00 (0.30 -– 3.41)	NR	NR	NR
Failure to achieve rise in CD4 count of >=100 cells/mm³ at 12 months after ART initiation	DRONDA [352]	<50 quarterly for 2 years	288	76	NR	NR	NR	NR	NR	NR	7 (9.2)	40 (18.9)	0.86 (0.41 – 1.80)
	NAKANJAKO [57]	<400 quarterly for 2 years	339	151	NR	NR	NR	14 (9.3)	9 (4.8)	1.94 (0.86 – 4.35)	NR	NR	NR
Failure to achieve rise in CD4 count of >=100 cells/mm³ at 12	NICASTRI [361]	<500 at 12 months	1117	336	NR	NR	NR	NR	NR	NR	Not reported	Not reported	Odds ratio 2.32 (1.36 – 3.95)

months after ART													
Failure to achieve an absolute CD4 count of >=174cells/mm³ at 6 months after ART initiation[1]	HUNT [307]	<1000 throughout follow-up	451	107	3 (2.8)	6 (1.7)	1.00 (0.26 – 3.92)	NR	NR	NR	NR	NR	NR
Failure to achieve an absolute CD4 count of >=350 cells/mm³ at 9 months after ART initiation	FALSTER [354]	<400 twice over one year	292	83	NR	NR	NR	NR	NR	NR	14 (3.5)	35 (2.2)	2.06 (0.89 – 4.79)
Failure to achieve an absolute CD4 count of >=200 cells/mm³ at 12 months after ART	ZOUFALY [313]	<50 throughout follow-up	1085	248	NR	NR	NR	18 (7.3)	11 (1.3)	2.70 (1.29 – 5.66)	NR	NR	NR
	LOUTFY [358]	<50 throughout	2028	404	NR	NR	NR	NR	NR	NR	14 (3.5)	35 (2.2)	1.61 (0.87 – 2.96)
Failure to achieve an absolute CD4 count of >=250	PACHECO [359]	<1000 quarterly for 2 years	147	40	5 (12.5)	4 (3.7)	3.23 (0.89 – 11.77)	NR	NR	NR	NR	NR	NR

cells/mm³ at 22 months after ART initiation													
Failure to achieve an absolute CD4 count of >=200 cells/mm³ at 36 months after ART initiation	ENGSIG [353]	<50 over 3 years	291	55	11 (20)	11 (4.7)	4.29 (1.96 – 9.38)	NR	NR	NR	NR	NR	NR
Failure to achieve an absolute CD4 count of >=500 cells/mm³ at 60 months after ART	KAUFMANN [357]	<1000 over 5 years	293	105	22 (21.0)	18 (9.6)	2.19 (1.23 – 3.89)	NR	NR	NR	NR	NR	NR

NR not reported. DIR discordant immune response IR concordant immune response. VL viral load.

Table 2-5 Effect of DIR on rate of clinical outcomes, according to DIR definitions, for 10 studies reporting incidence data

Definition of discordant immune response ***(time periods are length of time following ART initiation)***	**First Author**	**HIV viral load cut off**	**Number virologically suppressed**	**Number virologically suppressed with DIR**	**Effect of DIR on rate of Mortality**			**Effect of DIR on rate of AIDS**			**Effect of DIR on rate of AIDS or mortality**		
					DIR number of participants (per 100py)	IR number of participants (per 100 py)	Incidence rate ratio (min CI – max CI)	DIR number of participants (per 100 py)	IR number of participants (per 100 py)	Incidence rate ratio (min CI – max CI)	DIR number of participants (per 100 py)	IR number of participants (per 100 py)	Incidence rate ratio (min CI – max CI)
Failure to achieve rise in CD4 count of >=50 cells/mm³ after 6 months	BATISTA [363]	<50 at 6 months	657	102	NR	NR	NR	NR	NR	NR	47 (9.8)	202 (7.8)	1.21 (0.85 – 1.72)
	MOORE [310]	<500 at 6 months	1084	235	53 (5.7)	61 (1.8)	3.2 (3.9 – 12.7)	NR	NR	NR	NR	NR	NR
Failure to achieve an absolute CD4 count of >=200 cells/mm³	TAKUVA [364]	<400 at 6 months	4129	NR	NR	NR	2 (1.44 – 2.79)	NR	NR	1.67 (1.27 – 2.21)	NR	NR	NR

after 6 months													
Failure to achieve rise in CD4 count of >=100 cells/mm³ after 8 months	GILSON [309]	<50 twice over one year	2584	571	26 (3.5)	24 (0.5)	7.00 (3.9 – 12.7)	15 (2.0)	33 (0.7)	2.9 (1.4 – 5.4)	NR	NR	NR
Failure to achieve rise in CD4 count of >=50 cells/mm³ after 12 months	GRABAR [355]	<1000 at 6 months	1486	387	NR	NR	NR	NR	NR	NR	37 (6.6)	51 (1.8)	3.7 (2.3 – 5.7)
Failure to achieve an absolute CD4 count of >=200 cells/mm³ after 12 months	ZOUFALY [313]	<50 throughout	1085	248	18 (4.4)	11 (1.6)	2.8 (1.2 – 6.4)	NR	NR	NR	NR	NR	NR
	LOUTFY [358]	<50 throughout	2028	404	NR	NR	NR	NR	NR	NR	14 (1.1)	35 (0.8)	1.4 (0.7 – 2.6)

Failure to achieve an absolute CD4 count of >=250 cells/mm³ after 22 months	PACHECO [359]	<1000 quarterly for 2 years	147	40	5 (2.4)	4 (0.7)	3.2 (0.70 – 16.4)	NR	NR	NR	NR	NR	NR
Failure to achieve an absolute CD4 count of >=200 cells/mm³ after 36 months	ENGSIG [353]	<50 over 3 years	291	55	26 (3.5)	24 (0.5)	4.4 (1.7 – 11.3)	NR	NR	NR	NR	NR	NR
Failure to achieve an absolute CD4 count of >=200 cells/mm³ after 6 months	TAIWO [362]	<50 biannually throughout	NR	NR	NR	NR	5.96 (0.40 – 87.8)	NR	NR	HR 22.8 (1.89 – 275)	NR	NR	HR 10.7 (1.65 – 70)

NR not reported. DIR discordant immune response IR concordant immune response. VL viral load. py person years HR hazard ratio

2.4.6 Effect of DIR on risk of AIDS and serious non-AIDS events

Six studies reported AIDS events [57, 309, 311, 313, 351, 356]. The risk ratio for associations between DIR and AIDS events ranged from 0.96 (95% CI 0.53 – 1.76) to 2.70 (95% CI 1.29 – 5.66) (Fig 2B). One of these reported AIDS events in a low resource setting (Uganda) with a risk ratio of 1.94 (0.86 – 4.35). Five studies reported combined AIDS events or mortality [352, 354, 355, 358, 361] and risk ratios ranged from 0.86 (95% CI 0.41 – 1.80) to 2.06 (95% CI 0.89 – 4.79) (Fig 2C). One study from a low resource setting (Senegal) reported an incidence rate ratio of 1.21 (0.85 – 1.72).

Four studies detailed AIDS events. The most commonly reported pathologies were oesophageal candidiasis, tuberculosis, AIDS related cancers, *pneumocystis jirovecii* and bacterial pneumonia [57, 351, 356, 360]. Only Baker *et al* included serious non-AIDS events [351], reporting events in eight of 143 patients (5.6%) with DIR compared to 31 of 671 patients (4.6%) without.

2.5 Discussion

The main finding of this review was that we found definitions used to categorise DIR varied widely, with 14 different definitions used in the 20 included studies. Although meta-analysis was not performed due to heterogeneity in definitions as well as length of follow-up, mortality rates remain substantially and significantly elevated in patients with DIR in studies that reported rates adjusted for time. The relationship between DIR and AIDS is less clear and may be complicated by challenges in diagnosing or reporting AIDS conditions. Alternatively, other conditions such as serious non-AIDS events may be contributing to mortality and this warrants further investigation.

Heterogeneity in DIR definitions also greatly limits the ability to draw conclusions about clinical burden in this patient cohort. This chapter has synthesised existing data and suggested the definition for DIR to be a rise of less than 50 cells/uL at 6 months following ART initiation in those who have achieved virological suppression but with a CD4 count of less than 350 cells/uL. This provides a starting point for the development of consensus within the field. For the majority of included studies, mortality in patients with DIR was two to three times higher than in those with a satisfactory immune response. To our knowledge, this is the first review to systematically examine the fate of adults with DIR. Two further important gaps in the literature were identified: the large majority of current data reports on cohorts from high income countries; and only one study reported on the burden of serious non-AIDS events. Both the clinical burden of DIR in low income settings and the

global burden of serious non-AIDS events remain unclear. It is not possible to draw comparisons between the risks associated with DIR in low resource to high resource settings with the current literature.

In order to address the issues identified here around heterogeneity of definitions, we advocate the use of a standard definition. To define DIR based on a failure to achieve a rise in CD4 from baseline is more reflective of the amount of time spent at a lower CD4 count, which is an important predictor of poor outcomes [317]. In contrast, an absolute CD4 count at a given time point may only tell us about that point in time, when other factors such as co-existing infections may be affecting the CD4 count. The expected rate of CD4 reconstitution following ART initiation is 20 to 30 cells per month in the first 6 months and then 5 to 10 cells per month between 6 months and 24 months [38, 366]. Therefore, when choosing a time point to measure DIR, we believe that 6 months after ART initiation is logical. Further studies might also consider whether time taken to get to a pre-specified CD4 count may be more representative of total time spent below that value.

Many studies included in this review based their definition of DIR on a failure to achieve a rise of 50 cells/uL at 6 months' post ART initiation. Whilst this is a relatively strict CD4 cut off, these studies still reported a high proportion of virologically-suppressed patients with DIR. We would therefore recommend defining DIR as a rise of less than 50 cells/uL at 6 months following ART initiation in patients who have achieved virological suppression. This definition has the benefit of identifying a high risk group of patients early on in the course of their ART management to allow for increased benefit of any potential intervention. It is logical that this definition would only apply to those commencing ART with a CD4 <350 cells/uL so as not to over diagnose DIR in a population starting with higher CD4 counts. The heterogeneity in definitions for DIR and outcome measures means that it is not currently possible to meaningfully compare the utility of definitions to predict clinical outcomes. We recommend further studies to clinically validate a standardised definition.

This review should be interpreted in the light of several limitations. Firstly, the majority of studies were carried out using data collected from ongoing multicentre cohort studies, meaning cohorts are likely to be highly selected in terms of laboratory monitoring and attending follow-up visits. This limits the generalizability of the studies, and may mean that the risk of adverse clinical outcomes in individuals with DIR could be underestimated. Secondly, the HIV viral load limit defining virological suppression varied across studies. However, it remains unclear whether differences in viral load below 1000 copies/ml are biologically significant [367]. Lastly, individual studies did not distinguish

between early mortality in patients starting ART with advanced immunosuppression and long term mortality due to poor immune reconstitution. This could be addressed in future studies.

There are currently no effective therapeutic options to reduce the excess mortality associated with DIR and no difference has been demonstrated with newer ARVs including tenofovir compared to thymidine analogues. One approach under evaluation is to target underlying drivers of immune activation and inflammation. The addition of Raltegravir to standard two class regimes at ART initiation has the aim of decreasing viral set point but as yet only two small studies have shown any effect on immune responses [368, 369]. Similarly, a recent trial with valganciclovir to tackle ongoing CMV replication failed to show any improvement in CD4 count [370]. Although probiotics can improve the systemic pro-inflammatory profile, there is no evidence that this can improve CD4 counts [371]. To address generalised inflammation, anti-inflammatory agents such as statins and anti-rheumatic agents have been tested [372, 373]. Whilst statins reduced peripheral immune cell activation, there is no evidence that they can improve CD4 T cell count. Studies investigating the role of quinolones in reducing HIV related immune activation have shown only small decreases in inflammatory markers [374]. Immunomodulatoy agents such as IL-2 have shown limited success [375, 376] and current focus is being placed on IL-7 therapy [377, 378] with several ongoing trials in progress. Lastly, agents aimed at stimulating thymic output have also been tested in early studies [379, 380].

Practical management options may be more accessible in the short term. Standardised guidelines could recommend continuation of prophylactic therapies such as co-trimoxazole and isoniazid for patients with DIR, or could prompt investigation for subclinical opportunistic infections such as tuberculosis and CMV. Although prevention of DIR through early diagnosis of HIV infection and prompt treatment with ART is likely the most effective intervention [381-384], a large proportion of patients worldwide continue to present with advanced HIV infection [385, 386].

This systematic review highlights that a wide range of definitions have been used to characterise clinical outcomes in patients with DIR. These patients are at an increased risk of mortality and are in need of special attention, including integration into HIV clinical trials. We have suggested a definition for DIR based on the limited available data in order to help begin the process of arriving at a consensus definition that could be used to guide clinical care and in future research. We recommend that further studies validate this definition for DIR to aid the development of consensus guidelines.

3 CHAPTER 3: GENERAL STUDY METHODS

3.1 Recruitment of clinical cohort

3.1.1 Study design

The SHIELD study is a cohort study of HIV infected adults with advanced immunosuppression and HIV uninfected healthy volunteers. A cross sectional analysis of SHIELD enrolment data collected at 2 weeks post ART initiation answers objectives 2 – 5 (see Chapter 1). An analysis of longitudinal data up to 44 weeks post SHIELD enrolment answers objectives 6 and 7. Figure 3-1 gives an overview of how the SHIELD study was designed to answer each of the objectives.

3.1.2 Study site and patient management systems

3.1.2.1 QECH

Queen Elizabeth Central Hospital (QECH) is a tertiary referral hospital set within Malawi's second city, Blantyre. It is a receiving hospital for all district health clinics within urban Blantyre, and serves as a tertiary referral centre for the Southern Malawi region. QECH is a major teaching hospital affiliated with the University of Malawi, College of Medicine. Public health care in Malawi is free but resources within the public system are limited and many private fee paying facilities exist. ART is free and is supported by external funders, such as PEPFAR, making its supply reliable. The SHIELD study was conducted in three main locations within Queen Elizabeth Central Hospital: the adult medical inpatients wards, the ART clinic and the voluntary counselling and testing (VCT) clinic (Figure 3-2).

Figure 3-1 Overview of SHIELD study design and objectives

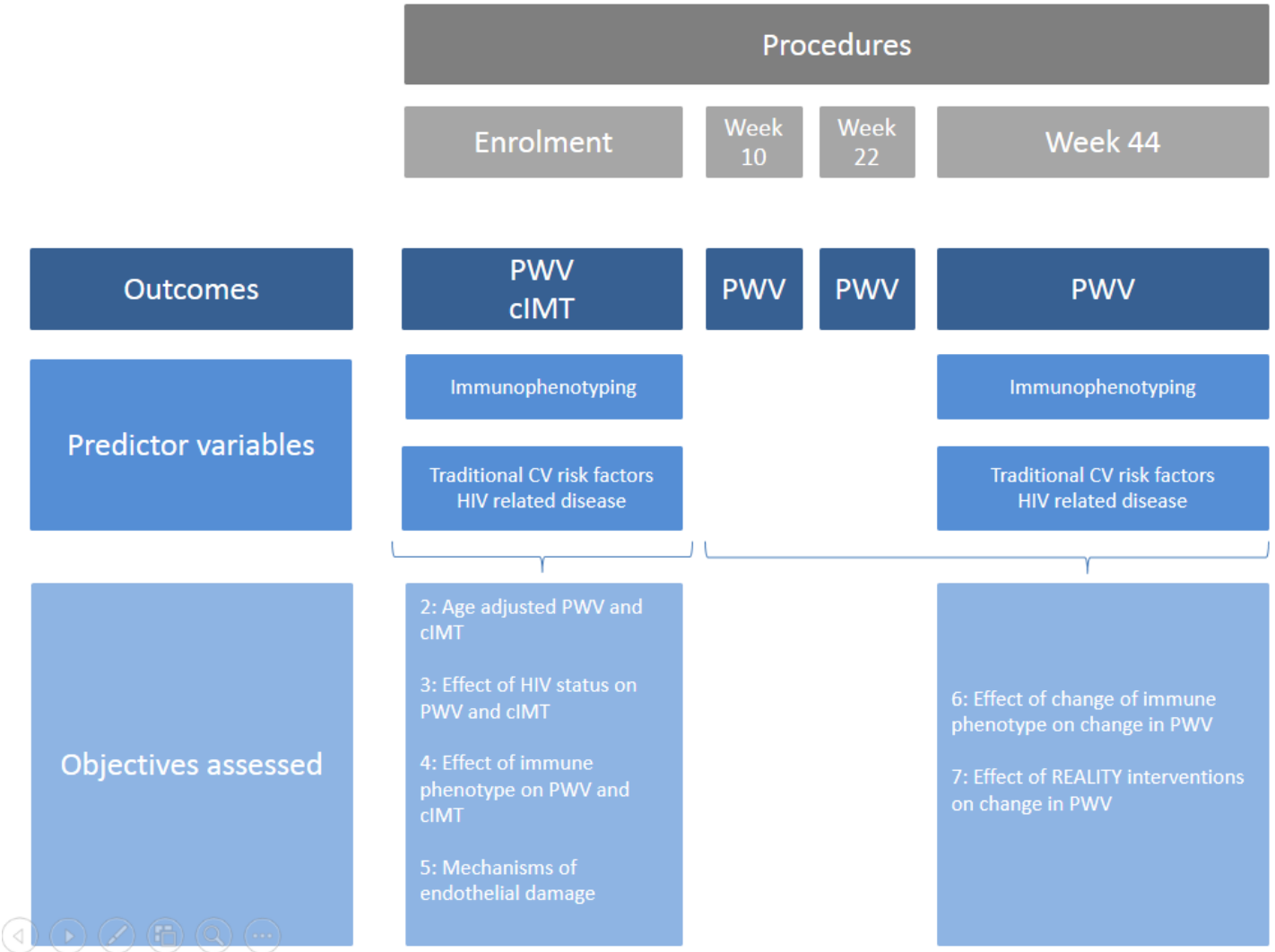


Figure 3-2 Map of QECH grounds, with locations of SHIELD study sites[14]

[14] From 387. https://www.google.co.uk/maps/place/Queen+Elizabeth+Central+Hospital, B., +Malawi/@-15.8030426,35.0168894,16z/data=!3m1!4b1!4m2!3m1!1s0x18d845947a5ca71d:0x143328d65dc6a84c. [cited 2016 22nd January]; Map of QECH].

3.1.2.2 Adult medical wards

Adult inpatients are admitted to medical wards through referrals from the Accident, Emergency and Trauma Centre (AETC). There are three general medical wards in QECH: Male ward, Female ward and TB ward. Together these wards care for approximately 220 patients. The medical take has an admission rate of around 10 to 20 patients per day, with one published study recording 4699 medical admissions in 2010 [388]. Approximately 70% of adult medical inpatients have HIV infection [389], with approximately one third of those not yet on ART, one third in the first 6 months of ART and one third on established ART (personal communication, Ingrid Peterson).

Routine HIV testing was not established on the wards prior to the initiation of the SHIELD and REALITY studies. HIV counsellors were employed by the studies to provide this service to the wards. As a result, the proportion of medical patients leaving hospital with a HIV diagnosis improved substantially. Patients with a new diagnosis of HIV are referred to the ART clinic as ART cannot be initiated or dispensed on the wards.

3.1.2.3 VCT clinic

The VCT clinic provides HIV testing and counselling for people requesting voluntary routine testing and also for some patients within QECH who present unwell and seek diagnostic services. In the last quarter of 2015, the VCT clinic tested 2181 new clients; 593 (27%) positive and 1580 negative (72%). All HIV counsellors are government registered through a two-week residential course and maintain skills through quarterly regional meetings. HIV tests are carried out strictly according to national guidance. Patients are counselled and then tested using the Determine HIV rapid test. A positive result using the Determine kit requires confirmation with a second test using the Unigold HIV rapid test. If the tests are discordant a result of inconclusive is given and clients are encouraged to reattend for testing after two weeks. The results of the HIV test are recorded in the patient's personal 'health passport', then stamped and signed. Yearly tests are recommended for those testing negative. For those testing positive, a referral is made to the ART clinic for further assessment. Figure 3-3 gives an overview of the HIV testing process through to ART initiation for adults at QECH.

3.1.2.4 ART clinic

Since its conception in 2004, the QECH ART clinic has initiated 25,000 patients on ART. It has three full time ART clinicians, four nurses trained in dispensing ART, and one physician from the department of medicine for each clinic session. At QECH ART clinic, over 10,000 patients are currently receiving ART and an average quarter sees 10 deaths and 35 defaults. The clinic is also a secondary referral centre, supporting 13 peripheral ART clinics. The ART clinic supports ongoing

research studies. Both REALITY and SHIELD studies had their own clinic rooms set within the ART clinic.

Every patient admitted to the clinic (whether for ART initiation or for pre-ART care) is given a mastercard and recorded onto an electronic data capture system. Essential information including height, weight, WHO stage, lab results and ART history are included for each patient. Paper records with this information are also filed within the clinic. Visits are recorded in the patient's health passport and when ART is dispensed or a non-ART visit occurs, essential information is summarised on a printed sticker which is also placed in the health passport.

A general overview of the ART initiation process is given in Figure 3-3. Every adult who tests positive for HIV either on the medical wards or at VCT is referred to QECH ART clinic. They are assessed by a clinician and given a WHO stage. Patients deemed as stage 3 or 4 automatically qualify for ART, without the need for CD4 measurement. Those at stage one or two have a CD4 count and go on to initiate ART if it is less than 500 copies/mm^3. Those with a CD4 of greater or equal to 500 copies/mm^3 are kept under surveillance at the clinic in 'pre-ART' care. Group counselling is mandatory for all patients who are eligible to start ART. Group counselling sessions are carried out twice a week at the ART clinic and patients must attend with a 'guardian', who also undergoes counselling. After successful completion of counselling, the patient reattends to see a clinician and starts ART according to national guidelines[390].

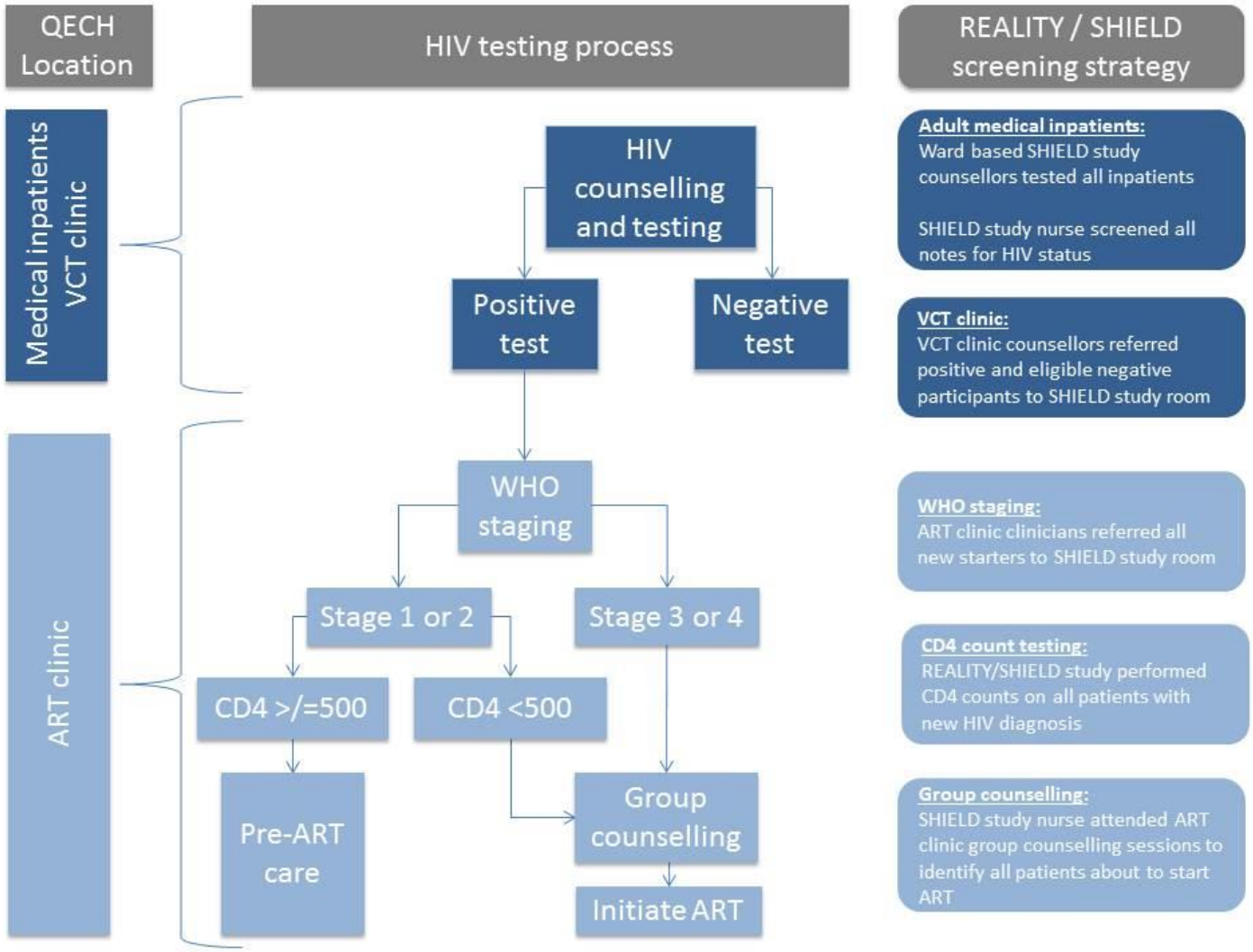
All patients with HIV infection are started on co-trimoxazole (960mg once daily formulation). As of 2013, the first line ART regime in Malawi has been Tenofovir 300mg, Lamivudine 300mg and Efavirenz 600mg (one co-formulation tablet once daily). Patients are usually given a two week course to begin, so that they can be reviewed early by a clinician for side effects. Prescriptions are then given monthly for the first three months and, depending on availability of drugs, up to 3 monthly prescriptions thereafter. Patients who present with symptoms are investigated and managed either by ART clinic staff or are referred to AETC at QECH.

3.1.2.5 MLW

MLW was established in 1995 and falls under the auspices of the College of Medicine, University of Malawi. It is partnered with the University of Liverpool, Liverpool School of Tropical Medicine and the University of Glasgow. These links are managed through the Liverpool-Glasgow-Wellcome centre for Global Health Research. MLW supports researchers from a broad range of research fields. MLW works closely with QECH and provides clinical services such as blood culture and CSF analysis. Clinical researchers working at MLW support clinical services in the hospital by undertaking regular ward rounds and clinics.

Clinical fellows at MLW are responsible for project managing their own studies, including line managing staff. They are supported by 12 operational departments including HR, finance and supply, data and IT.

Figure 3-3 Overview of HIV testing process through to ART initiation at QECH and REALITY/SHIELD screening strategy



3.1.3 Study populations

3.1.3.1 Overview of SHIELD study populations

The SHIELD study is comprised of three patient cohorts: HIV-infected patients co-recruited from REALITY, HIV-infected patients not recruited to REALITY and HIV-uninfected participants recruited from the VCT clinic. HIV infected adults were recruited exclusively to the SHIELD study from the REALITY trial between January 2014 and May 2015. After enrolment to REALITY was completed, further HIV infected participants were recruited into SHIELD directly from the standard ART system from May until August 2015. Great efforts were employed to ensure integration of REALITY and SHIELD studies, especially with regards to screening for identification of eligible patients. REALITY patients were identified from within the standard QECH care pathway for HIV infected patients. This same strategy was continued after REALITY completed enrolment to recruit the additional HIV infected non-REALITY SHIELD patients. The REALITY HIV infected cohort was subject to additional eligibility criteria that were not required for the non-REALITY HIV infected cohort (see section 3.1.3.2.4).

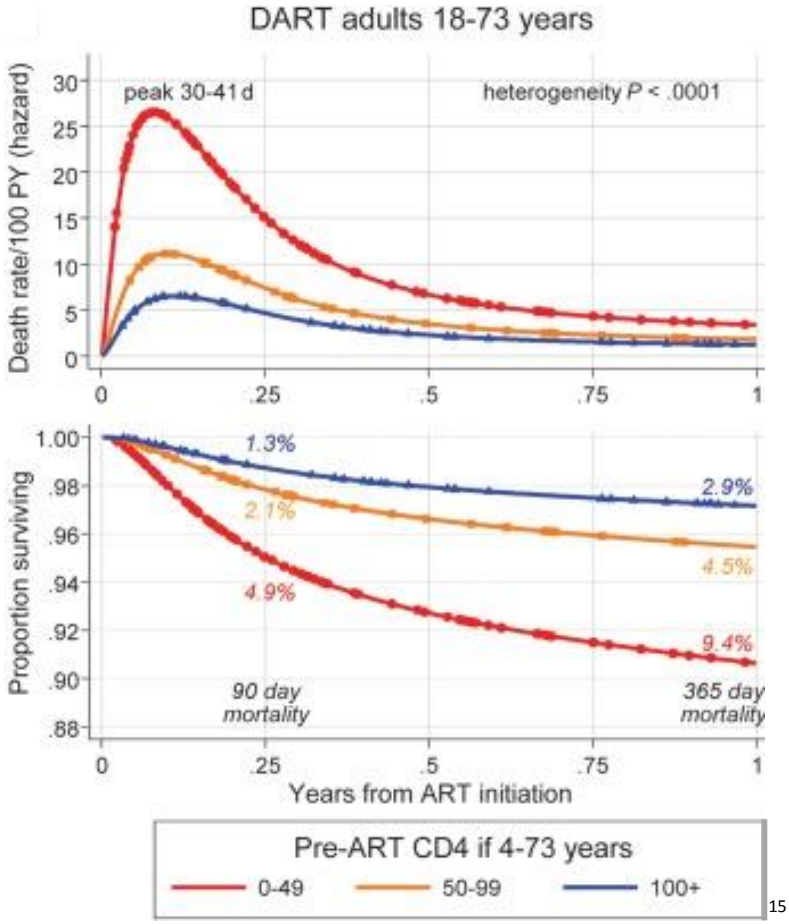
3.1.3.2 REALITY study

The REALITY study was conducted to assess potential interventions to decrease early mortality following ART initiation in adults and children with a CD4 count of less than 100 cells/mm^3. The SHIELD study aimed to assess the relationship between immune activation and cardiovascular risk in this same population of patients with CD4 count less than 100 cells/mm^3. Therefore, the SHIELD study was different from the REALITY study in that REALITY did not assess either immune activation nor cardiovascular risk.

3.1.3.2.1 Background and Aims

Analysis from the DART trial showed a high 3 month mortality in patients starting ART with a CD4 count of less than 100 cells/mm^3 (Figure 3-4)[391]. The primary aim of the REALITY study was to test interventions aimed at decreasing 3-month mortality in patients starting ART with a CD4 less than 100 cells/mm^3. Secondary objectives fall into three domains and aimed to identify: the cost-effectiveness of interventions to reduce early mortality; the mechanisms of action of effective interventions; and the acceptability of interventions at a community level. Analysis of underlying mechanisms of action include measurement of HIV viral load and resistance, molecular diagnostics, measures of immune activation, immune responses to pathogens, microbial translocation and enteropathy and body composition (REALITY trial number NCT01825031, www.clinicaltrials.gov).

Figure 3-4 Mortality following ART initiation according to nadir CD4 count in the DART cohort



[15]

3.1.3.2.2 Interventions

The REALITY trial assessed three potential interventions to reduce 3-month mortality in patients starting ART with CD4 less than 100 cells/mm^3.

Early mortality may be related to direct effects of the HIV virus and a more rapid reduction in viral load may help to reduce mortality. Integrase inhibitors confer an additional decrease in viral load at ART initiation when combined with the standard two class regime. Arm A therefore added Raltegravir (400mg twice daily) as a third ART class for 3 months.

Arm B is the addition of augmented prophylaxis against opportunistic infections, bacterial infections and helminths. Co-infections have been shown to be a major cause of early mortality following ART initiation. The REALITY study tested an anti-infective package which includes co-trimoxazole (960mg

[15] From 391. Walker, A.S., et al., *Mortality in the Year Following Antiretroviral Therapy Initiation in HIV-Infected Adults and Children in Uganda and Zimbabwe.* Clin Infect Dis, 2012. **55**(12): p. 1707-18.

once daily), isoniazid (300mg once daily with pyridoxine 25mg once daily) and fluconazole (100mg once daily) for 3 months; azithromycin (500mg once daily) for 5 days; and a one off dose of albendazole (400mg).

Individuals with low BMI have higher mortality on ART and this is thought to be related to a catabolic state which ensues following ART initiation. Ready to Use Therapeutic Food (RUTF) is currently recommended only for those with the lowest BMI (<18.5). Arm C randomised patients to receive Ready to Use Supplementary Food (RUSF), which can be used in any patient starting ART with a CD4 <100 cells/mm^3 regardless of BMI.

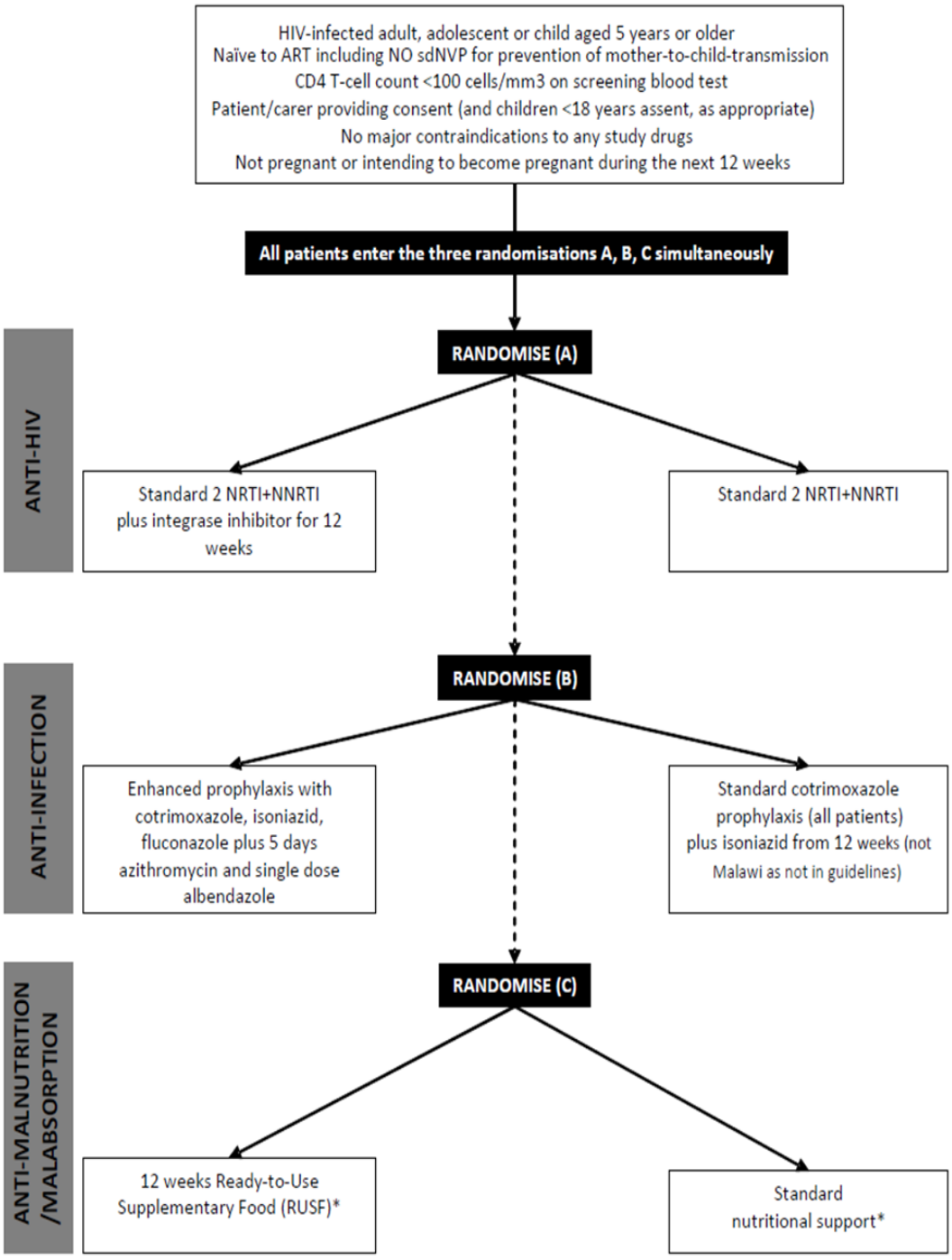
3.1.3.2.3 Study design

The REALITY trial was an open label randomised control trial with a factorial design. Patients were randomised simultaneously to all three arms (Figure 3-5). Patients may have been randomised to all intervention groups, no intervention groups, or any combination of these. For each arm, patients not randomised to an intervention received the standard of care. QECH, Blantyre, Malawi is one of 8 REALITY sites across low income sub-Saharan Africa. The sample size was 1800 participants over 18 months across the 8 sites totalling 600 children and 1200 adults. 450 participants were initially projected for the Blantyre site.

3.1.3.2.4 Study population

Patients were eligible for inclusion in the REALITY trial if they met the following criteria: aged 5 years or older, documented HIV infection, ART naïve, CD T cell count <100 cells/mm^3 at REALITY screening, results of haematology and biochemistry tests available, no contra-indication to planned ART according to national guidelines and provision of informed consent. Patients were excluded if they met any of the following criteria: contraindications to any proposed drug; pregnant, breastfeeding or intending to become pregnant within 3 months of starting ART; ever received single dose Nevirapine.

Figure 3-5 Schematic of REALITY trial randomisation process



3.1.3.2.5 Follow-up and ascertainment of outcomes

The schedule of follow-up for the REALITY trial is shown in Figure 3-6. Patients were brought back at week 2 to monitor clinical progress. In addition to the scheduled visits, patients were also encouraged to attend in the event of new signs or symptoms.

Figure 3-6 Overview of REALITY study schedule and procedures

	EVENTS	WEEKS IN TRIAL										
	Doctor/Nurse visit* ■ Nurse visit □	Screening ‡	0	(2)	4	8	12	18	24	36	48	Any acute event (9)
	Drug supply to next visit (4/6/12 weeks)*		X		X	X	X	X	X	X	X	
	Isoniazid prophylaxis may be given (irrespective of randomisation)†						X	X	X	X	X	
	Routinely de-worm all children (irrespective of randomisation)								X		X	
ALL PARTICIPANTS	Patient information sheet and consent for screening	X										
	Symptom screen for active tuberculosis	X	X									
	Informed consent for trial enrolment		X									
	History & Physical (1)	X	X		X		X		X	X	X	
	Grip strength (measured by handgrip dynamometer)		X		X		X				X	
	Symptom check list and EQ5D		X	X	X	X	X	X	X	X	X	
	Peripheral neuropathy assessment		X								X	
	Socioeconomic questionnaire		X								X	
	Acceptability assessment (2)				X		X		X			
	Adherence assessment (3)				X	X	X	X	X	X	X	
	Pregnancy Test (4)	X			(X)		(X)		X		X	
	Haematology 1.5-2mls (5)	X			X		X		X		X	(X)
	Biochemistry 1.5mls (6)	X			X						X	(X)
	Lymphocyte Subsets (same draw as haematology except w0) (7)	X	X		X		X		X		X	
	Plasma storage 3.5-7ml (8)	X	X		X		X		X	X	X	
	Plasma storage 2-4ml (9)											X
	Store smear/any organism isolated from standard culture (9)											X

Clinical events were managed by the REALITY trial team using resources available within the QECH public service and, where necessary, private resources. Examples of private services used include pathology services for lymph node tissue examination and purchasing of drugs such as amphotericin. Any clinical event was recorded and reported on a separate case report form.

3.1.3.3 SHIELD patient recruitment

3.1.3.4 Co-recruitment of HIV infected patients from REALITY study

Due to the procedural burden during the REALITY enrolment visit, SHIELD enrolment was carried out at the week 2 REALITY visit. Potential REALITY participants were informed about the SHIELD study and provided with information during the screening process so that they had time to consider whether they wanted to participate or not. They were then approached during the week 2 REALITY visit to ascertain whether they were interested in taking part. If the potential participant was agreeable, they underwent a separate informed consent process with SHIELD study staff. All REALITY participants aged 18 or older were eligible for recruitment to the SHIELD study. REALITY participants were excluded from taking part in the SHIELD study if the patient, guardian or clinician felt that the patient was too unwell to take part in a second study or if the patient was clinically severely anaemic (due to additional blood draws for SHIELD).

3.1.3.4.1 HIV infected participants recruited outside REALITY

The same screening process was used to identify potential REALITY participants and HIV infected SHIELD participants not recruited to REALITY. However, some inclusion and exclusion criteria could be removed for the HIV infected non-REALITY participants because they were not participating in a clinical trial. Haematology or biochemistry results were no longer required prior to enrolment and there were no exclusions based on contra-indications to drugs or breastfeeding. The inclusion criteria for this group were: aged 18 years or older; documented HIV infection; ART naïve; CD T cell count <100 cells/mm^3 at screening; no contra-indication to planned ART according to national guidelines; and provision of informed consent. Patients were now only excluded if they were pregnant at the time of enrolment. Pregnant women were excluded due to possible perturbations of immune phenotype during pregnancy as well as challenges with longitudinal measurements of PWV, given that it is measured over the abdomen.

3.1.3.4.2 HIV uninfected participants

HIV uninfected participants were identified from the voluntary counselling and testing clinic. Clients were eligible if they had a documented negative HIV test within the past two months but were excluded if they had any symptoms or signs of an acute infection within the previous past two weeks. Patients referred for HIV testing through the adjacent STI clinic were excluded. HIV

uninfected participants were not age matched to HIV infected participants, but an early look at the demographics showed that HIV infected participants were almost exclusively over 30 years old so an additional inclusion criterion of 30 years or older was imposed.

Following analysis of baseline immunophenotyping data showing high immune activation in the HIV uninfected Malawian comparison group, ethical approval was sought to recruit 10 non-Malawian HIV uninfected healthy volunteers to provide normative data for immune activation studies. Volunteers were eligible for this arm of the study if they were over 18 and had lived in a high income setting until at least the age of leaving secondary school. They were excluded if they had any signs or symptoms of an acute infection within the past two weeks. This cohort was recruited by placing posters within the hospital and MLW. Following informed consent, participants underwent HIV testing at the VCT clinic and underwent a blood draw to test for immune activation parameters.

3.1.4 Study procedures

3.1.4.1 SHIELD study schedule and procedures

A summary of the SHIELD study schedule is given in Figure 3-7.

3.1.4.1.1 Informed consent

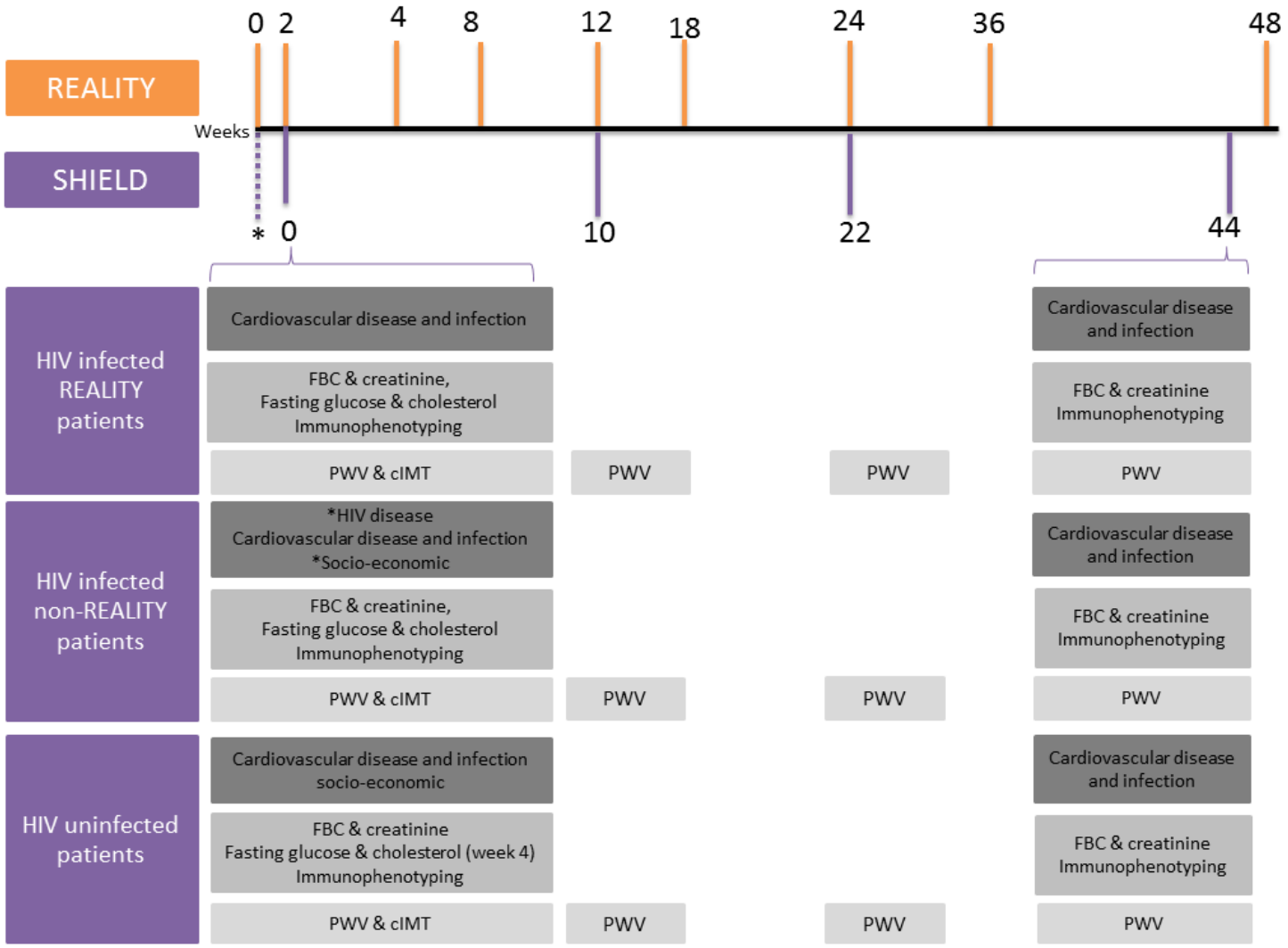
After having been identified as eligible during the screening process, participants were invited to provide informed consent. Patients were counselled in any available ART clinic room, away from the study clinic room. Patients who had not already been able to review the information leaflet were provided with time to do so. Where both the patient and guardian were unable to read, the information leaflet was read to them by an independent member of the ART clinic staff who then also co-signed the consent form. Finger prints were taken for those unable to sign the consent form. Patients were encouraged to ask questions and their understanding of what was involved was checked.

An amendment was made during the study to request additional consent for the export of patient samples and to approach participants for future studies. For this, participants were offered an additional information leaflet and consent form to review and sign - if they felt happy to - during their routine clinic visits.

3.1.4.1.2 Enrolment

Three types of procedure were carried out at SHIELD enrolment and exit visits: questionnaires, blood draw and cardiovascular tests. Each patient had the following questionnaires administered at enrolment: cardiovascular disease and infection history, HIV disease and socio-economic.

Figure 3-7 Overview of SHIELD study schedule and procedures



The HIV disease and socio-economic forms were carried out as part of REALITY enrolment and so were not repeated during the SHIELD enrolment visit for these participants. To ensure time points of questionnaire delivery were comparable between HIV infected REALITY and non-REALITY participants, the HIV disease and socio-economic questionnaires for non-REALITY HIV infected participants were carried out when they were initiating ART (as would have been the case for REALITY participants). Cardiovascular disease and infection questionnaires were administered two weeks following ART initiation (as was the case for REALITY participants). The HIV disease questionnaire was not relevant for HIV uninfected participants, who had cardiovascular disease and infection as well as socio-economic questionnaires administered at enrolment. HIV infected participants had fasting bloods for cholesterol and glucose measured during the 2 week visit if they attended fasting (defined as no food or drink other than water in the previous 6 hours). If on the day of the enrolment visit patients were not fasted, they were advised to come fasting for the next visit in two weeks' time and fasting blood was tested for cholesterol and glucose at that later visit. Unfasted HIV uninfected participants were advised to return the next day. Otherwise all enrolment bloods reported for HIV infected patients (including immunophenotyping) were carried out 2 weeks following ART initiation. PWV and cIMT were carried out in the clinic during the patient visit.

3.1.4.1.3 PWV and Exit visits

During the week 10 and 22 follow-up visits only PWV was recorded. There were no questionnaires administered and no blood was drawn. The socio-economic and HIV disease questionnaires were not repeated during the SHIELD exit visit. The cardiovascular disease and infection questionnaire was modified to pick-up any new diagnoses that the patients had received during the time they had been in the study.

3.1.4.1.4 Handling of missed appointments

Tracing was initiated for any participant who was more than one week overdue for their appointment. Firstly, attempts were made to contact the participant by telephone. If unsuccessful, the field-worker would carry out a visit to the participant's home. Any participants who withdrew or who were untraceable were discussed in the weekly team meeting to learn appropriate lessons from this. A loss to follow-up form was completed for any participant who was no longer able to attend visits. This could have been because of death, participant withdrawal or an inability to trace the participant. In the case of a death, hospital records and patient health passports were reviewed where possible to provide a cause of death. For participant withdrawal, the reason was recorded. In

cases where the study team was unable to trace the participant, the efforts that were made to trace the participant were recorded.

3.1.5 Study timescale

The SHIELD study was planned to recruit from January 2014 until March 2015, with 44 weeks of follow-up running until January 2016. However, due to initial challenges with REALITY recruitment rates, SHIELD enrolment continued from January 2014 until August 2015 and follow-up ran until March 2016. For participants recruited between June and August 2015, follow-up was truncated by a maximum of 14 weeks.

3.2 Outcome measures – physiological measurements of endothelial damage

3.2.1.1 Calculation of PWV

There are three methods for measuring PWV. Firstly, it can be measured using pressure and flow characteristics. The Moens-Kortweg equation calculates PWV based on using elastic modulus, viscosity, and vessel diameter. It assumes that $PWV=\sqrt{(Eh/2\rho R)}$ where E is Young's modulus of the arterial wall, h is wall thickness, R is arterial radius at the end of diastole and ρ is blood density [222]. The Bramwell & Hill equation uses some additional assumptions to modify the original Moens-Kortweg equation for the calculation of PWV, specifically relating PWV to distensibility: $PWV=\sqrt{(\Delta PV/\Delta V\rho)} = \sqrt{(1/\rho D)}$ where $\Delta PV/\Delta V\rho$ is the relative volume elasticity of vessel segment, ρ is the density of blood and D is distensibility [392]. The Bramwell Hill equation can be used to calculate PWV through ultrasound methods to establish the required pressure and flow characteristics but this can be quite cumbersome.

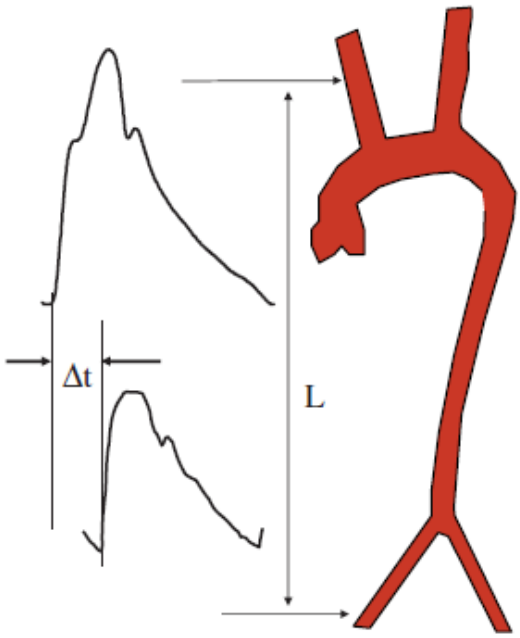
Secondly, PWV can be measured by calculating the velocity of the forward wave. Technically, PWV is the distance travelled by a wave divided by the time for the wave to travel that distance ($\Delta x/\Delta t$). However, because this assumes that the forward travelling wave is constant and there are no reflections, to measure PWV based on the time for a wave to get from one point to another, the measurement would also have to be adjusted for flow characteristics and for wave reflections. This would also require ultrasound methods.

Lastly, these challenges can be overcome by measuring two pulse waves simultaneously. By using the 'foot to foot' technique, the measurement is made at a time when there are minimal reflected waves (Figure 3-8). This approach makes the measurement of PWV much simpler and can be carried out using non-invasive approaches such as mechanical tonometers or pulse detection devices [218].

However, the measurement of PWV is open to errors and most notably measurement of the length between two points [393]. Some evidence also suggests that PWV measurement may be affected by plasma viscosity [394] .

Figure 3-8 Calculation of Pulse Wave Velocity[16]

$$PWV = \frac{\Delta L}{\Delta t} = \sqrt{\frac{dP}{\rho .} \cdot \frac{V}{dV}}$$



3.2.1.2 PWV measurement

3.2.1.2.1 Regions

PWV can be measured over several different sites including carotid-femoral, brachial-ankle, carotid-radial. Different sites provide different types of information, for example, carotid-radial gives information about the more peripheral muscular arteries, whereas carotid femoral reflects the large elastic arteries. PWV values also differ between sites and brachial-ankle PWV may be around 20% higher than carotid-femoral PWV [395]. Overall, strong evidence now exists to support the measurement of PWV along the aorta as the gold standard method for determining arterial stiffness as a biomarker for cardiovascular disease. Firstly, it is of major clinical relevance as the arteries branching from the aorta are responsible for the main cardiovascular complications (carotid, coronary, and renal vessels). It is also the region most predictive of cardiovascular events. Lastly, the

[16] From [18]

change in PWV with age over the aorta is larger than with other arteries and is therefore more discriminatory [396].

3.2.1.2.2 Length

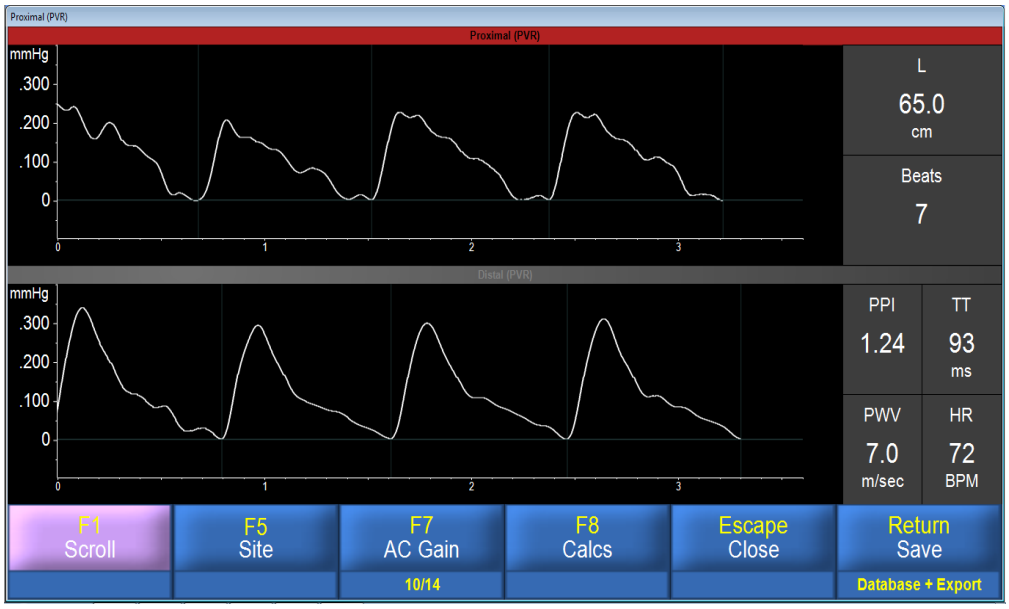
The measurement of the distance for calculation of transit time is the major source of error in measurement of PWV. Various different methods of measuring distance have been compared and associated with clinical outcomes, but validated cut offs differ significantly according to how the distance measured. This limits comparability across studies. The expert consensus document on measurement of aortic stiffness was updated in 2012 by the European Society of Hypertension Working Group on Vascular Structure and Function and the European Network for Noninvasive Investigation of Large Arteries [223, 224] to specify guidance on measurement of length. They proposed that the most accurate measurement method was to calculate the distance from the carotid artery to femoral artery and multiply by 0.8. Recently, various methods for calculating length in PWV were compared with invasive techniques and found that the proposed 'direct' measurement (carotid to femoral length x0.8) overestimated the invasive aortic stiffness measurement by 1.7m/s in older patients but showed good agreement in patients aged between 50 and 70 years [397]. Whichever technique is used, it has been highlighted that it is important that the approach used to measure the length for the PWV calculation is reported [398].

3.2.1.2.3 Vicorder

For the SHIELD study we chose to use the Vicorder system due to evidence showing that it was possible to train operatives with limited experience, because of its low intra- and inter-operator variability, and because of the ease of use in clinical settings making it preferable for use in a low income setting [399, 400]. The Vicorder is an automatic system which uses oscillometry to simultaneously detect the carotid and femoral pulse. The equipment consists of the Vicorder hardware system (Skidmore Medical) which has two blood pressure measurement channels and two photoplethysmography channels. This system is connected to a laptop with Vicorder software via a USB cable. Two colour coded pneumatic hoses are then attached to the blood pressure and photoplethysmography channels. These hoses are in turn connected to a 30mm pad which is placed around the neck, with the detector positioned over the carotid artery and a larger 100mm wide cuff which is placed around the thigh, at the highest possible point (Figure 3-9). The cuffs are inflated to 65mmHg at which point a waveform is detected at each site and displayed on the screen (Figure 3-10).

Figure 3-9 PWV being performed on a practice volunteer

Figure 3-10 A screen shot from the pulse wave analysis software showing carotid (top) and femoral (bottom) waveform traces



3.2.1.3 Intra-operator variability

An intra-operator variability assessment was carried out for the PWV operator. For 10 participants, the PWV was repeated 20 minutes after the initial exam according to the same protocol.

3.2.1.4 SHIELD study PWV measurement protocol

The study PI was trained in PWV technique during a 2 week course in PWV and cIMT assessment at the University College London Cardiovascular Physiology Unit, London prior to study commencement. The SHIELD clinical officer was in turn trained onsite by the study PI. The SHIELD clinical officer was responsible for carrying out PWV on the patients recruited to the study.

PWV was measured after the patient had been lying flat for at least 10 minutes. After the cuffs were applied to the right carotid and femoral regions, the distance would be measured from the sternal notch to the umbilicus and then from the umbilicus to the middle of the top of the femoral cuff. This measurement was taken with one length of the tape measure and was recorded as the distance for the calculation of PWV (it was taken to be the equivalent of carotid sensor to femoral sensor x0.8 as recommended in the University College London (UCL) Cardiovascular Physiology Unit Guidelines). For repeat or follow-up measurements on the same patient, the operator ensured that the same length that was taken during the first procedure was used for all subsequent assessments of PWV.

Length was inputted into the Vicorder software and when both the carotid and femoral waveforms appeared acceptable for 6 sequential beats, the PWV value was recorded. SHIELD protocol required that three measurements within 0.5 m/s of each other be reported and the average of those three measurements was taken as the final result.

The PWV measurement procedure was overseen by the study PI for the first 30 patients. This included a post-analysis check of both the carotid and femoral artery pulse waveforms. Thereafter, intermittent checks were carried out throughout the study period to ensure that the waveforms were still of acceptable quality.

3.2.1.5 SHIELD cIMT protocol

The SHIELD study adopted the same protocols for cIMT scanning and measurement as the vascular biology unit, UCL. This in turn follows the Mannheim Consensus [291].

3.2.1.5.1 Acquisition of cIMT images

cIMT was measured using B-mode ultrasonography (SIUI CTS 7700, Trisonics) (Figure 3-11). A 7hz linear array transducer was used at a gain of 60dB and frequency of frame rate of 15Hz. The carotid artery was visualised in longitudinal view and in the lateral position so that the distinctive parallel

double line appearance was captured on both near and far carotid walls, indicating that the cross sectional image was taken through the middle of the artery (Figure 3-12).

Figure 3-11 cIMT being performed on a SHIELD practice volunteer

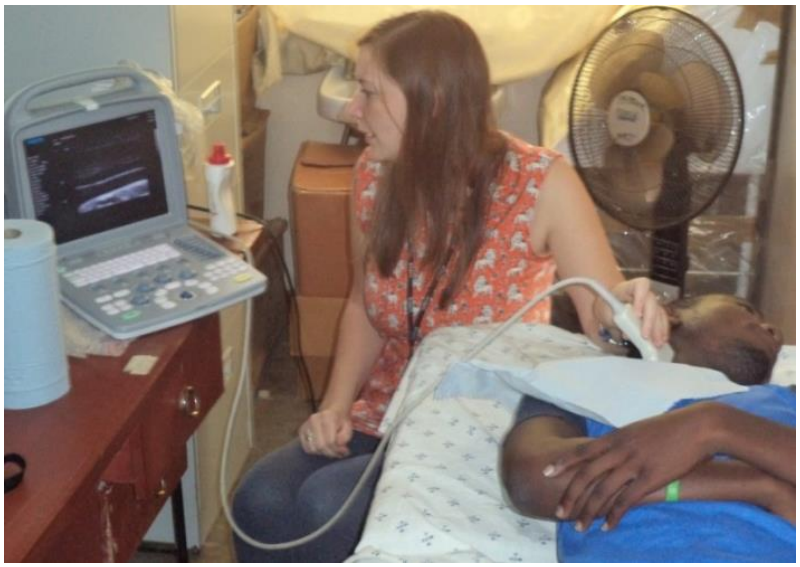
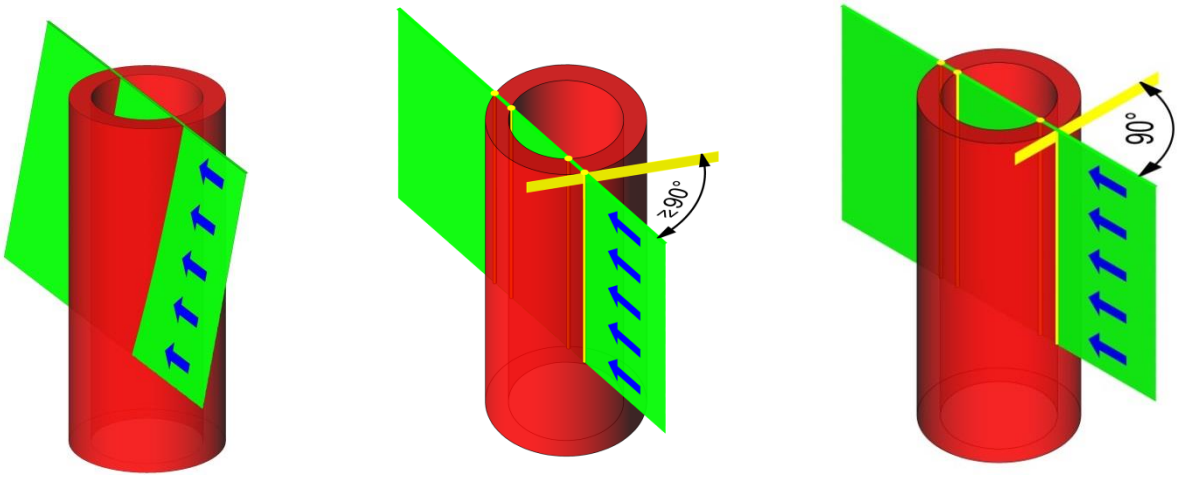


Figure 3-12 Correct angle for ultrasound beam to ensure double line appearance in common carotid artery

A Double lines short **B** Double lines not seen **C** Correct angle

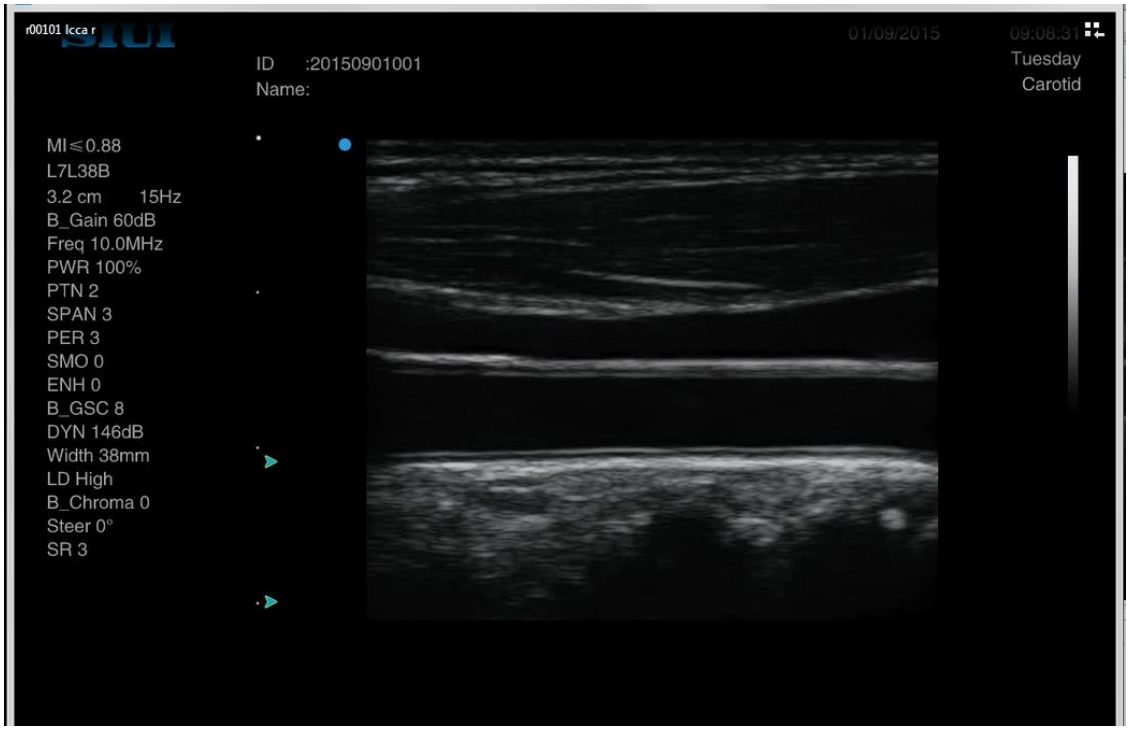


cIMT was measured by one of two technicians. The study PI was trained at UCL department of cardiovascular physiology before study commencement. Further training was then provided onsite in Malawi to both the study PI and the study sonographer (who had previous research experience in

measuring carotid cIMT). This onsite training was delivered by two sonographers experienced in carotid cIMT assessment from the Royal Liverpool University Hospital.

The SHIELD procedure required that a minimum of 10 seconds cineloop be acquired at each one of 6 different regions: right and left common carotid, right and left carotid bulb and right and left internal carotid artery. Minimum requirements for the common carotid artery were that i) double lines were visible for both the near and far walls ii) double lines were visible for at least 10mm length iii) the start of the carotid bifurcation was visible on the scan iv) the artery was horizontal in the image (Figure 3-13).

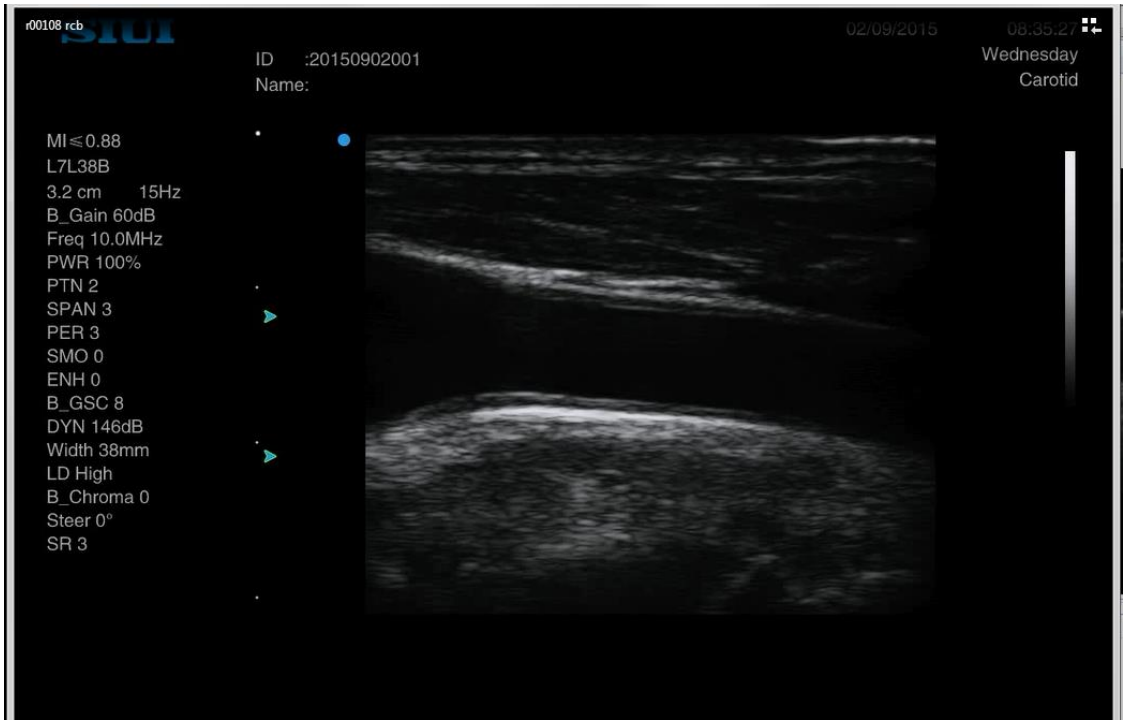
Figure 3-13 Image of requirements for common carotid artery[17]



For the carotid bulb scans, it was required that at least 1mm proximal to the carotid bulb was visualised but only far wall double lines were required to be visible for at least 5mm of length (Figure 3-14).

[17] From a SHIELD participant

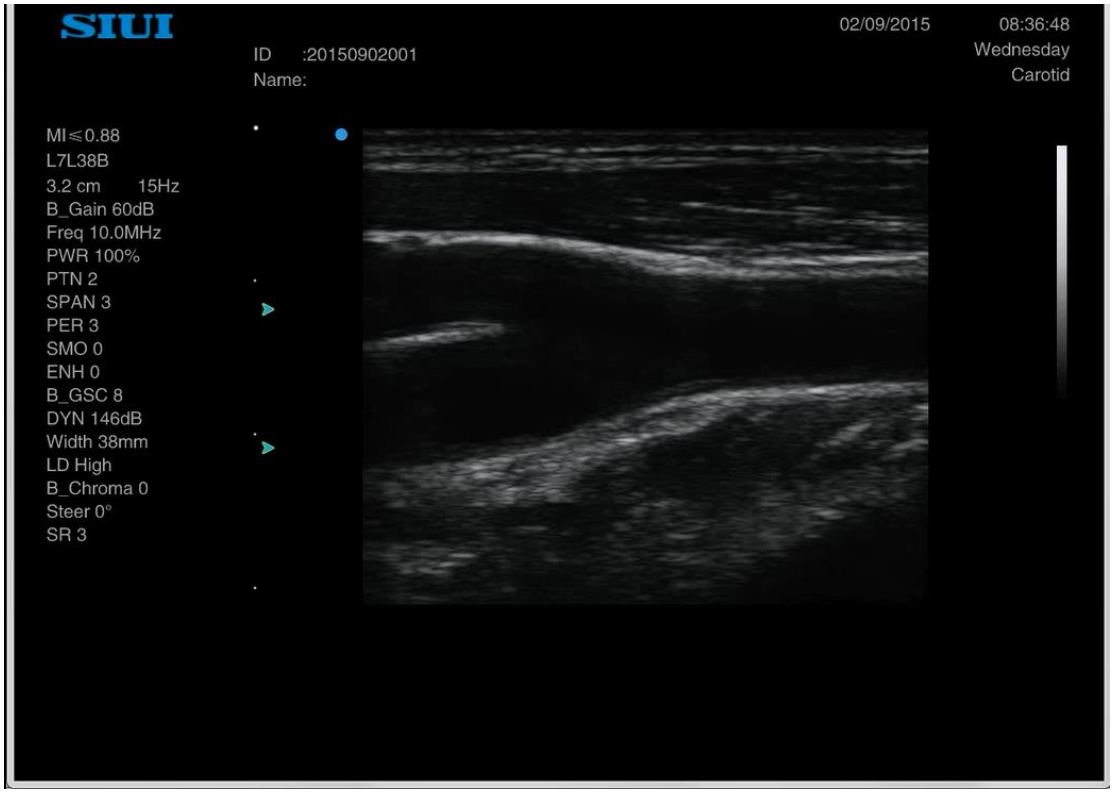
Figure 3-14 Image of requirements for carotid bulb[18]



For the ICA, it was required that the flow divider was visible and that double lines were seen at some point along the internal carotid aspect of the flow divider (Figure 3-15).

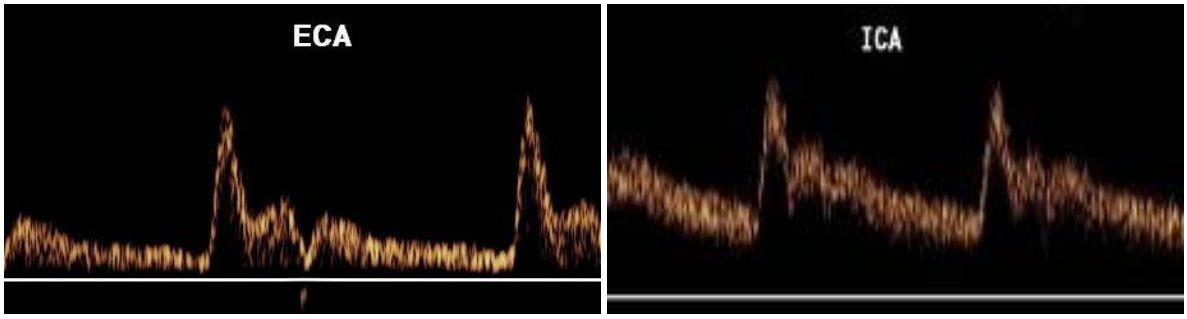
[18] From a SHIELD participant

Figure 3-15 Image of requirements for internal carotid artery[19]



To confirm that it was the internal carotid artery that was being measured, doppler flow was assessed along the artery to confirm it was consistent with the wide waveform of the internal carotid artery (Figure 3-16). When the technician was satisfied that the best possible cineloop had been acquired, it was saved under patient study numbers to the ultrasound machine as well as an external hardware device.

Figure 3-16 Comparison of waveforms for the ECA and ICA



The quality of the cIMT images was reviewed regularly by the study PI and by an experienced cIMT analyst at the department of vascular physiology at UCL.

[19] From a SHIELD participant

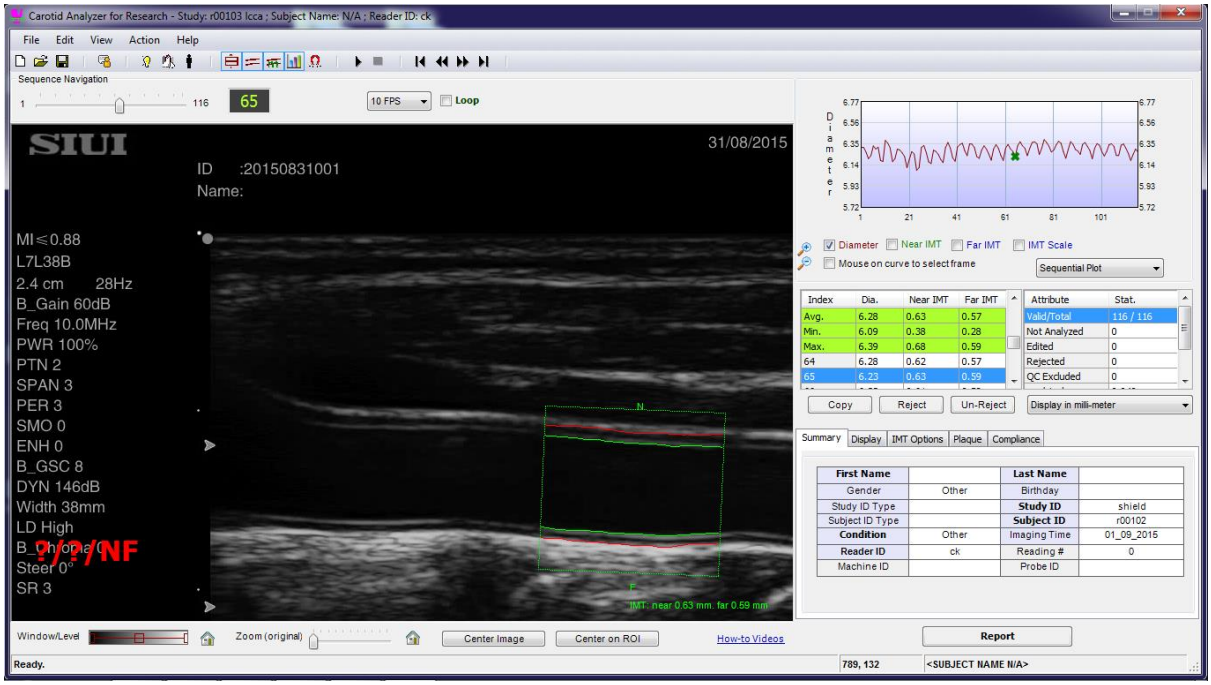
3.2.1.5.2 Semi-automated cIMT measurement

Use of semiautomated edge detection software for making the measurement of the cIMT is recommended by the Mannheim consensus [291]. For the SHIELD protocol we used Carotid Analysis for Research software (mia-llc, Iowa, USA). The SHIELD study PI was trained on use of the software at the unit of vascular biology, UCL before study initiation. Analysis of a random selection of images was also performed by an experienced operator at the same department following the SHIELD protocols to ensure high quality scan readings.

Cineloops were imported into the software and were first calibrated then played through to visualise the target region for assessment. Six separate scans (one for each region of interest) were analysed for each participant. For each scan, cIMT was measured at the far wall as measurements from the far wall are more repeatable [291], where a region of interest was defined by the operator (study PI). If, after edges had been automatically detected, the operator felt that the edges detected by the software were in error, they were able to manually adjust the placement of the detection lines. An average of three measurements that were within 0.05mm was taken for each region.

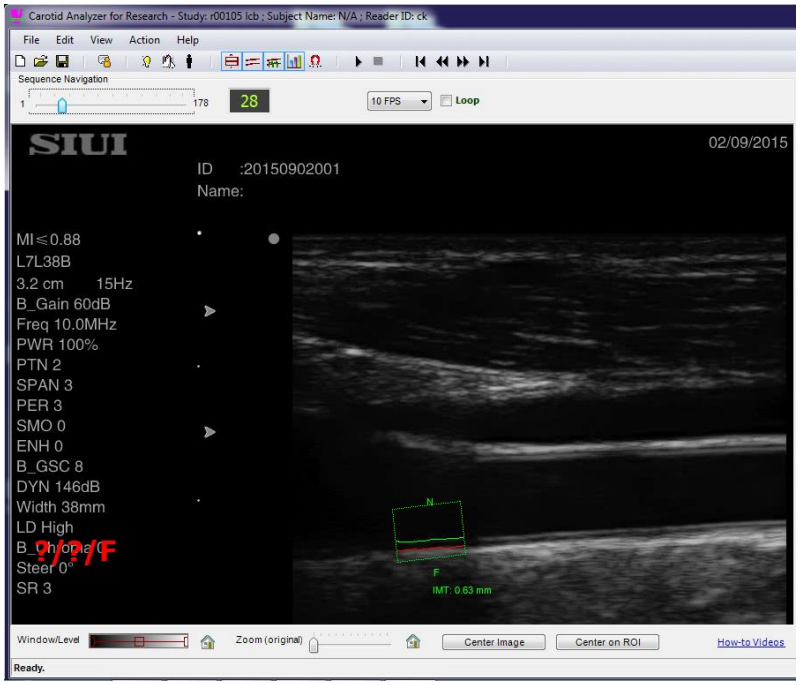
For the common carotid artery, the region of interest was placed at least 1cm proximally to the start of the bifurcation and over a 1cm section (Figure 3-17). Edges were then detected automatically over the duration of the cineloop. As well as the far cIMT, vessel diameter was also measured and graphed on the software. The operator then chose three cycles based on the narrowest vessel diameter (correlated with cardiac diastole) to read the cIMT.

Figure 3-17 Demonstration of cIMT measurement using edge detection software for common carotid artery



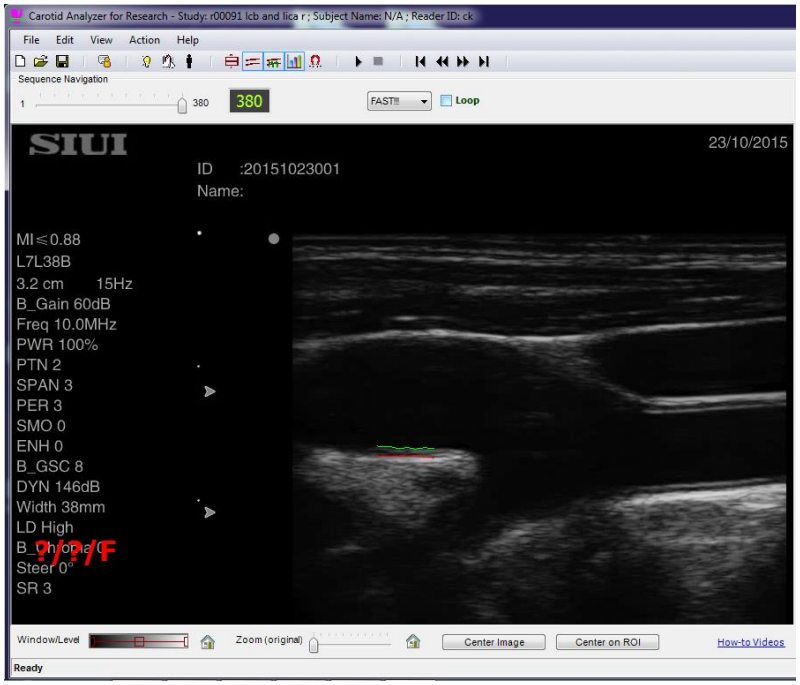
For the carotid bulb, the region of interest was 5mm in length and was placed at the start of the bifurcation over the far wall (Figure 3-18).

Figure 3-18 Demonstration of cIMT measurement using edge detection software for carotid bulb



For the ICA, the region of interest was placed within 10mm distally to the start of the flow divider, at any point where the double line pattern was visible (Figure 3-19). Because the ICA measurements were more challenging, a minimum length of region for assessment of cIMT was not defined but was left to the operator's discretion.

Figure 3-19 Demonstration of cIMT measurement using edge detection software for internal carotid artery



3.2.1.5.3 Intra-operator and inter-operator variability

For cIMT both intra and inter-operator variability were assessed as two operators carried out the scanning. Each operator was required to scan a minimum of 10 extra participants to establish individual scores for intra-operator variability. In each case, the operator would wait a minimum of 20 minutes before repeating the scan according to the same procedure. A further 10 participants were scanned by both operators at separate times on the same day to ascertain inter-operator variability. When cIMT edge detection analysis was being undertaken, the assessor was blinded to scanning operator and to which scans were taken for the purposes of variability studies.

3.3 General Laboratory methods

3.3.1.1 Biochemistry and haematology

Blood samples for fasting glucose, fasting cholesterol, creatinine, full blood count and CD4 count were processed by the MLW CORE laboratory facility. Each patient was provided with a unique barcode which was linked to the patient study number and date of birth. Completed blood samples were taken to the MLW CORE reception where they were booked in electronically onto the LIMS lab management system. Biochemistry tests were then processed on AU480 chemistry analyser (Beckman Coulter), full blood count on the ACT5 Diff (Beckman Coulter) and CD4 count on a

FACScount flow cytometer (Becton Dickinson, BD Biosciences, San Jose, USA). Left over serum and plasma from enrolment bloods (biochemistry and full blood count) was stored at -80C in the MLW CORE laboratory freezer facilities. The MLW laboratories participate in the UK National External Quality Assessment Scheme (NEQAS).

3.3.1.2 Immunology and plasma biomarkers

Blood was taken in citrate and EDTA bottles separately and was transported directly to the MLW immunology lab under the care of the SHIELD laboratory technician. All samples were manually logged and processed according to the SHIELD immunology standard operating procedure which is further detailed in Chapter 5. In brief, the EDTA sample was spun to identify the buffy coat which was stored in freezing medium at -80C. The remaining plasma was also stored at -80C in the MLW freezer archive. Citrate samples were spun with lymphoprep to identify the PBMC layer which was removed with a plastic pipette and then stained for real time surface immunophenotyping. Remaining PBMCs were frozen in liquid nitrogen. Electronic records were maintained for all samples kept in freezer archives and liquid nitrogen. These records were checked for accuracy periodically throughout the study.

3.4 Data management

The data collection process for the SHIELD study followed good clinical practice (GCP) guidelines on data handling as well as MLW standard operating procedures for data collection and management. The SHIELD study was audited and regulated by the MLW Clinical Trials and Research Unit. A paper screening log was maintained to record all patients who were being assessed for study eligibility during the screening process. Patients who were eligible to enter the study were assigned a unique study identification number. Data were collected in the study clinic using paper forms which were scanned into Intelligent Charter Recognition (ICR) scanning software (Cardiff Teleform Version 10.7, Vista, CA). Once scanned, a quality assurance step was performed by the study PI to correct any queries highlighted by the software. An additional step was undertaken whereby the original and scanned documents were compared to look for important errors in recognition. The data were then committed to a password protected access database which was stored on MLW central servers. On completion, the data were downloaded directly into Stata 13.1 (Statacorp, USA) for analysis. For flow cytometry and cIMT data, results were entered into an excel file and then merged with the CRF data in Stata. Paper CRFs were stored in the MLW research office and were only accessed by the SHIELD study team. Consent forms containing patient identifiable information were stored separately within the MLW research premises. The CRFs will be archived at MLW for 5 years after which time they will be destroyed in accordance with MLW policy.

3.5 Statistical analysis

3.5.1 Variable management

Data was downloaded directly from the database into STATA files and was then checked alongside source CRFs for missing data and inaccurate outliers. Following cleaning, databases were then merged. Outcome variables (cIMT and PWV) were examined for normality and any true outliers greater than 97.5th or less than 2.5th centiles were truncated to take the value of the 97.5th or 2.5th centile value respectively for the purposes of regression analysis to avoid undue influence. Those values that did not follow the normal distribution were transformed according to the best fit distribution. Statistical analysis for each study objective is described in the relevant chapters. All analysis was undertaken using Stata v13.1 (Statacorp, USA).

3.6 Sample size calculations

Data on cIMT and PWV from low resource SSA were not available to inform the sample size calculation for this study. Therefore, calculations were based on available data from high resource settings. For cIMT, approximately 25% of patients have a cIMT of greater than 1.0mm which represents approximately a 2 fold increased risk of cardiovascular events [401]. A recent meta-analysis found that patients who are HIV positive are 50% more likely to have a cIMT greater than 1.0mm[402].

For PWV, the greatest mortality occurs in the top quartile of patients which equates to a cfPWV of approximately 12 m/s. Changes in arterial stiffness occur over a few months [403, 404]. Orlova et al showed that 50% of patients with high cardiovascular risk did not have an improvement in PWV after 6 months of coronary prevention therapy and were 4 times more likely to experience a serious cardiac event [405].

Variables of interest and outcome measures used to inform sample size calculations for each specific objective are outlined in Table 3-1.

Table 3-1 Overview of variables used to perform sample size calculations for each specific objectives

Objective	Variable of interest	Outcome	Type of analysis
2	HIV status	cIMT values cfPWV values	Descriptive
3	HIV status	cIMT >1.0mm*	Statistical; regression^{+}
4	sCD14 >2.91x10^{8} pg/mL*	cfPWV >12 m/s*	Statistical; regression^{+}

5	Endothelial microparticles	cfPWV not decreased	Exploratory
6	Decrease in proportion of activated monocytes <50%* as measured by CD163 and HLA-DR expression	cfPWV not decreased	Statistical; regression[+]
7	Randomisation arm	cfPWV as continuous variable	Exploratory

**Values are an estimation of the worst affected quartile; actual values used for the final analyses will be based on the distribution within the SHIELD study. [+]Objectives 3, 4 and 6 require sample size calculations as outlined below.*

3.6.1 Sample size calculation for the determinants of endothelial dysfunction at ART initiation (objectives 3 and 4)

As per table 3-1, objectives 3 and 4 require sample size calculations. The calculation for objective 3 is based on cIMT as the main outcome measure with HIV status as the variable of interest. Calculations are based on a HIV positive population of 330 patients in line with recruitment targets for the REALITY trial. The table below therefore calculates the possible power that can be achieved for various different sizes of HIV uninfected adults with a fixed number of HIV infected participants. The proportion of HIV uninfected patients with outcome cIMT of >1.0mm is calculated at 25% as the literature consistently reports significantly higher rates of clinical outcomes in the top quartile of patients for these variables. A similar principle of 25% of adults with a low soluble CD14 having a pathological PWV of over 12 m/s at baseline was used to calculate sample size for objective 4.

Ratio of exposed to unexposed	***Estimated OR***	***α***	***β***	***Sample size required***	***Total sample size accounting for 10% loss to follow-up***
2:1	1.5	0.05	0.12	450	495
3:1			**0.21**	**400**	**440**
4:1			0.33	375	413
6:1			0.48	350	385
3:1	1.75		0.07	400	440

4:1		0.15	375	413
6:1		0.28	350	385
3:1	2.0	0.01	400	440
4:1		0.03	375	413
6:1		0.09	350	385

Therefore, in order to achieve a power of at least 80% and detect an OR of 1.5, we would need a total sample size of 440, with 330 HIV positive patients and 110 HIV negative patients.

3.6.2 Sample size calculation for determinants of endothelial dysfunction over time (objective 6)

For objective 6, the outcome is a failure to decrease PWV over the study period, with the variable of interest being a high monocyte count. For this power calculation, a fixed sample size of 330 patients was used as derived above. Taking the quartile of patients with the smallest decrease in activated monocytes following ART as the most at risk, the calculations are based on 25% of patients having a low decrease in activated monocytes and 75% having a large decrease in activated monocytes. The table below shows three different potential proportions of patients with a low decrease in monocyte activation who do and do not experience a decrease in PWV during the study period. For each of those proportions, the potential OR possible with this fixed number of patients is demonstrated. For example, this sample size would give us 75% power to detect an OR of 1.5.

Proportion patients in the exposed group with and without outcome of interest	***Estimated OR***	***α***	***β***
1:1	**1.5**	0.05	**0.25**
	1.75		**0.08**
	2.0		**0.02**
2:3	1.5		0.44
	1.75		0.18
	2.0		0.06
1:2	1.5		0.52
	1.75		0.29

2.0	0.14

Therefore, with the expected proportion of patients with and without a decrease in PWV to be 1:1 in the group with higher cardiovascular risk (those exposed to higher monocyte activation), we would have reasonable power (75%) to demonstrate an odds ratio approximating 1.5.

3.7 Ethical considerations

Ethical approval for the SHIELD study was granted by the College of Medicine Research and Ethics Committee (COMREC), University of Malawi and the University of Liverpool Research and Ethics Committee. Written informed consent was obtained from all participants.

4 CHAPTER 4: ENDOTHELIAL DAMAGE IN ADULT MALAWIANS AND ASSOCIATION WITH HIV

4.1 Introduction

The risk of cardiovascular disease in adults living in low resource sub-Saharan Africa countries has not been well characterised. The global burden of disease study demonstrated an increase in the contribution of non-communicable diseases relative to infectious disease in the region [192], and cardiovascular disease is the leading cause of death from non-communicable disease globally. Urbanisation in SSA is predicted to result in changes in the epidemiology of CVD, as traditional cardiovascular risk factors such as hyperlipidaemia, diabetes and obesity increase [193]. This is superimposed on a HIV epidemic which is also evolving in many parts of SSA to become a chronic disease, affecting an aging population[50].

HIV infection has been associated with an approximately 2 to 3-fold increased risk of cardiovascular events in high income settings [128]. However, debate still exists on the limitations of comparing cohorts of HIV infected and uninfected individuals in these settings due to inherent differences in traditional cardiovascular risk factor profiles of these patient groups [406]. Carrying out research into cardiovascular disease in HIV in a low income country like Malawi could contribute unique information to the international research effort for two reasons. Firstly, the HIV epidemic in Malawi is generalised and therefore the HIV infected population will be broadly more similar to the HIV uninfected control groups. Secondly, there are fewer traditional cardiovascular risk factors (such as hyperlipidaemia, diabetes and obesity) in the general population in Malawi owing to the fact that it is at an earlier stage of the epidemiological transition than other SSA countries, again reducing potential for confounding [207].

Regionally it remains unclear whether HIV is a risk factor for cardiovascular disease in the low income SSA setting, given the low prevalence of other risk factors. Identifying an association early, could help to prevent a clash of these two epidemics as urbanisation increases. Healthcare systems across SSA are geared towards prevention and treatment of infectious disease. Resources and capacity do not exist to tackle large scale chronic morbidity. Simple interventions and management guidelines for the prevention of cardiovascular disease in HIV could be feasible within the HIV care system, and it is therefore imperative that any additional risk of cardiovascular disease that may affect people infected with HIV in SSA is identified early so that health care systems and prevention programmes could be implemented in time.

4.2 Specific objectives

This chapter will address specific objectives 2 and 3:

2. Establish the range of age adjusted carotid intima medial thickness (cIMT) and arterial stiffness values in HIV negative patients and HIV positive patients with advanced HIV

3. Establish to what extent advanced HIV is a risk factor for increased cIMT and arterial stiffness in Malawi

4.3 Methods

4.3.1 Study procedures

The study procedures for Objectives 2 and 3 are as described in the general methods.

4.3.2 Sample size calculation

Objective 2 was descriptive and the sample size calculation for objective 3 is described in general methods (subsection 3.6.1).

4.3.3 Statistical analysis

4.3.3.1 Variable management

Variables measured can be loosely categorised into demographic variables, traditional cardiovascular risk variables, infection related variables and immunological variables. Level of primary school education was used as an indicator of educational status and was divided into a binary outcome of primary education or less versus any secondary education or more. This distinction was made because primary school education is currently free of charge in Malawi. Waist – height ratio was also calculated as an indicator of central obesity because this has been shown to be a more specific risk factor for cardiovascular disease than BMI. Data collected on smoking included whether the patient was a current smoker or an ex-smoker, the number of years spent smoking and the number of cigarettes per day. For the purposes of analysis, smoking status was categorised into 'ever smoked' (current and ex-smokers) or 'never smoked'. Similarly, for alcohol, data was collected on current or ex-drinker, how many years spent drinking and how many days of the week. For the purposes of analysis this was categorised into 'ever drank alcohol' or 'never drank alcohol'. For a participant to be classified as having a pre-existing cardiovascular diagnosis (myocardial infarction, stroke, heart failure or transient ischaemic attack (TIA)), diabetes, or hypertension), this diagnosis must have been recorded in the hand held notes or in the clinic file. Both cholesterol and glucose samples were taken when the patient had been fasting for a minimum of 6 hours. A current infection was defined as an infection which occurred within 1 month of the

clinic visit (for longer term infections such as TB this was taken to be within one month of completion of treatment).

4.3.3.2 Objective 2: Establish the range of age adjusted carotid intima medial thickness (cIMT) and arterial stiffness values in HIV negative patients and HIV positive patients with advanced HIV

cIMT and PWV measurements were first assessed for intra and inter-operator variability using Bland-Altman analysis for linear concordance. Raw data were then plotted against age and according to HIV status. cIMT and PWV values for our cohort were both categorised into 10-year age bands and reported in the form of linear regression adjusted for age.

4.3.3.3 Objective 3: Establish to what extent advanced HIV is a risk factor for increased cIMT and arterial stiffness in Malawi as compared to HIV negative volunteers

Firstly, we performed univariate analysis of all variables measured at baseline (demographic variables, traditional cardiovascular risk factors, and other clinical factors) according to HIV status. Wilcoxon Ranksum was used to analyse associations for categorical data and Spearman rho for continuous data.

Next, we asked the question: "Is HIV independently associated with PWV or cIMT at ART initiation?" We aimed to answer this by building a model where HIV status was included as a forced variable whilst adjusting for potential confounders. Direct Acyclic Graph (DAG) diagrams (see Figure 4-1 and Figure 4-2) were constructed to identify potential confounders. Any variable identified as being on the causal pathway on the DAG was assessed in univariate analysis. Any of these variables that had univariate p value <0.2 were carried forward for inclusion as confounders in the model. However, where one or more variables were strongly co-linear (eg systolic and diastolic BP or weight and BMI), the variable that was most strongly associated on univariate analysis was chosen. Backwards elimination was used to sequentially exclude variables with a p value of >0.2.

Lastly, we asked the question: "Which traditional risk factors are associated with PWV and cIMT in Malawian adults, and does the addition of HIV status improve the traditional risk factor model?". We identified all variables which were associated with the outcome on univariate analysis with a p value of <0.2. For those variables that were strongly correlated with each other (eg weight and BMI), we chose the variable that had the most significant association with the outcome. Backwards elimination was used to sequentially exclude variables with a p value of >0.2.

Figure 4-1 Direct Acyclic Graph of associations between measured variables and Arterial Stiffness

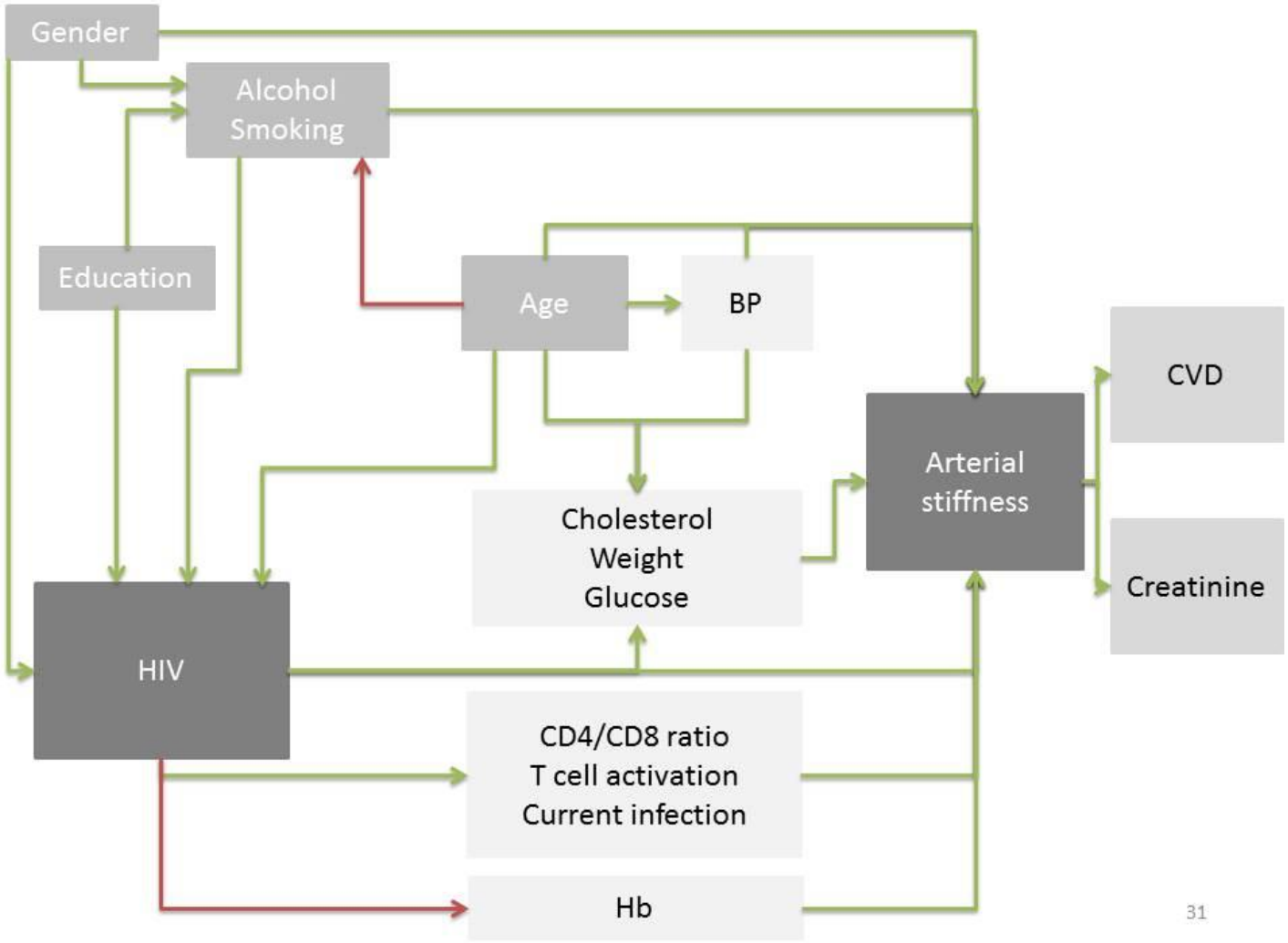
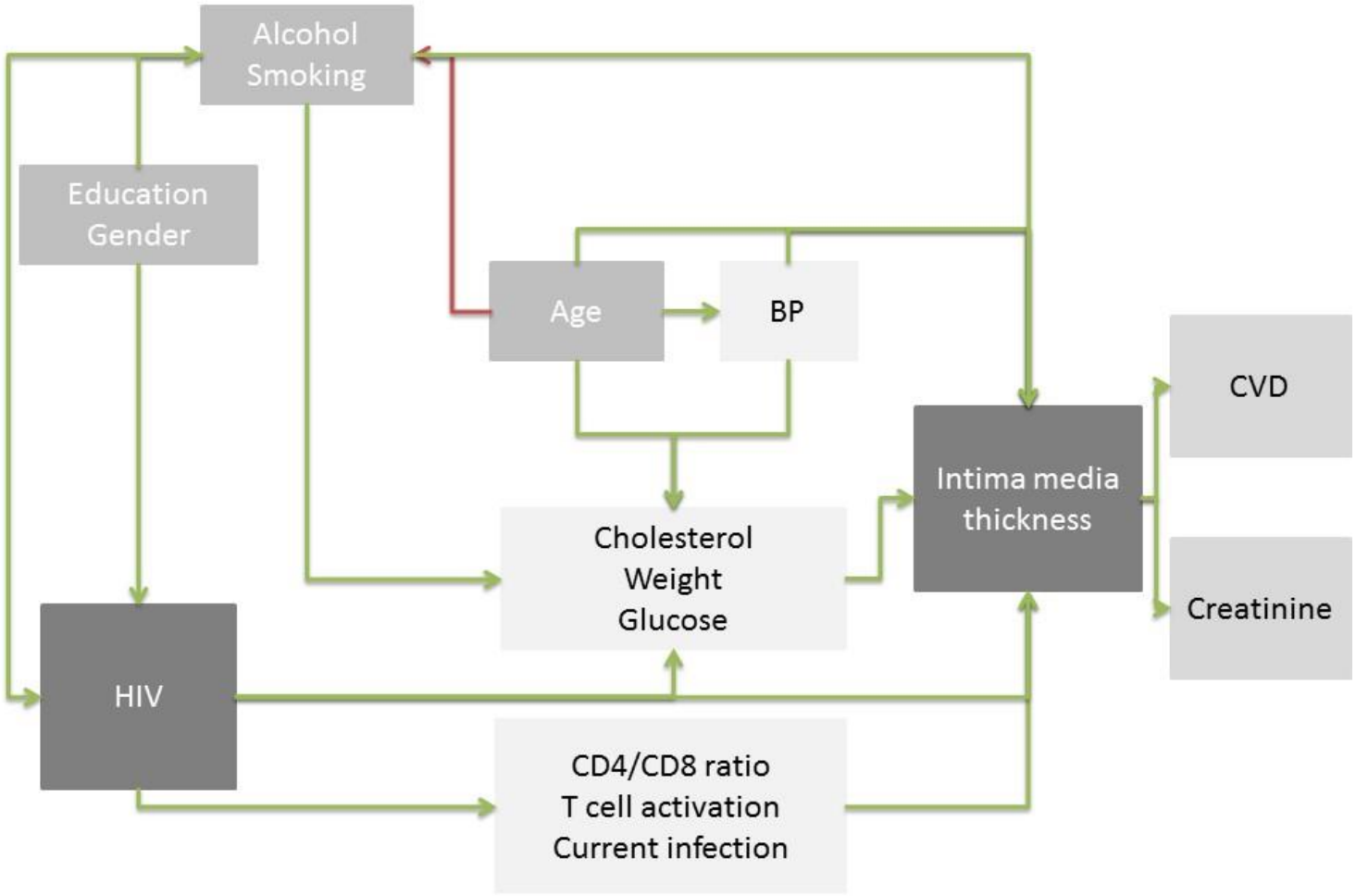


Figure 4-2 Direct Acyclic Graph of associations between measured variables and Intima Media Thickness



4.4 Results

4.4.1 Description of cohort

4.4.1.1 Patient Flow

In total the SHIELD study recruited 279 HIV infected participants (including 170 co-recruited from the REALITY study) and 110 HIV uninfected participants (See Figure 4-3). The 279 HIV infected participants were identified following screening of 2106 patients with a new diagnosis of HIV. Of those screened who were not enrolled, 1477 (73%) patients were found to have a CD4 count that was too high (>/= 100 cells/uL), 117 (6%) were eligible but declined participation, 42 (2%) died before they could be recruited, 39 (2%) were started on ART by the national program before they could be recruited, 23 (1%) were not resident in Blantyre and therefore could not attend follow-up, 16 (1%) did not receive results in a timely manner due to machine failures, 14 (1%) were lost after screening, 12 (1%) were already taking part in another clinical trial, 9 (0.4%) were too sick to consent or participate, 8 (0.4%) did not meet the REALITY study eligibility criteria, 2 (0.1%) opted for treatment in a private clinic and for 68 (3%) patients the reasons for not being recruited were unclear (Figure 4-4). Overall, 626 (30%) of the 2106 patients screened had a CD4 less than 100 cells/uL. Breaking this down into site of HIV testing, 499 of 1845 (27%) patients screened from VCT had a CD4 count less than 100 cells/uL compared to 127 of 254 (50%) of patients screened from medical inpatient wards. Data on characteristics of patients who declined participation were not collected.

Figure 4-3 Summary of recruitment of SHIELD study participants

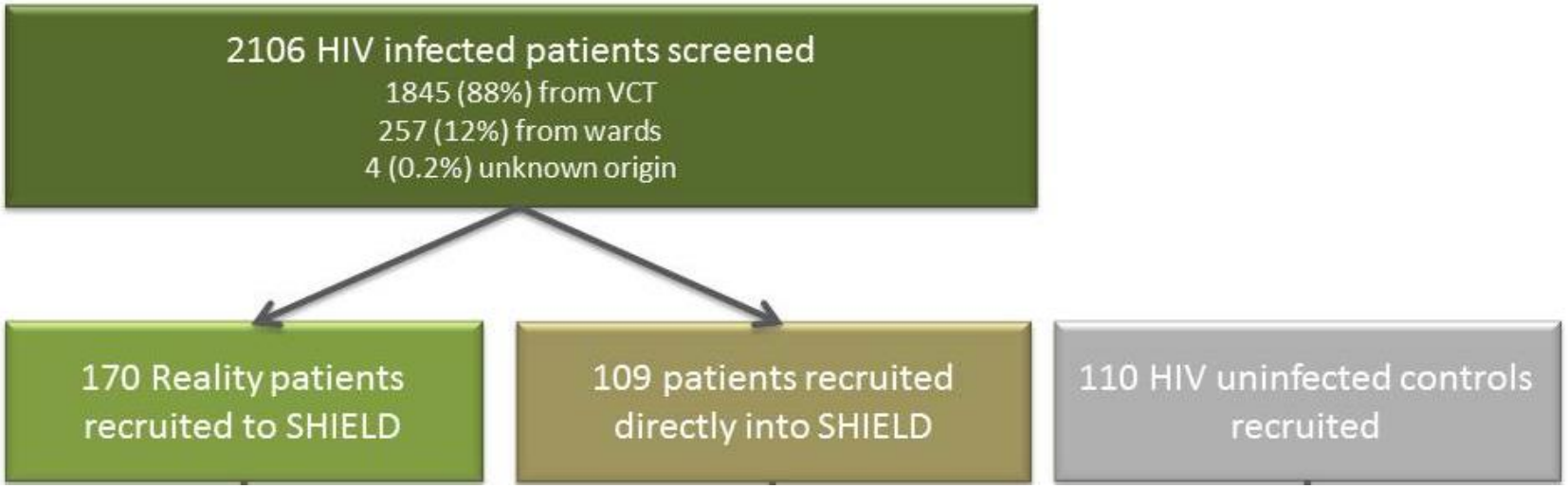
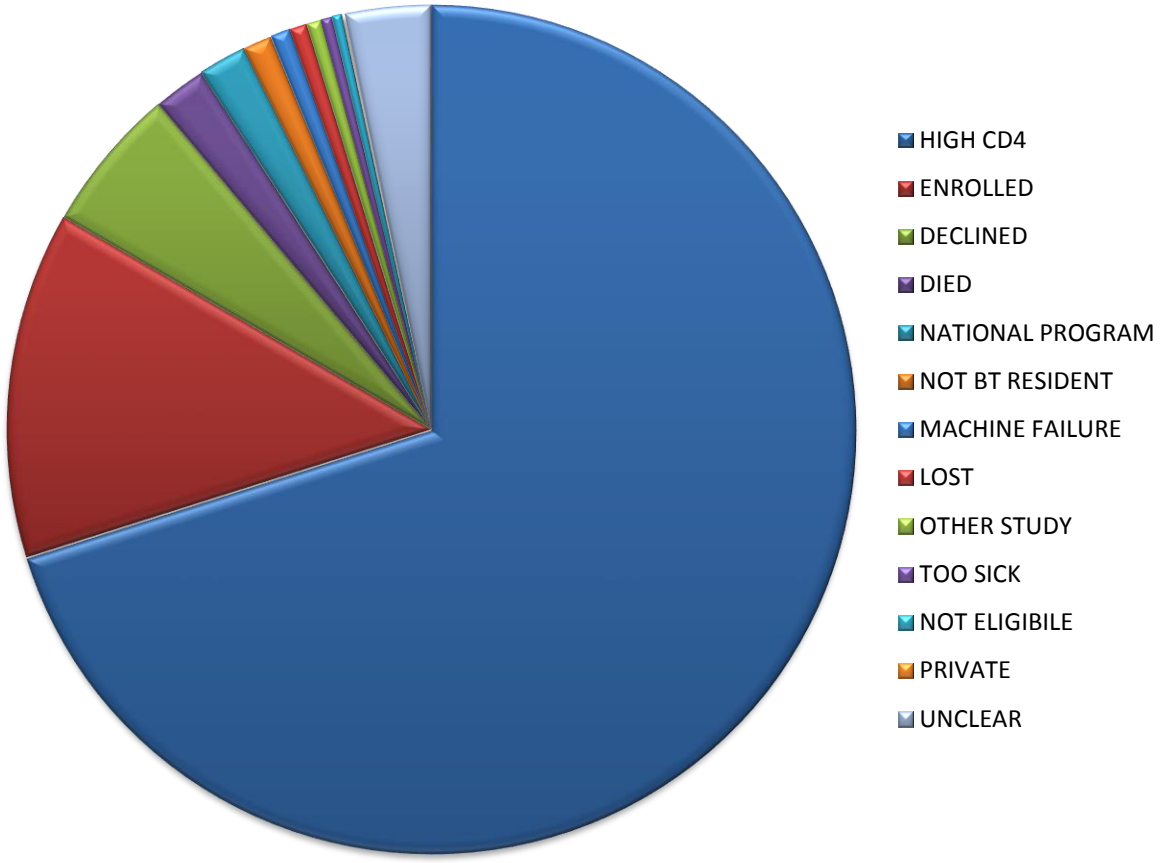


Figure 4-4 Screening outcomes for 2016 patients screened for REALITY and SHIELD studies



4.4.1.2 Baseline demographic and clinical characteristics

An overview of the demographic and clinical variables of the 389 participants recruited, according to HIV status, is given in Table 4-1. The HIV infected cohort showed a higher proportion of males and patients with primary school education or less. Amongst the traditional cardiovascular risk variables, the HIV infected cohort had significantly lower weight, BMI, waist: height ratio, systolic blood pressure and fasting cholesterol, were marginally more likely to have been previously prescribed cardiovascular medications and were more likely to have taken alcohol in the past, although not reaching statistical significance. A higher fasting glucose was also noted. For the remaining variables, the heart rate was higher in the HIV infected group and haemoglobin and lymphocyte counts were lower. Three HIV uninfected participants had suffered with a malaria infection within one month prior to enrolment.

Table 4-1 Baseline demographic and clinical characteristics according to HIV status

		HIV uninfected n=110	**HIV infected N=279**	**P value**
Demographic variables	Age	36.8 (+/- 9.8)	37.5 (+/- 9.8)	0.41
	No. Male	44 (40%)	157 (56%)	0.004
	Primary school education or less	38 (40%)	136 (53%)	0.02
Traditional CV risk factor variables	Weight	61.7 (+/- 11.4)	54.6 (+/ -9.8)	<0.0001
	Waist: height ratio	0.49 (+/- 0.07)	0.46 (+/- 0.06)	<0.001
	BMI	23.3 (+/- 4.6)	20.6 (+/- 3.5)	<0.0001
	Systolic BP	125 (+/- 14)	119 (+/- 15)	<0.001
	Diastolic BP	76 (+/- 10)	74 (+/- 9)	0.27
	History of smoking	16 (15%)	56 (20%)	0.21
	History of alcohol	28 (25%)	119 (43%)	<0.01
	Pre-existing cardiovascular diagnosis	1 (1%)	1 (0.4%)	0.47
	Prescribed CV drugs	5 (5%)	4 (1.5%)	0.08
	Pre-existing diabetes	1 (1%)	1 (0.4%)	0.65
	Pre-existing Hypertension	3 (3%)	5 (2%)	0.40
	New diagnosis of hypertension	46 (42%)	88 (32%)	0.055
	Fasting cholesterol	4.0 (+/- 1.0)	3.7 (+/- 1.1)	0.049
	Fasting glucose	4.7 (+/- 0.9)	5.0 (+/- 1.1)	0.01
	Creatinine	63.5 (+/- 13.6)	69.1 (+/- 23.6)	0.13
Infection related variables	Heart rate	74 (+/- 11)	86 (+/- 18)	<0.0001
	Haemoglobin	13.7 (+/- 1.8)	11.5 (+/- 2.1)	<0.0001
	Current infection	3 (3%)	57 (21%)	<0.0001

	TB	0 (0%)	2 (1%)	
	Cryptococcal meningitis	0 (0%)	0 (0%)	
	Pneumonia	0 (0%)	10 (4%)	
	Gastroenteritis	1 (1%)	17 (6%)	
	Malaria	2 (2%)	3 (1%)	
Immune related variables	Lymphocytes	2.1 (+/- 0.8)	1.3 (+/- 0.7)	<0.0001
	Monocytes	0.47 (+/- 0.84)	0.52 (0.54)	0.054
	Absolute CD4 count cells/uL	NA	41 (18 – 62)	NA
	HIV viral load $x10^6$ copies	NA	1.1 (0.4 – 2.9)	NA

4.4.1.3 Description of HIV related disease

Enrolment for HIV infected participants took place over a 2-week period, to minimise the burden for this sick patient group. Of the 279 participants recruited with HIV infection, 2(0.7%) withdrew after initially giving consent (both after discussing with family members), 8(3%) patients died, 3(1%) did not re-attend and were lost to follow-up. Of those remaining the mean (sd) CD4 count was 41(38.6) cells/uL: 39(54.6) for the SHIELD cohort and 41(27.2) for the REALITY cohort. The median (IQR) HIV viral load was 1.1 $x10^6$ (0.4 – 3.0) $x10^6$: 2.2 $x10^6$ (0.6 – 4.3) $x10^6$ for the SHIELD cohort and 1.1 $x10^6$ (0.4 – 2.1) $x10^6$ for the REALITY cohort. The number of patients in WHO stages 1, 2, 3 and 4 were 82(30%), 130(48%), 50(18%) and 10(4%) respectively. Pulmonary or disseminated TB was diagnosed in 30 patients, chronic diarrhoea in 29, severe weight loss in 8, moderate weight loss in 3, cryptococcal meningitis in 5, oesophageal candidiasis in 1, Kaposi's sarcoma in 1 and severe bacterial infection in 1. All patients were commenced on standard first line ART (Tenofovir, Lamivdine, Efavirenz) apart from one patient who was commenced on Zidovudine, Lamivudine and Nevirapine.

4.4.2 Age adjusted PWV and cIMT values

4.4.2.1 Variability studies

4.4.2.1.1 PWV: Intra-operator variability

PWV was performed by one operator, the study clinical officer, and so the intra-operator variability was calculated for this operator. The concordance correlation co-efficient was 0.986 (95% CI 0.957 –

0.995) based on 13 observations. This correlation is displayed in Figure 4-5. Figure 4-6 shows that the difference between readings does not change according to the PWV value in the limits of agreement plot, and this was associated with a small correlation between difference and mean value of -0.29 using the F-test for correlation. Slight bias for the second reading to be lower is likely due to a few outliers and a small sample size.

Figure 4-5 Intra-operator concordance for 13 paired PWV measurements

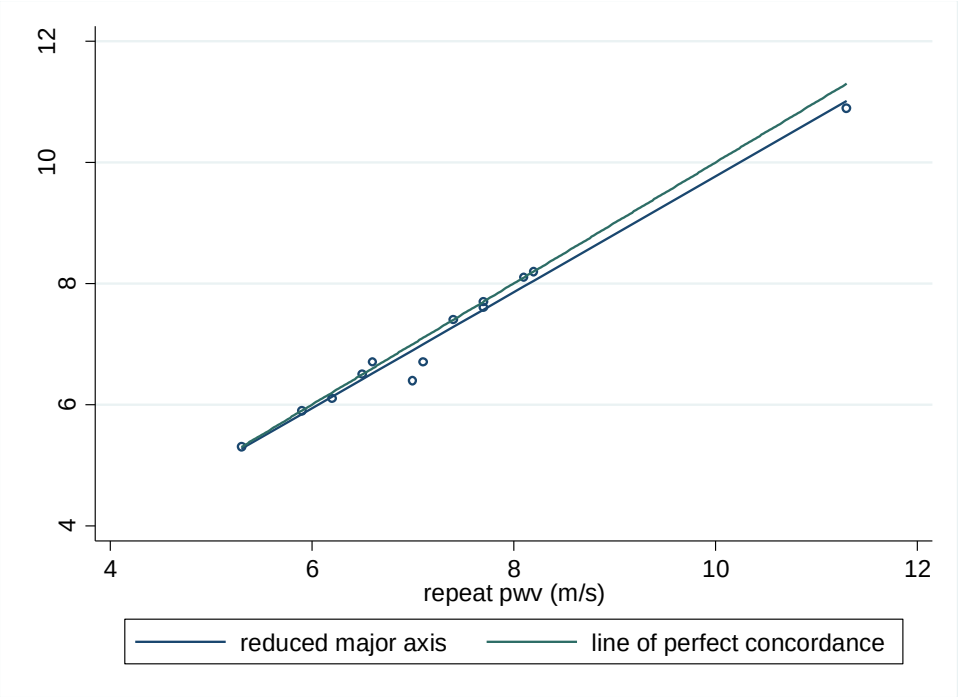
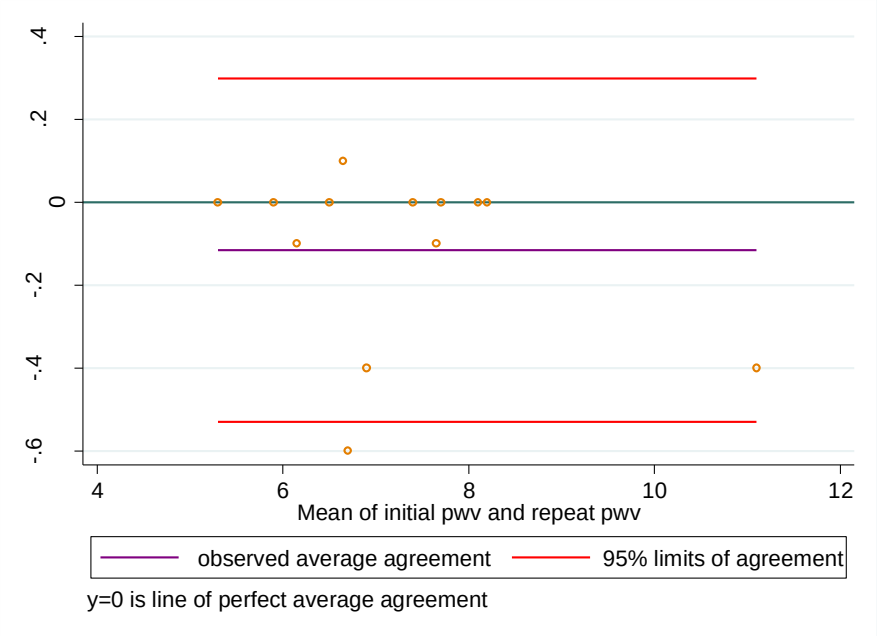


Figure 4-6 Plot of mean difference for 13 paired PWV measurements



4.4.2.1.2 cIMT: intra-operator variability

For cIMT measurements there were two operators (sonographer and study PI). Intra-operator variability is presented separately for each operator. Each operator was given a score for each of the 3 cIMT sections measured in the protocol (common carotid artery, carotid bulb and internal carotid artery), using an average value of the left and right sides. For the sonographer, the concordance correlation coefficient (95% CI intervals) for the common carotid artery, carotid bulb and internal carotid artery regions were 0.964 (0.883 – 0.989, Figure 4-7), 0.963 (0.895 – 0.987, Figure 4-8) and 0.363 (-0.129 – 0.711, Figure 4-9) respectively. As with PWV, there was no change in mean differences according to cIMT values and so these graphs were not repeated.

Figure 4-7 Intra-operator variability for sonographer, for 12 paired common carotid artery measurements

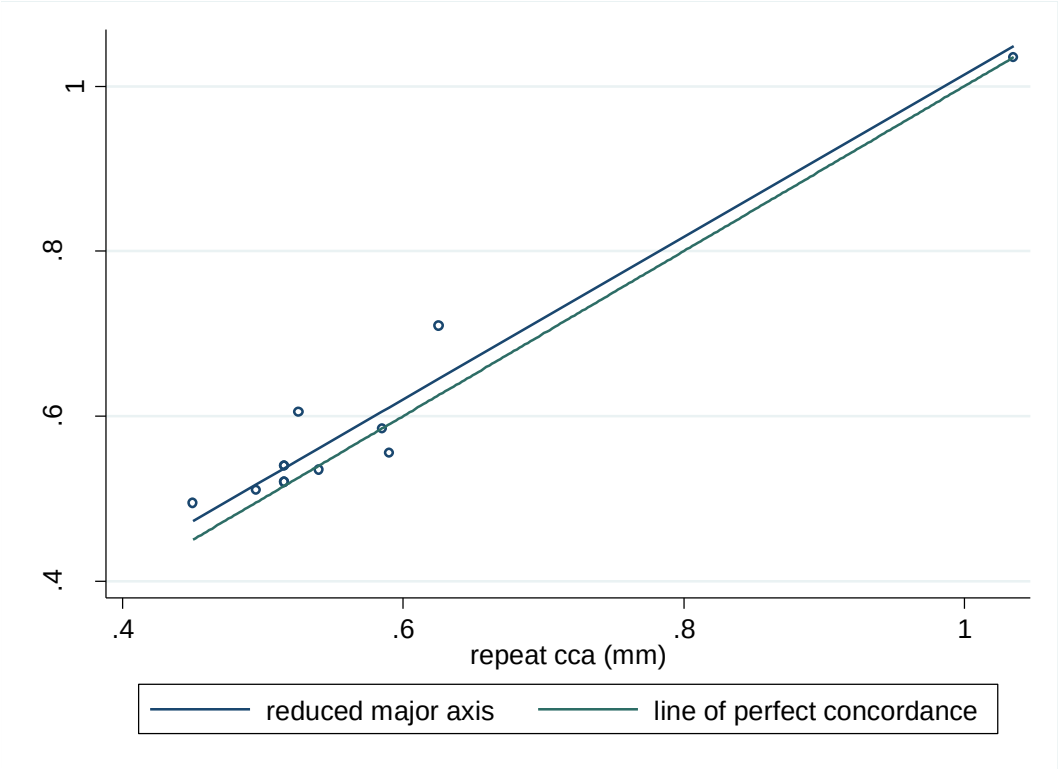


Figure 4-8 Intra-operator variability for sonographer, for 12 paired carotid bulb measurements

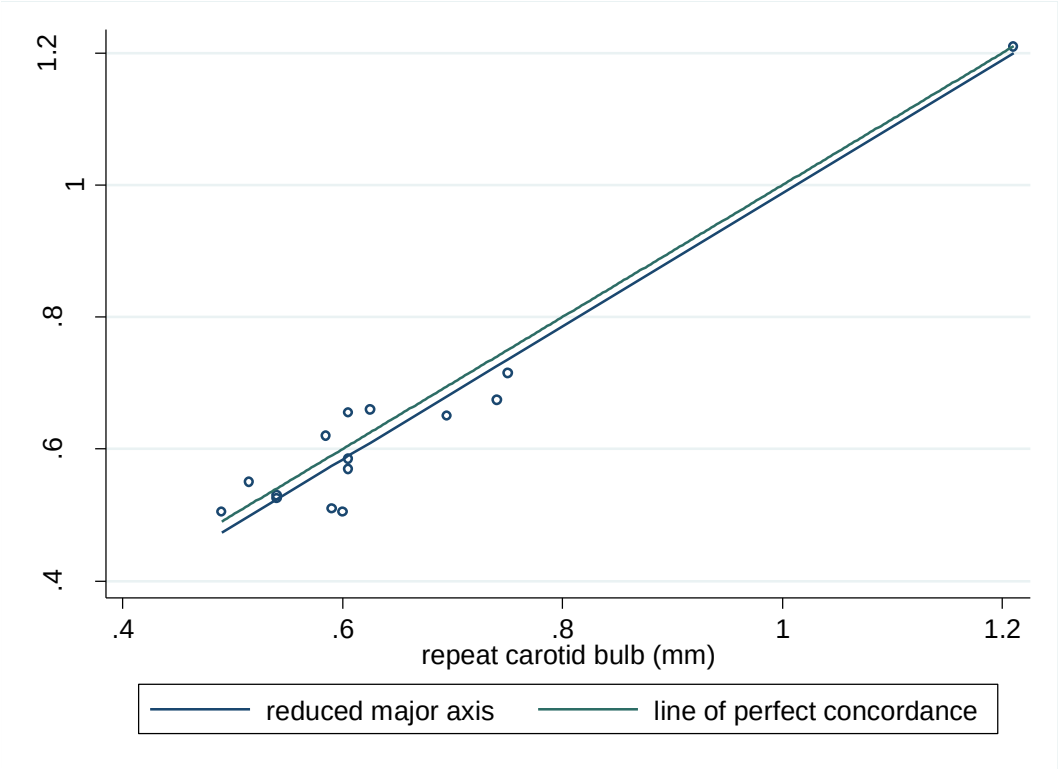
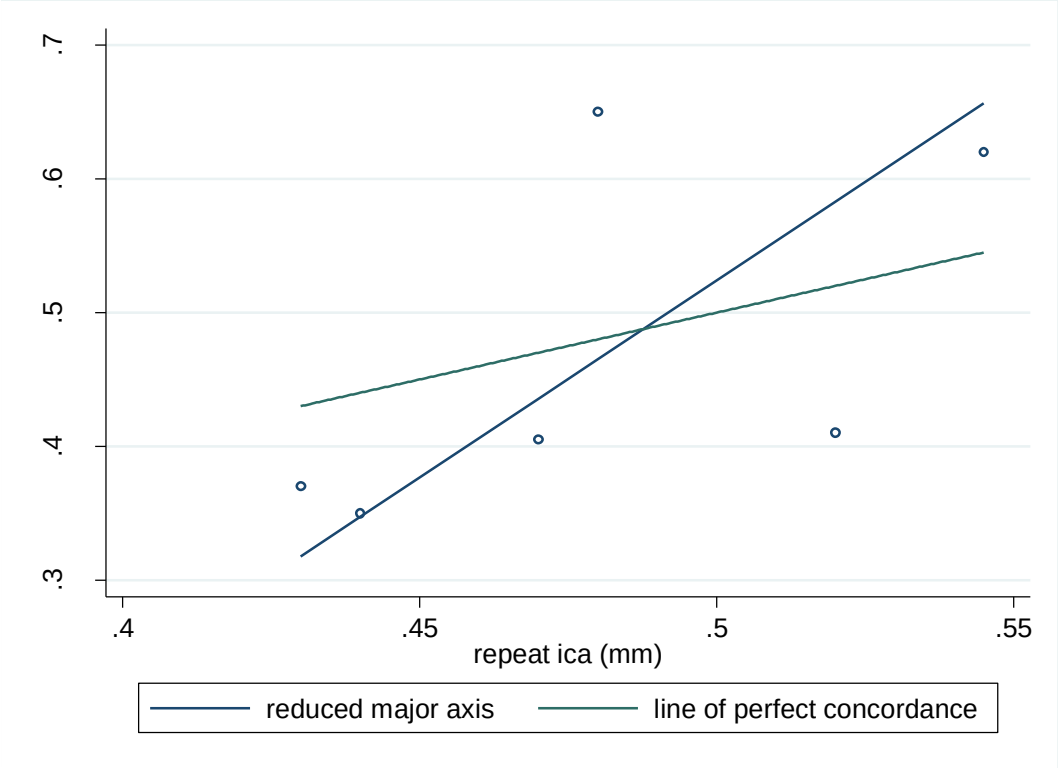


Figure 4-9 Plot of mean difference for 12 paired carotid bulb measurements for sonographer



For the study PI, the concordance correlation coefficient (95% CI intervals) for the common carotid artery, carotid bulb and internal carotid artery regions was 0.853 (0.554 – 0.957, Figure 4-10), 0.171 (-0.316 – 0.586, Figure 4-11) and 0.298 (-0.535 – 0.837, Figure 4-12) respectively. This was based on 11 paired measurements for common carotid artery, 9 paired measurements for carotid bulb and 6 paired measurements for internal carotid artery.

Figure 4-10 Intra-operator variability for study PI, for 11 paired common carotid artery measurements

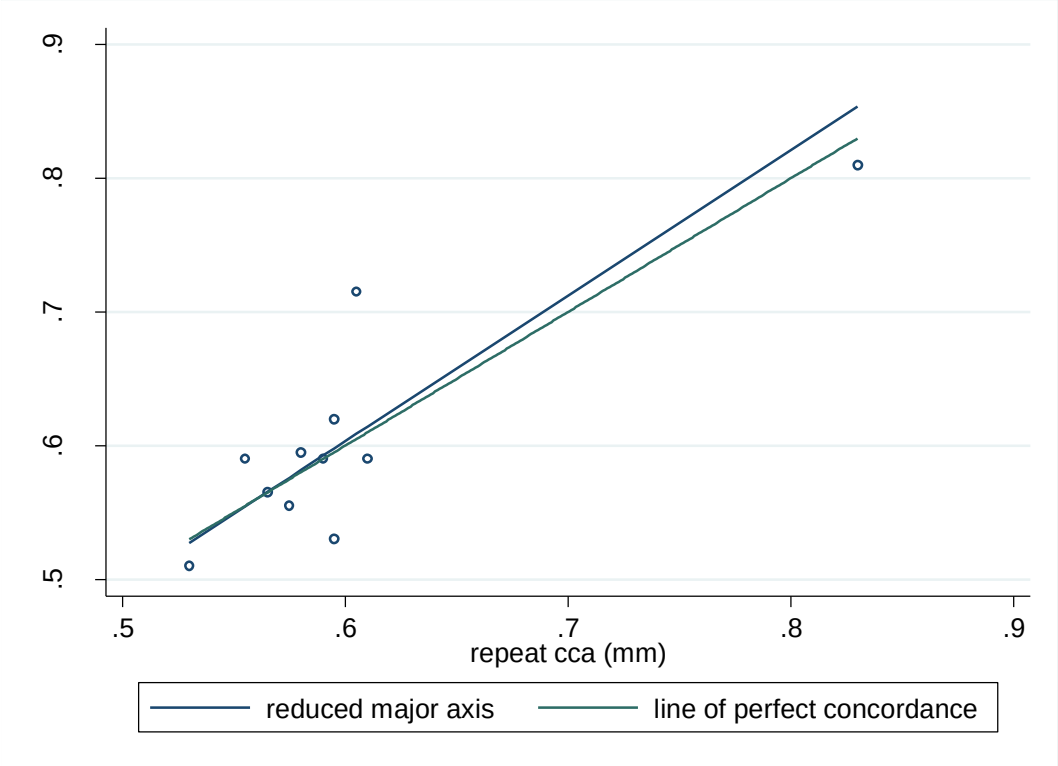


Figure 4-11 Intra-operator variability for study PI, for 9 paired carotid bulb measurements

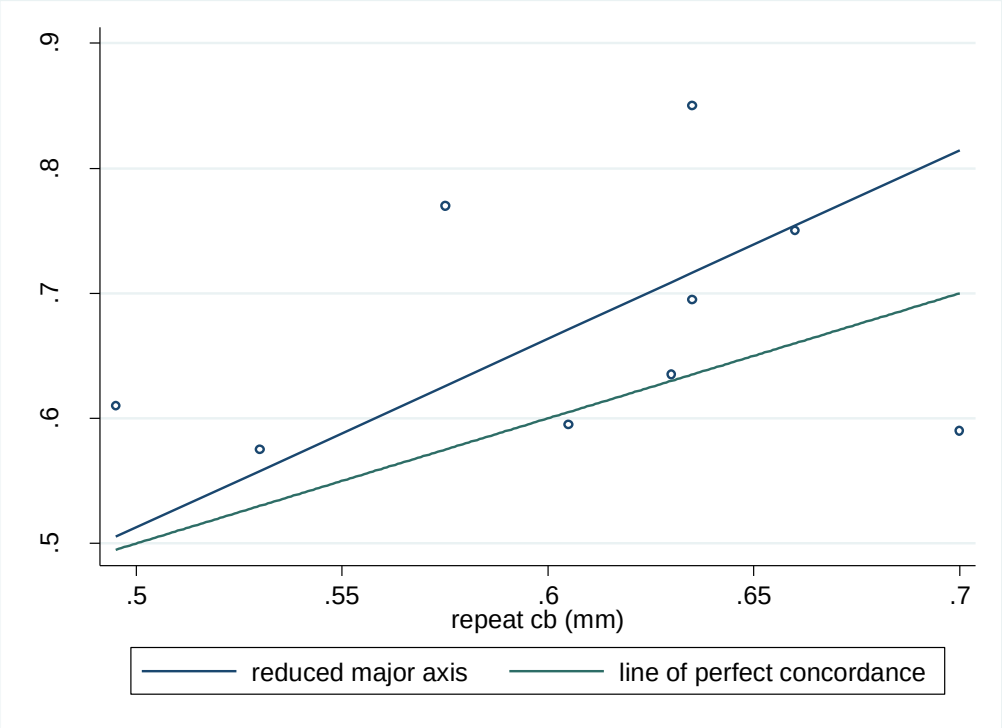
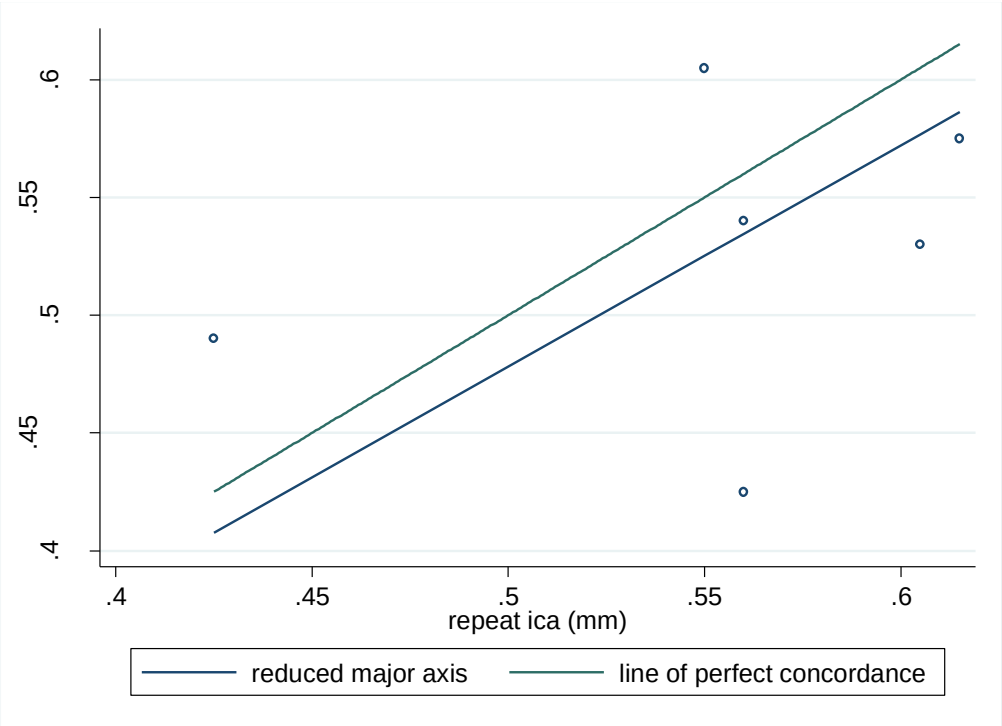


Figure 4-12 Intra-operator variability for study PI, for 6 paired internal carotid artery measurements



4.4.2.1.3 cIMT: Inter-operator variability

Ten participants were scanned by both operators to derive inter-operator variability for the three cIMT regions. The concordance correlation coefficient between the two operators for the common carotid artery, carotid bulb and internal carotid artery cIMT was 0.683 (0.112 – 0.915, Figure 4-13), 0.814 (0.40 0 – 0.952, Figure 4-14) and -0.174 (-0.674 – 0.436, Figure 4-15). This was based on 9 paired measurements for common carotid artery, 9 paired measurements for carotid bulb and 5 paired measurements for internal carotid artery.

Figure 4-13 Inter-operator variability for 9 paired common carotid artery measurements

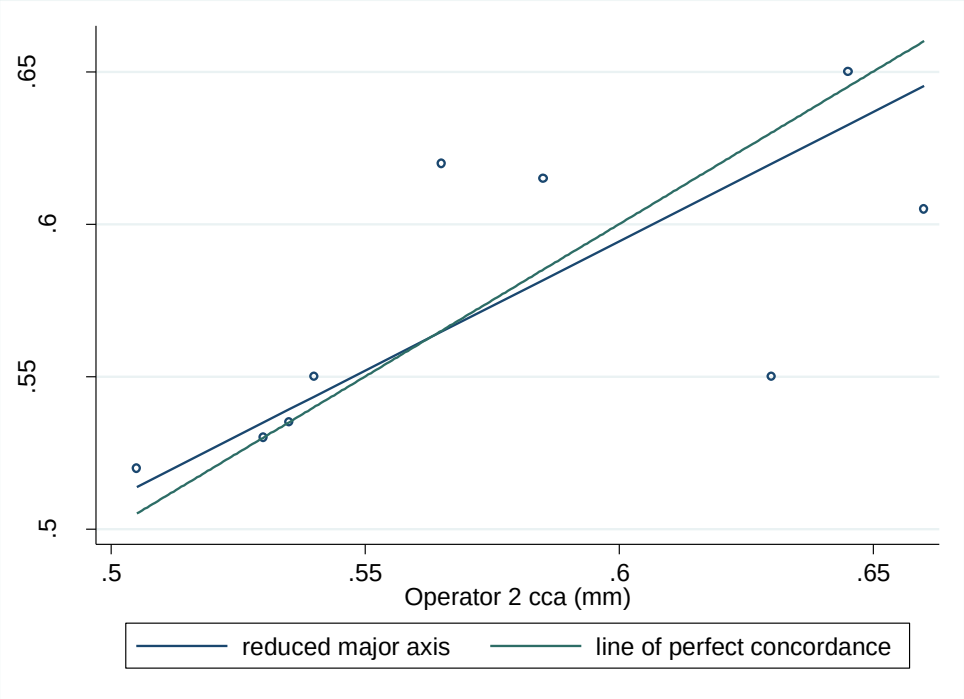


Figure 4-14 Inter-operator variability for 9 paired carotid bulb measurements

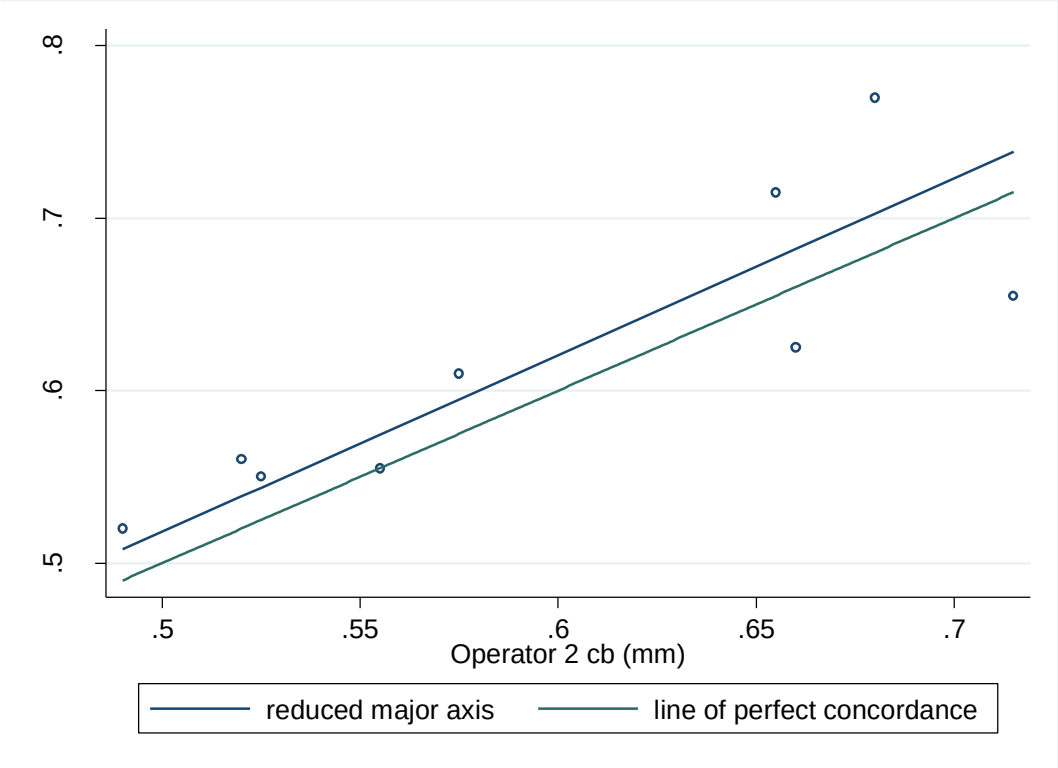
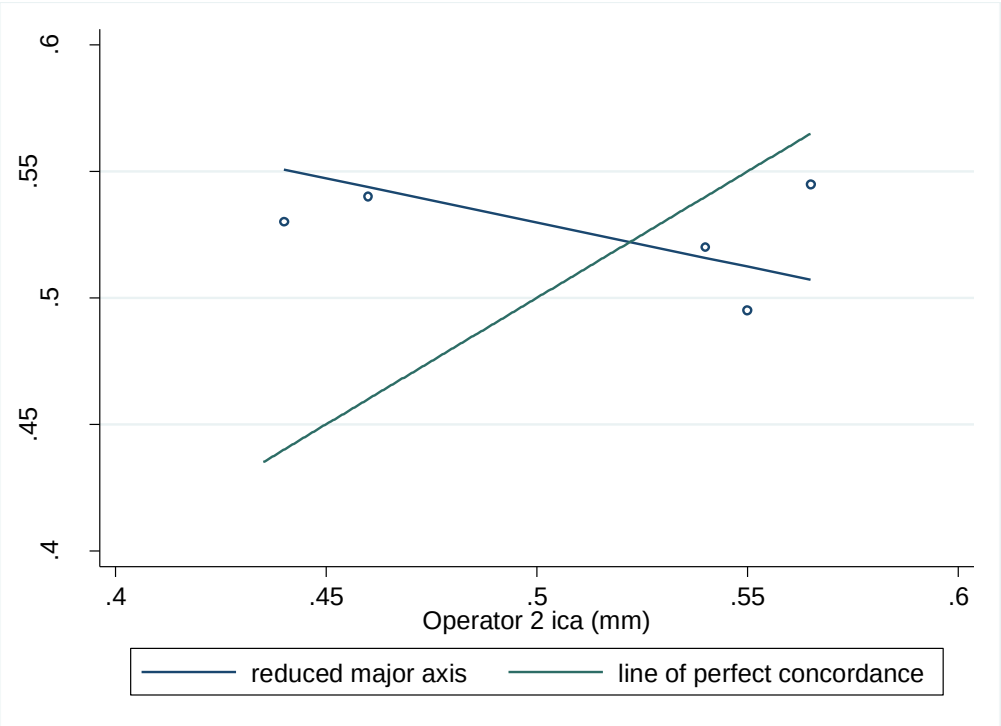


Figure 4-15 Inter-operator variability for 5 paired internal carotid artery measurements



4.4.2.2 Distribution of PWV and cIMT values

The distributions of PWV and cIMT were examined because regression methods assume a normal distribution. The distribution of PWV values are shown in Figure 4-16. The Shapiro-Wilk W test for normality gave a W value of 0.98 ($p<0.0001$), indicating that a log distribution is the best transformation to achieve a normal distribution. Figure 4-17 shows the histograms of PWV data by different possible transformations. This confirms that the log transformation is the best fit.

Figure 4-16 Distribution of raw PWV values

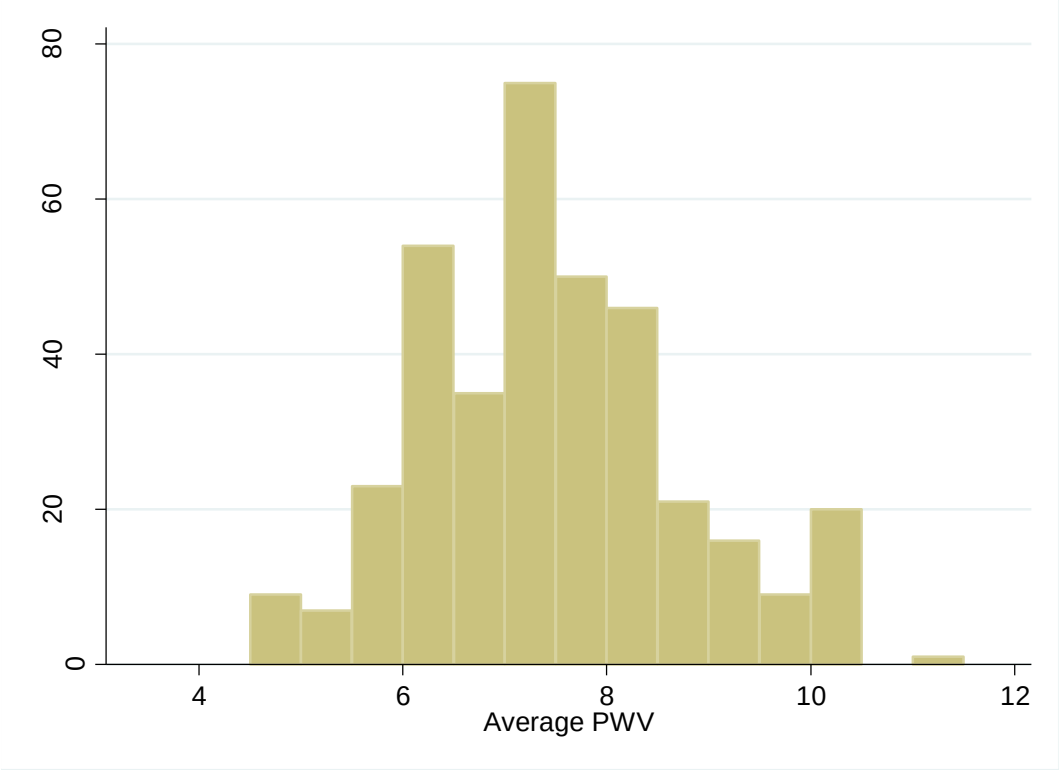
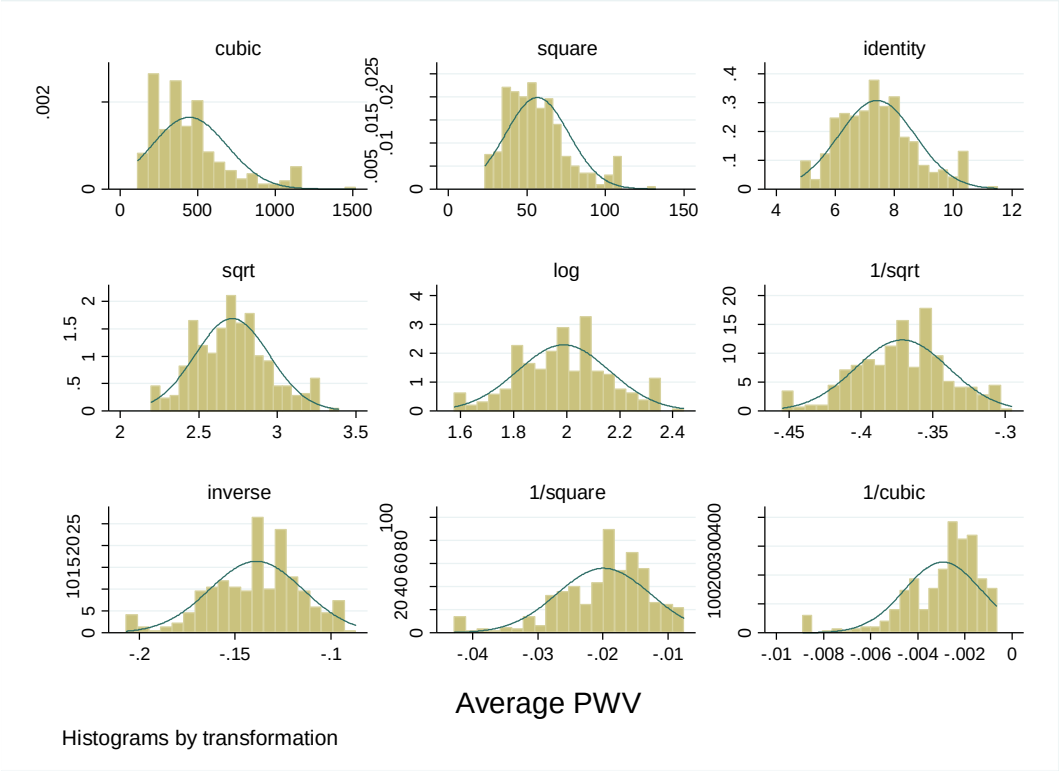


Figure 4-17 Distributions of transformed PWV values



PWV was therefore log transformed for inclusion in modelling throughout this analysis. Coefficients are presented after having been back transformed into linear values for ease of interpretation.

CCA cIMT values were also not normally distributed (Figure 4-18). For CCA cIMT, the Shapiro-Wilk W value was 0.922 (<0.000001), with a theta coefficient of -1.54, indicating that an inverse square transformation would be most appropriate. This was confirmed when visualising the distributions in histograms of the transformed values using the gladder command in Stata version 13.1 (Figure 4-19). To be more specific, the theta value was used instead of the inverse square, so CCA cIMT data was transformed using the formula: **1/(cca_imt^1.54)**. As before these values were back transformed from model outputs to linear coefficients.

Figure 4-18 Distribution of raw CCA cIMT values

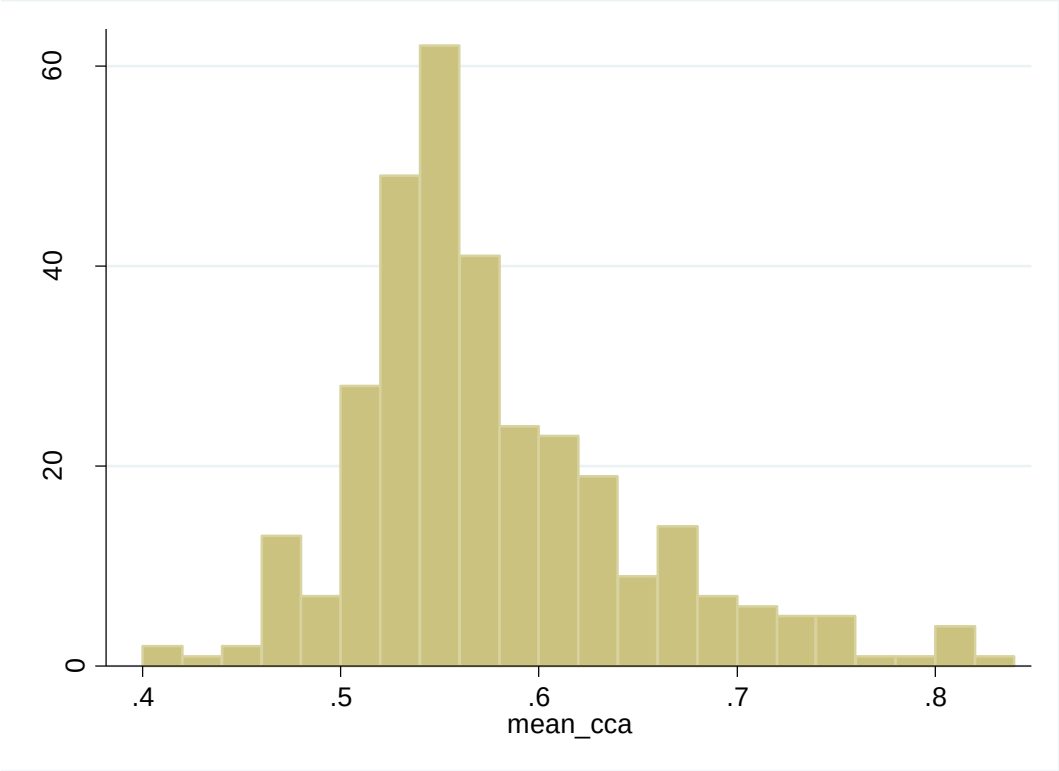
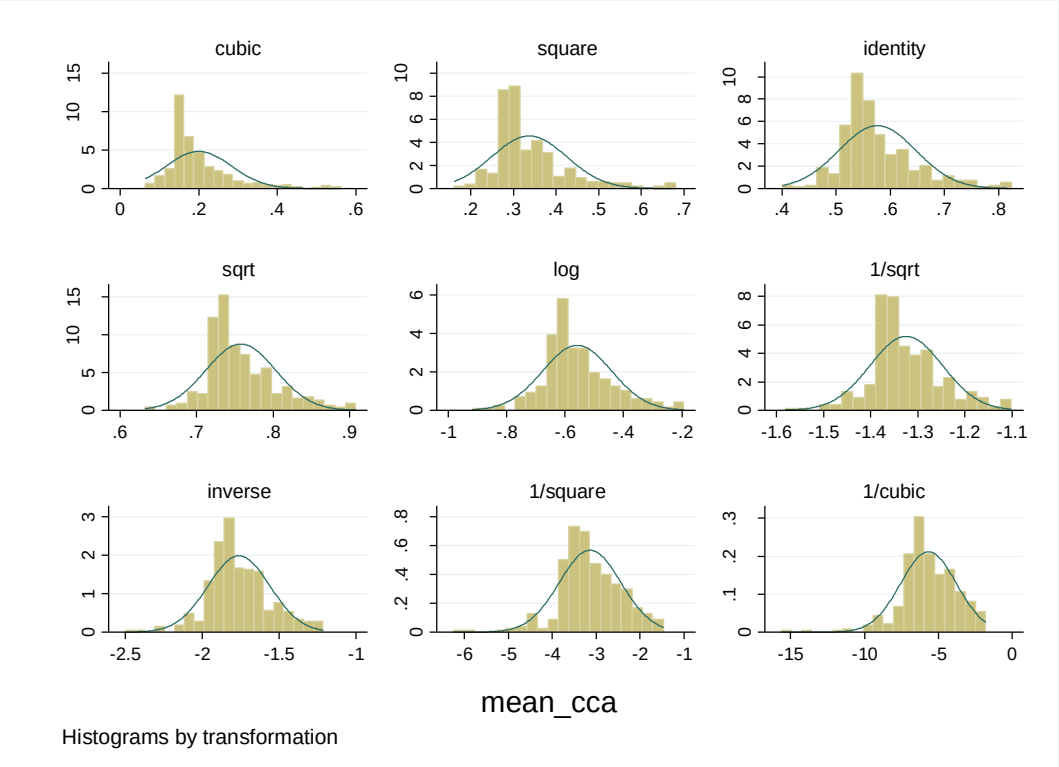


Figure 4-19 Distribution of transformed CCA cIMT values



4.4.2.3 PWV and cIMT values according to HIV status and age category

Enrolment visit PWV values were available for 259 (93%) patients with HIV infection and 107 (97%) without. PWV values are presented according to 5-year age bands for patients with and without HIV infection in Table 4-2 and Table 4-3 respectively. For both cohorts, the mean PWV increases steadily through increasing age bands. These categories are compared according to HIV status in Figure 4-20.

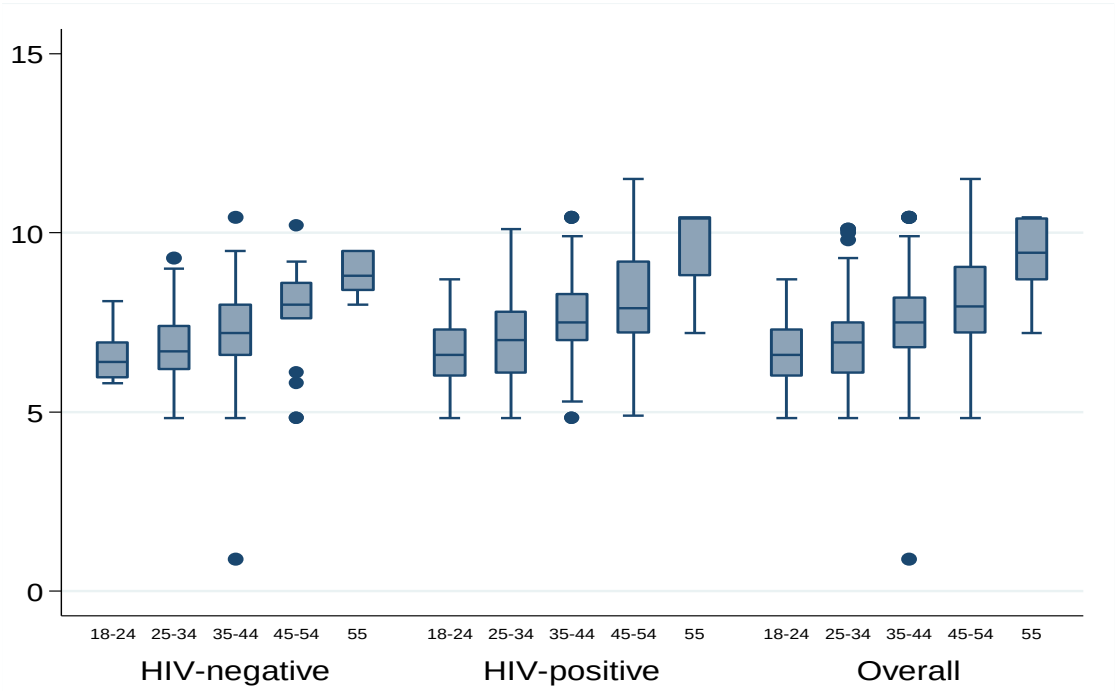
Table 4-2 PWV values for 259 participants with HIV infection

Age category	Number of participants	Mean PWV (m/s)	Min PWV (m/s)	Max PWV (m/s)	SD
18 - 25	21	6.7	4.8	8.7	1.00
25 - 35	90	7.0	4.8	10.1	1.12
35 - 45	98	7.6	4.8	10.4	1.10
45 - 55	39	8.2	4.9	11.5	1.40
>55	11	9.6	7.2	10.4	1.20
All	259	7.5	4.8	11.5	1.32

Table 4-3 PWV values for 107 participants without HIV infection

Age category	Number of participants	Mean PWV (m/s)	Min PWV (m/s)	Max PWV (m/s)	SD
18 - 25	12	6.5	5.8	8.1	0.71
25 - 35	42	6.8	4.8	9.3	0.98
35 - 45	29	7.4	4.8	10.4	1.16
45 - 55	17	7.7	4.8	10.2	1.48
>55	7	8.9	8	9.5	0.59
All	107	7.2	4.8	10.4	1.22

Figure 4-20 PWV according to age category and HIV status



After assessing whether cIMT image quality met the quality requirements stipulated in SHIELD SOPs, 356 (92%) participants had a common carotid artery scan that could be interpreted, 359 (92%) had a carotid bulb scan that could be interpreted and 326 (84%) had an internal artery carotid scan that could be interpreted. The sonographer carried out 293 (75%) of scans and the study PI carried out 96 (25%) of scans. The study was designed to have one main cIMT operator (sonographer) and one substitute operator (PI) because the sonographer could not be available full time due to clinical duties. Common carotid artery cIMT values are shown in Table 4-4 and Table 4-5, carotid bulb values in Table 4-6 and Table 4-7, and internal carotid artery values in Table 4-8 and Table 4-9 for patients with and without HIV infection respectively.

Table 4-4 Common carotid artery IMT values according to age category for 237 participants with HIV infection

Age category	Number of participants	Mean CCA IMT (m/s)	Min CCA IMT (m/s)	Max CCA IMT (m/s)	SD
18 - 25	16	0.53	0.44	0.61	0.05

25 - 35	84	0.55	0.42	0.80	0.06
35 - 45	89	0.58	0.49	0.76	0.05
45 - 55	38	0.65	0.53	0.83	0.08
>55	10	0.64	0.56	0.80	0.07
All	237	0.58	0.42	0.83	0.07

Table 4-5 Common carotid artery IMT values according to age category for 87 participants without HIV infection

Age category	**Number of participants**	**Mean CCA IMT (m/s)**	**Min CCA IMT (m/s)**	**Max CCA IMT (m/s)**	**SD**
18 - 25	8	0.51	0.48	0.58	0.51
25 - 35	33	0.54	0.40	0.63	0.54
35 - 45	27	0.57	0.48	0.67	0.57
45 - 55	14	0.65	0.55	0.74	0.65
>55	5	0.70	0.59	0.81	0.70
All	87	0.57	0.40	0.81	0.57

Table 4-6 Carotid bulb IMT values according to age category for 214 participants with HIV infection

Age category	**Number of participants**	**Mean CB IMT (m/s)**	**Min CB IMT (m/s)**	**Max CB IMT (m/s)**	**SD**
18 - 25	17	0.55	0.49	0.66	0.05
25 - 35	72	0.60	0.47	0.81	0.07
35 - 45	83	0.67	0.51	0.95	0.10
45 - 55	31	0.72	0.54	1.06	0.12

>55	11	0.76	0.60	0.98	0.12
All	214	0.65	0.47	1.06	0.11

Table 4-7 Carotid bulb IMT values according to age category for 82 participants without HIV infection

Age category	**Number of participants**	**Mean CB IMT (m/s)**	**Min CB IMT (m/s)**	**Max CB IMT (m/s)**	**SD**
18 - 25	8	0.57	0.44	0.82	0.12
25 - 35	34	0.64	0.54	0.86	0.07
35 - 45	22	0.66	0.54	0.87	0.09
45 - 55	14	0.73	0.55	0.93	0.12
>55	4	0.87	0.75	0.98	0.10
All	82	0.66	0.44	0.98	0.11

Table 4-8 Internal carotid artery IMT values according to age category for 149 participants with HIV infection

Age category	**Number of participants**	**Mean ICA IMT (m/s)**	**Min ICA IMT (m/s)**	**Max ICA IMT (m/s)**	**SD**
18 - 25	8	0.43	0.35	0.53	0.07
25 - 35	53	0.49	0.31	0.65	0.07
35 - 45	58	0.53	0.37	0.71	0.07
45 - 55	25	0.57	0.44	0.69	0.06
>55	5	0.57	0.49	0.64	0.05
All	149	0.52	0.31	0.71	0.08

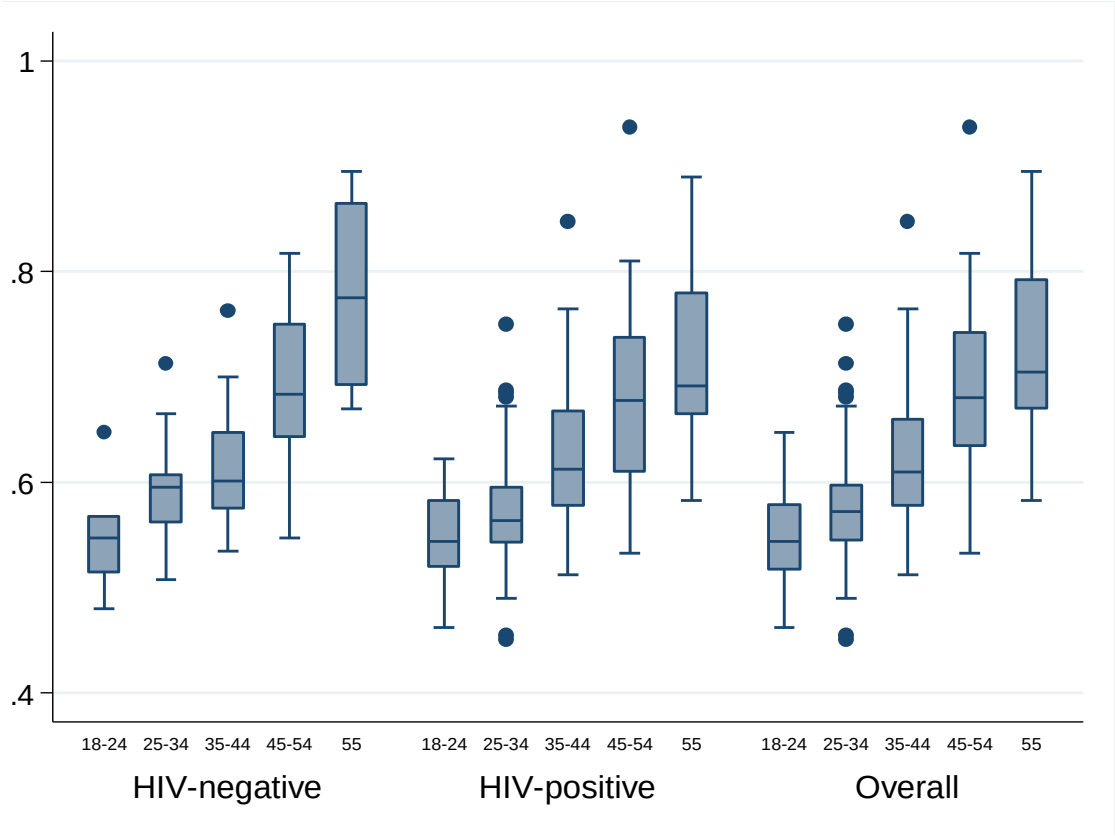
Table 4-9 Internal carotid artery IMT values according to age category for 43 participants without HIV infection

Age category	Number of participants	Mean ICA IMT (m/s)	Min ICA IMT (m/s)	Max ICA IMT (m/s)	SD
18 - 25	3	0.47	0.40	0.51	0.06
25 - 35	20	0.52	0.38	0.66	0.07
35 - 45	10	0.53	0.39	0.68	0.08
45 - 55	8	0.62	0.55	0.80	0.08
>55	2	0.60	0.56	0.65	0.06
All	43	0.54	0.38	0.80	0.08

During study design, three carotid regions were chosen to study whether HIV infection might have a differential effect on cIMT in different sections of the carotid tree, compared to the effects of traditional cardiovascular risk factors. However, cIMT was not significantly higher at any of the three regions in HIV infected participants. Furthermore, absolute mean cIMT values for HIV participants were lower for the bulb and the internal carotid artery when compared to common carotid artery.

In addition, variability was higher at the carotid bulb and internal carotid artery. Although the absolute numbers were small in these analyses, this is consistent with published literature on higher variability in these regions. With this lack of evidence for an increased effect of HIV at the bulb or the internal carotid, combined with the low reproducibility, the mean common carotid artery cIMT is now used as the cIMT outcome measure. The common carotid artery cIMT was the most reproducible and is the most highly cited in published literature. Figure 4-21 compares CCA cIMT values for each age category according to HIV status.

Figure 4-21 Mean common carotid artery IMT according to age category and HIV status



4.4.2.4 Continuous models adjusted for age

Using linear regression, every 10-year increase in age is associated with a 0.23 m/s increase in PWV (95% CI 0.17 – 0.29) for participants with HIV infection (Figure 4-22) and a 0.22 m/s increase in PWV (95% CI 0.14 – 0.30) for participants with HIV infection (Figure 4-23). Every 10-year increase in age was also associated with a 0.1 mm increase in common carotid artery cIMT (95% CI 0.08 – 0.12) for participants with HIV infection (Figure 4-24) and a 0.13 mm increase (95% CI 0.11 – 0.16) for participants without HIV infection (Figure 4-25).

Figure 4-22 PWV according to age for adult Malawians with advanced HIV infection

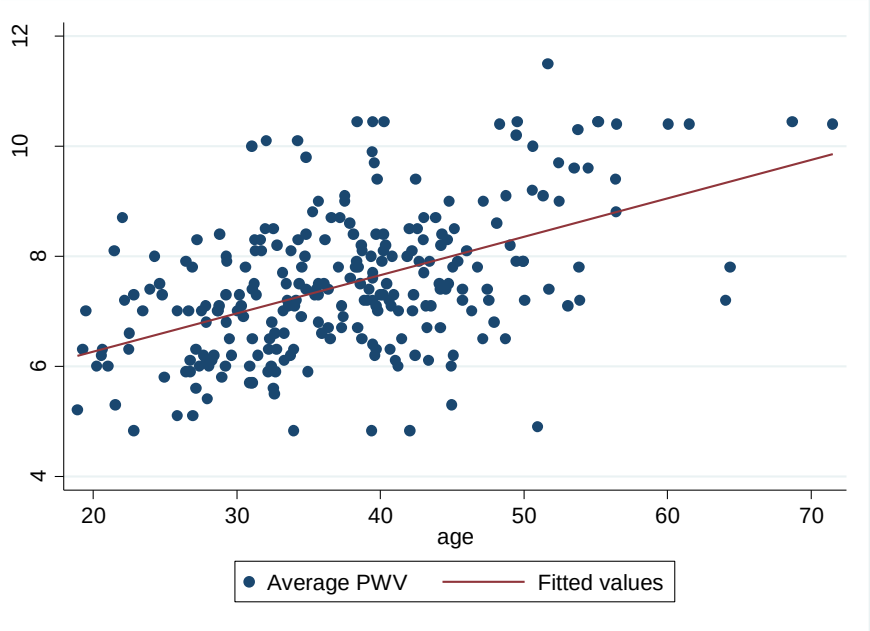


Figure 4-23 PWV according to age for adult Malawians without HIV infection

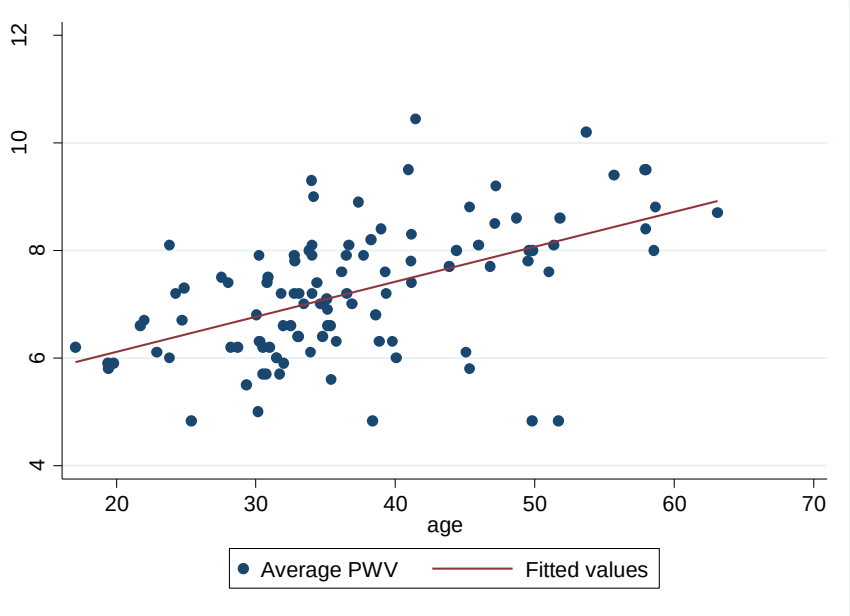


Figure 4-24 Mean CCA IMT values according to age for adult Malawians with advanced HIV infection

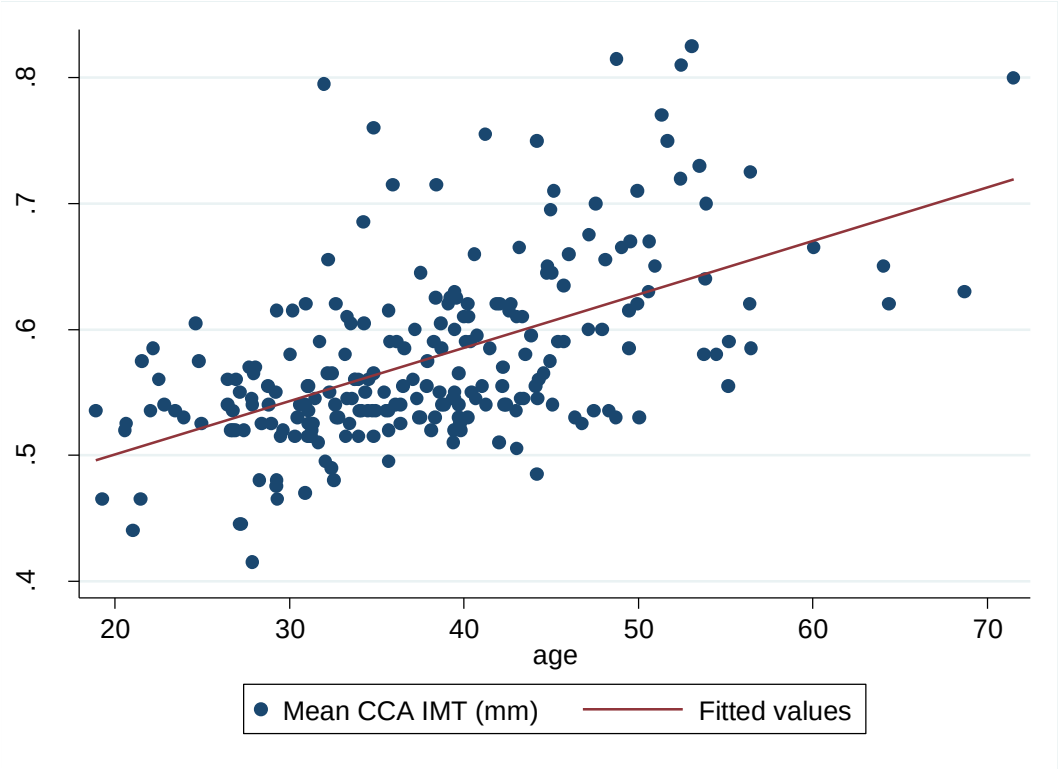
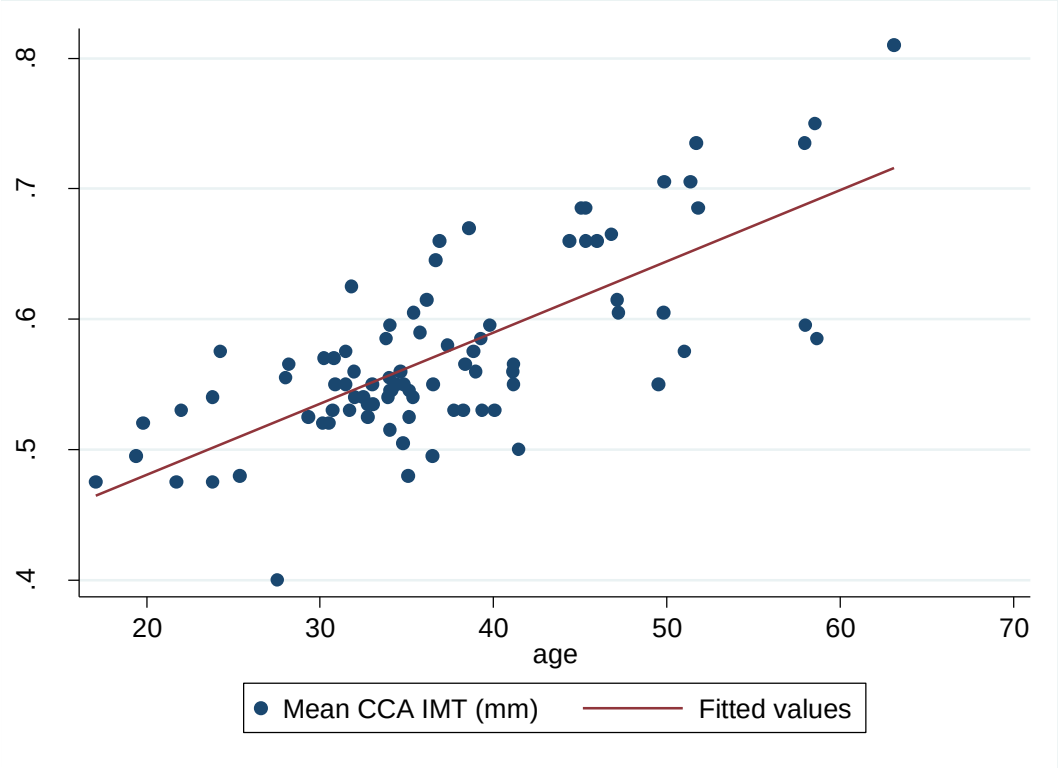


Figure 4-25 Mean CCA IMT according to age for adult Malawians without HIV infection



4.4.2.5 Analysis for HIV and age effect modification

An effect modification analysis was carried out to test for 'accelerated aging' in participants with HIV infection. The concept of accelerated aging is based on the premise that people with HIV experience age related comorbidities at an earlier age than those without HIV. We therefore sought to assess whether there the effect of HIV on PWV was greater in older age categories.. A linear regression model for PWV with an interaction term for age and HIV found that the addition of HIV increased the effect of age on PWV from a coefficient of 0.065 to 0.070 m/s (p value 0.73), indicating that HIV did not significantly modify the effect of age on PWV. The same analysis performed for mean CCA cIMT found that the addition of HIV resulted in a non-significant reduction of the effect of age on cIMT from a coefficient of 0.005 to 0.004 (p=0.11).

4.4.3 Is HIV independently associated with PWV or cIMT at ART initiation?

4.4.3.1 PWV

Univariate analysis for PWV risk factors is shown in Table 4-10 for continuous variables and Table 4-11 for categorical variables. Those variables with a p value of <0.2 on univariate analysis were considered for inclusion in the multivariate model if they had been identified as a potential confounder (See Figure 4-1 Direct Acyclic Graph of associations between measured variables and Arterial Stiffness). If two or more variables had a p value less than 0.02 but were represented a similar risk factor and would display high co-linearity, the variable with the strongest association with the outcome measurement was chosen to be included in the model. HIV status was forced into the model as this was the variable of interest. Age and sex were also forced into the model as obligate confounders because they are important a priori factors in cardiovascular risk. Diastolic BP, fasting cholesterol, weight and current infection status were entered as important potential confounders. The final model for the effect of HIV on PWV after adjusting for potential confounders is shown in Figure 4-12. HIV is associated with a 12% adjusted increase in PWV in patients initiating ART with CD4<100 cells/uL compared with healthy HIV uninfected participants.

Table 4-10 Univariate analysis of continuous variables and PWV

	Spearman's rho	**P value**	**Included in model**
Age	0.49	<0.0001	X
Weight	0.13	0.01	X
Waist: height ratio	0.10	0.07	
BMI	0.10	0.07	

Heart rate	0.07	0.21	
Systolic BP	0.22	<0.0001	
Diastolic BP	0.31	<0.0001	X
Haemoglobin	0.09	0.06	X
Fasting Cholesterol	0.12	0.03	X
Fasting Glucose	0.07	0.24	
Creatinine	0.14	0.008	
Lymphocytes	-0.03	0.52	
Monocytes	-0.01	0.82	

Table 4-11 Univariate analysis of categorical variables and PWV

	Median PWV (m/s)	**P value**	**Included in model**
HIV infected	7.3		
HIV uninfected	7.2	0.10	X
Male	7.45		
Female	7.1	0.0003	X
Primary school education or less	7.4		
Greater than primary school education	7.2	0.26	
Smoker or ex-smoker	7.3		
Never smoked	7.3	0.49	
Drinks alcohol or past alcohol	7.2		
Never drank alcohol	7.4	0.80	
Pre-existing cardiovascular diagnosis	8.2		
No previous cardiovascular diagnosis	7.3	0.001	
Prescribed CV drugs	8.0		
Never prescribed CV drugs	7.3	0.02	
Clinically hypertensive	7.8		
Not clinically hypertensive	7.2	0.0004	
Current infection	7.5		
No current infection	7.3	0.12	X

Table 4-12 Final multivariate model for the effect of HIV on PWV after adjusting for confounders

Variable	Fold change in PWV	P value	95% CI (min)	95% CI (max)
HIV	1.12	0.02	1.02	1.23
Age (per 10-year increase)	1.18	<0.0001	1.13	1.23
Female sex	0.92	0.07	0.85	1.01
Diastolic BP (per 10 mmHg increase)	1.07	<0.0001	1.03	1.13
Haemoglobin	1.02	0.09	1.00	1.04

4.4.3.2 cIMT

Using the same methodology, the relationship between HIV and common carotid artery cIMT was examined. In addition to HIV, age and sex which were included as forced variables, waist: height ratio, fasting cholesterol, fasting glucose, smoking history and alcohol history were entered into the final model to adjust for potential confounders. Univariate analysis is presented in Table 4-13 and

Table 4-14. There was a small increase in fold change of CCA cIMT of 2% but this was not statistically significant (p=0.26, Table 4-15). Sex was not an important adjusted risk factor for cIMT and although the decision had been made a prior to include age and sex as forced variables, a sensitivity analysis found that performing the model without adjusting for sex did not affect the effect size of the other variables and therefore the pre-defined strategy was adhered to.

Table 4-13 Univariate analysis of continuous variables and common carotid artery IMT

	Spearman's rho	P value	Included in model
Age	0.57	<0.001	X
Weight	0.29	<0.001	
Waist: height ratio	0.30	<0.001	X
BMI	0.30	<0.001	
Heart rate	0.85	0.04	
Systolic BP	0.22	<0.001	
Diastolic BP	0.24	<0.001	
Haemoglobin	0.51	0.24	

Fasting Cholesterol	0.24	<0.001	X
Fasting Glucose	0.08	0.17	X
Creatinine	0.19	<0.001	
Lymphocytes	0.01	0.90	
Monocytes	0.00	0.98	

Table 4-14 Univariate analysis of categorical variables and common carotid artery IMT

	Median CCA IMT	**P value**	**Included in model**
HIV infected	0.56 (0.53 – 0.62)		
HIV uninfected	0.56 (0.53 – 0.61)	0.87	
Male	0.56 (0.53 – 0.61)		
Female	0.56 (0.54 – 0.61)	0.83	
Primary school education or less	0.56 (0.53 – 0.62)		
Greater than primary school education	0.56 (0.53 – 0.61)	0.48	
Smoker or ex-smoker	0.56 (0.54 – 0.61)		
Never smoked	0.55 (0.52 – 0.61)	0.06	X
Drinks alcohol or past alcohol	0.55 (0.53 – 0.61)		
Never drank alcohol	0.56 (0.54 – 0.52)	0.05	X
Pre-existing cardiovascular diagnosis	0.60 (0.56 – 0.71)		
No previous cardiovascular diagnosis	0.56 (0.53 – 0.61)	0.007	
Prescribed CV drugs	0.71 (0.62 – 0.74)		
Not prescribed CV drugs	0.56 (0.53 – 0.61)	0.001	
Clinically hypertensive	0.58 (0.55 – 0.63)		
Not Clinically Hypertensive	0.55 (0.53 – 0.60)	<0.001	
Current infection	0.56 (0.52 – 0.61)		
No current infection	0.56 (0.53 – 0.61)	0.41	

Table 4-15 Final multivariate model for the effect of HIV on CCA IMT after adjusting for confounders

Variable	**Fold change in CCA IMT**	**P value**	**95% CI (min)**	**95% CI (max)**

HIV	1.02	0.26	0.98	1.06
Age (per 10-year increase)	1.11	<0.0001	1.09	1.13
Female sex	1.01	0.72	0.97	1.05
Waist: height ratio	1.50	<0.01	1.11	2.00
Current or previous alcohol	0.96	0.046	0.92	1.00

4.4.4 Which traditional risk factors are associated with PWV and cIMT in Malawian adults, and does the addition of HIV status improve the traditional risk factor model?

A full model was constructed for both outcomes including major traditional cardiovascular risk factors, as identified from the literature: age, sex, history of smoking, systolic BP, waist height ratio, fasting cholesterol and fasting glucose. No participants had a family history of cardiovascular disease and so this factor was not included. This may represent challenges identifying family history of CVD in this setting, or a low rate of previous CVD in the population.

4.4.4.1 PWV

Age, male sex and systolic BP were important risk factors for PWV in the final traditional risk factor model (Table 4-16). Age was the most important factor, with a 21% increase in PWV for every 10-year increase in age. When HIV status was added to the model, HIV infection was associated with an additional 9% increase in PWV (Table 4-16).

Table 4-16 Final model for risk factors associated with PWV in adult Malawians with and without the inclusion of HIV status

Variable	**Traditional risk factors for PWV**				**Traditional risk factors model with HIV status added**			
	Fold change in PWV	P value	95% CI (min)	95% CI (max)	Fold change in PWV	P value	95% CI (min)	95% CI (max)
Age (per 10-year increase)	1.21	<0.0001	1.16	1.26	1.19	<0.0001	1.15	1.24

Female sex	0.91	0.03	0.84	0.99	0.92	0.02	0.85	0.99
Systolic BP	1.04	<0.01	1.01	1.07	1.04	<0.001	1.02	1.07
HIV infection	NA	NA	NA	NA	1.09	0.045	1.00	1.18

4.4.4.2 cIMT

Only age and waist: height ratio were important independent risk factors for cIMT in this cohort of adult Malawians. When HIV was added to this final traditional cardiovascular risk factor model it was not retained.

Table 4-17 Final model for risk factors associated with CCA IMT in adult Malawians

Variable	Fold change in CCA IMT	P value	95% CI (min)	95% CI (max)
Age (per 10-year increase)	1.11	<0.0001	1.09	1.13
Waist: height ratio (per 0.1 cm increase)	1.06	<0.0001	1.03	1.08

4.5 Discussion

In this relatively young cohort of Malawian adults, advanced HIV infection was an important risk factor for elevated PWV two weeks following ART initiation. The risk associated with being HIV positive was higher than that associated with a 10mmHg increase in diastolic BP.

PWV amongst healthy, HIV uninfected participants in this study was higher than in comparable cohorts previously published. For the 30 to 40 age category, median PWV from cohorts in Angola, Europe and USA was approximately 0.9 m/s lower than in HIV uninfected Malawian adults [407-409]. Amongst a community sample of HIV uninfected South Africans, average PWV was 1.3 m/s lower than in this Malawian HIV uninfected cohort and with a similar average age [410]. This may be related to methodological issues in the selection of the HIV uninfected cohort for our study. It may be that by identifying some participants from the VCT clinics, some people, although appearing to be healthy from clinical assessment at time of enrolment, did have some reason for presenting for HIV testing. Or, this discrepancy could be related to the measurement of PWV itself including the use of

different devices. Alternatively, it may be that in this urban cohort of Malawian adults, arterial stiffness may be higher due to background inflammation or other as yet unidentified factors.

In studies that have compared PWV in HIV infected and uninfected participants, HIV has not been identified as an independent risk factor for increased arterial stiffness. Fourie et al found that there was no difference in PWV between HIV infected and uninfected participants and that PWV did not differ within the HIV infected group according to treatment status. However, the HIV treatment naïve group had a much higher average CD4 count than participants in the SHIELD study and participants in the treatment group had received ART for a median of nearly 3 years [411]. Ngatchou et al in Cameroon had a similar average PWV in untreated HIV infected adults but again, the CD4 count was higher and the cohort was an average of 7 years older [160]. Several studies however have demonstrated that low nadir CD4 counts are independently associated with PWV (rather than HIV status itself) [412-414].

Within the HIV infected cohort, one third of patients screened had a CD4 less than 100 and those recruited had a high mortality rate and overall evidence of being more clinically unwell (lower BP, higher heart rate and lower haemoglobin). The clinical application of increases in PWV to the risk of cardiovascular disease in this setting is currently limited. However, we have shown that PWV is feasible and repeatable in low income sub-Saharan Africa.

In contrast, no association was found between HIV and cIMT. There are several potential reasons for this. Firstly, it is likely that the phenotype of cardiovascular disease in the majority of Malawians is still that of hypertension and inflammatory driven disease, as opposed to atherosclerotic disease which would be associated with urbanisation related traditional risk factors such as high cholesterol, diabetes and obesity. Secondly, it may be more likely to see differences in levels of atherosclerosis between those with and without HIV infection in older age groups. Next, it is possible that the pathogenesis of HIV in this setting does not necessarily promote wall thickening, but could, in fact lead to thinning of the artery wall [415]. Lastly, the measurements may not have been sufficiently accurate to find small differences in this relatively small group of patients given the limited reproducibility of cIMT in this study

When comparing out cIMT data to other settings, cIMT seems to be higher in our HIV uninfected Malawian group when compared to healthy European controls assessed in a study by a South African group comparing cIMT readings from the common carotid in a group of HIV infected South African participants to a group of healthy Dutch controls [416]. They found the South African group to reach a cIMT of 0.78mm (termed subclinical atherosclerosis) 10 years younger than their

European counterparts (age 76 versus 66). The age at which our Malawian HIV infected cohort reached this threshold was age 71, lying in between the two cohorts. This would suggest that there may be some acceleration of time to subclinical atherosclerosis amongst Malawian adults with HIV, where there are few traditional cardiovascular risk factors apart from hypertension, but not as much as with South African patients with HIV, where urbanisation related risk factors are more prevalent.

cIMT was much more reliable when measured at the common carotid which is consistent with existing literature [417]. Although the repeatability for the bulb and internal carotid was less reliable in this study, there was no clear evidence that cIMT was higher in these regions for patients with HIV infection than for those without. Both PWV and cIMT were strongly correlated with age and although there was no interaction between age and HIV in predicting PWV in this study, the numbers of older patients were small and this interaction could be further assessed in an older cohort.

The INTERSTROKE study showed that hypertension was the most important risk factor in Africa for all causes of stroke in contrast with other global regions where risk factors such as diet, diabetes mellitus and smoking were important [418]. This is in keeping with our current finding that hypertension remains the most prevalent traditional cardiovascular risk factor in this low income SSA setting.

PWV was closely associated with blood pressure and haemoglobin. High blood pressure is known to be an important factor in the development and propagation of arterial stiffness. The measurement of arterial stiffness is influenced by both blood pressure and plasma viscosity. Blood pressure was measured at the time of PWV assessment and used to produce the calculation, meaning that measurements were adjusted for acute readings. Therefore, the relationship between PWV and blood pressure should reflect accumulated exposure to blood pressure over time. PWV measurements can also be influenced by plasma viscosity, which is the likely explanation for why a higher PWV was associated with higher haemoglobin levels.

Waist height ratio (ratio of waist circumference in cm to height in cm) was the only traditional risk factor associated with cIMT. This would support the hypothesis that atherosclerosis is associated with factors traditionally related to urbanisation in this setting such as dyslipidaemia, diabetes or obesity. However, it is important to note that this study was restricted in only having fasted total cholesterol and not cholesterol differentials. Perturbations may have existed with LDL and HDL values even with normal total cholesterol and these may have given stronger associations with cIMT.

Despite the fact that this is a young cohort with relatively few traditional risk factors 12% of participants were found to have a PWV >10 m/s, and 14% had a cIMT>0.78 (corresponding to pathological cut off values [291, 417]). If a threshold of 8 m/s is taken, as suggested in South African cohort, then one third of participants would qualify as having an elevated PWV [410].

This is the first comparison of arterial stiffness and cIMT in HIV infected and uninfected adults from a low income SSA setting. Further, we present the first data to specifically demonstrate an increase in PWV in HIV infected individuals. It is likely that this difference may be due to the advanced immune suppression in our HIV infected cohort as PWV has never specifically been measured in a cohort of participants with such advanced immune suppression. PWV correlates strongly with some previously documented risk factors and, together with high repeatability, demonstrates both feasibility and utility in this setting.

Clinical application of this increased risk is limited by the lack of validation of PWV in the SSA setting. Clinical validation of both PWV and cIMT may provide a useful and efficient resource for quantification of cardiovascular risk and its management in low income SSA. Lastly, it is recommended that further consideration is given to routine measurement of BP in ART clinics as, along with HIV, this is the main risk for increases in arterial stiffness and is modifiable.

5 CHAPTER 5: IMMUNE ACTIVATION IN ADULT MALAWIANS AND ASSOCIATION WITH ENDOTHELIAL DAMAGE

5.1 Introduction

Chronic immune activation is predominantly characterised by the expansion of T cells expressing an activated cell surface phenotype including HLA-DR and CD38 [72, 419]. It is closely associated with T cell exhaustion (measured by PD-1 expression) and T cell senescence (measured by CD57 expression), which are a result of exposure to a high antigenic load and repeated cell cycling of activated cells [82, 420, 421]. Chronic immune activation is a feature of many non-communicable diseases including auto-immune disorders such as rheumatoid arthritis and SLE [422]. Persistent stimulation of the immune system in these disorders leads to a continued state of systemic inflammation which predisposes patients to endothelial damage and cardiovascular disease.

Most infectious pathogens that are encountered by the immune system are cleared away so that the immune system returns to an inactivated state. However, in the case of HIV (and other infections such as CMV, Hepatitis B and Hepatitis C), the pathogen can continue to replicate despite the initial immune response. Chronic immune activation can persist in people with HIV infection even after they have achieved viral suppression on ART [423, 424]. This state of systemic inflammation has been linked to endothelial damage and cardiovascular disease in high resource settings but the extent to which it contributes to cardiovascular disease in a resource poor sub-Saharan Africa setting is unclear [425].

This chapter aims to assess whether there is any relationship between immune activation and endothelial damage in adult Malawians initiating ART with a CD4 count less than 100 cells/uL and in HIV uninfected healthy controls.

5.2 Specific objectives

This chapter addresses objective 4: “To establish to what extent immune activation is a risk factor for higher cIMT and arterial stiffness”.

5.3 Methods

5.3.1 Study procedures

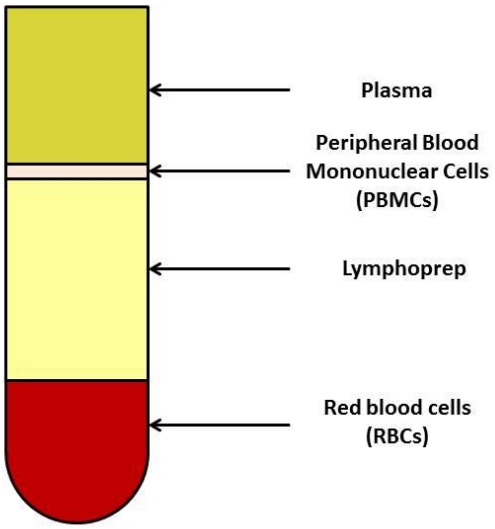
Markers of immune activation were assessed in the same cohort described in Chapter 4.

5.3.2 Characterisation of surface immunophenotype

5.3.2.1 PBMC separation

Blood collected in sodium citrate was diluted in a 1:2 dilution with phosphate buffered saline (PBS) and then slowly added to a 50ml falcon tube containing Lymphoprep (Axis-Shields-Diagnostics) in a 2:1 ratio ensuring that the blood and Lymphoprep did not mix. The samples were then centrifuged at 500g for 25 minutes, with the lowest possible brake speed to prevent mixing of the sample, to generate 4 distinct layers as shown in Figure 5-1. PBMCs were carefully aspirated using a plastic pipette and then added to 10mls PBS.

Figure 5-1 Layers produced following centrifugation of whole blood with lymphoprep



PBMCs were then washed twice in 50mls PBS at 500g for 10 minutes to remove any remaining lymphoprep. After PBS was discarded, 5mls RPMI-1640 culture medium was added. 10uL of PBMC/RPMI solution was mixed with tryptan blue at a 1:10 dilution and 10uL of that was added to both sides of a counting chamber. The number of unstained cells within one large square was then entered into the following equation to estimate the concentration of PBMCs in the sample:

Number of cells in one large square * dilution factor * 10^4 = number of cells/ml

5.3.2.2 Staining

5.3.2.2.1 Overview of staining process

Two staining panels were analysed: one for T cell phenotyping and one for monocyte phenotyping. Each panel consisted of one sample of 50uL of PBMCs in RPMI that was stained with all the

fluorochromes for that panel. For the purposes of compensation, each panel also included one tube for an unstained sample and one single stain tube for each fluorochrome being used in that panel. The unstained and single stained samples were conducted using anti-mouse Igk and negative control compensation particles (BD biosciences) in order to minimise usage of cells for compensation purposes. A staining volume of 100uL was maintained for both cells and compensation tubes. All tubes were incubated in the dark at room temperature for 15 minutes and then the tubes containing cells were washed twice in 100uL PBS at 500g for 10 minutes. Cells were acquired on a 3 laser, 9 colour CyAn Flow Cytometer (Beckman Coulter) in 300uL PBS.

5.3.2.2.2 T cell panel staining and optimisation

For T cells, a staining concentration of $1x10^6$ cells in 50uL was required and cells were spun down and resuspended in RPMI to acquire this concentration. The final T cell panel is shown in Table 5-1. For each antibody, the volume displayed in the table was added to the 50uL PBMCs suspended in RPMI. For the T cell panel, this brought the staining volume up to slightly over 100uL (107.5uL) and therefore no PBS was added.

Table 5-1 Final T cell panel

Target cell surface marker	Fluorochrome	Antibody volume (uL)	Flow cytometer Channel	Voltage
CD57	FITC	10	1	750
CD38	PE	5	2	800
CD8	PE Cy7	0.4	4	600
CD4	V450	10	6	720
CD3	BV510	10	7	720
PD-1	APC	12.5	8	850
HLA-DR	APC Cy7	10	9	900

The panel was designed based on two main factors: the emission spectra detected by the 9 CyAn filter channels, and pairing the brightness of the fluorochromes with the density of cell surface marker expression.

The three CyAn lasers excite fluorochromes at 488nm (Blue laser), 405nm (Violet laser) and 640nm (Red laser). Emissions from these excited fluorochromes are then detected by 9 filters as outlined in Table 5-2. Although one laser can excite several different fluorochromes, only one fluorochrome/antibody combination can be assigned for detection at each channel. The

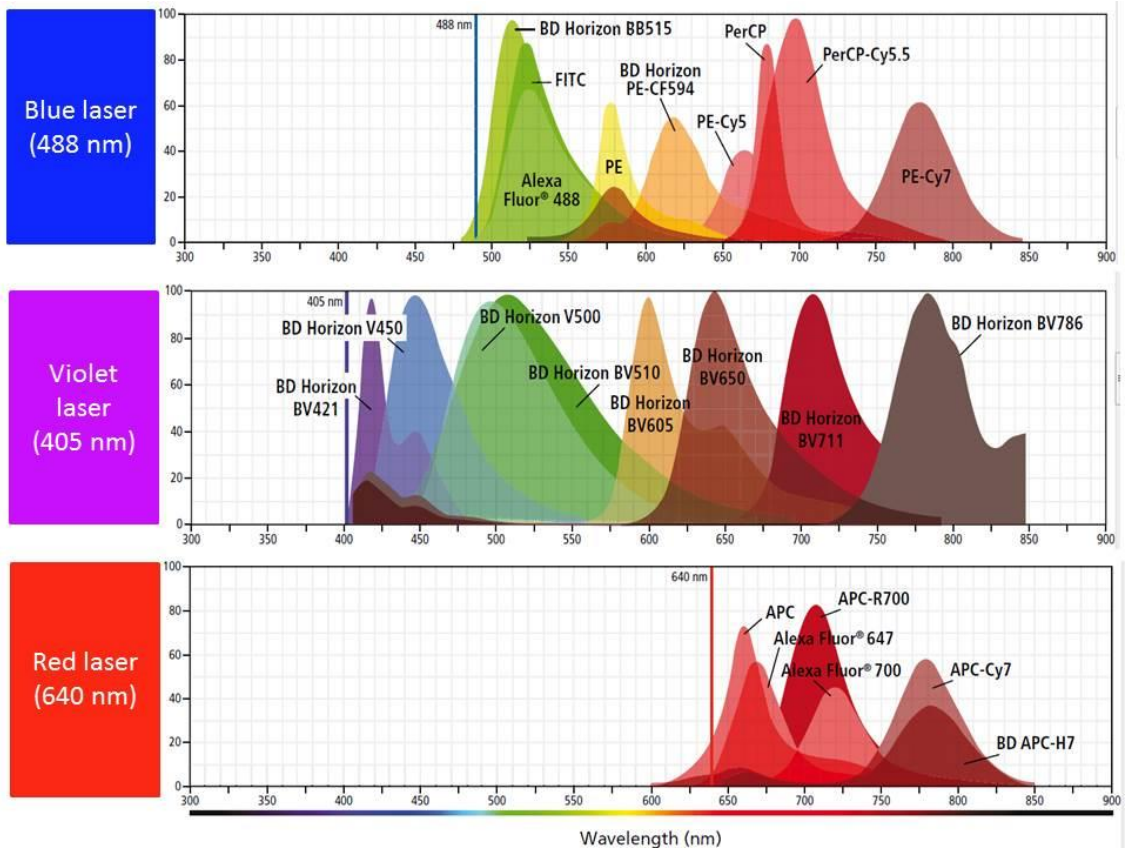
fluorochromes detected for each filter channel are also listed in Table 5-2. Therefore, one of the possible fluorochromes from each channel should be paired with one cell surface antibody of interest. In principle, it is best to choose filter channels which are furthest away from each other to reduce the degree of spectral overlap. The overlap in emission spectra for common fluorochromes according to each CyAn laser is presented in Figure 5-2. For larger panels using more channels, there will invariably be overlap between emission spectra and compensation techniques can be employed to adjust for it (see 5.3.2.3.2).

Table 5-2 CyAn Flow Cytometer laser and filter properties[20]

Laser Light Source	Parameters	Fluorochromes Detected with Standard CyAn Configuration
488 nm	FL1	FITC, GFP, CFSE, Alexa Fluor 488, YFP*
	FL2	PE
	FL3	ECD, PE-Alexa Fluor 610, PI, Qdot605*
	FL4	PC5, PerCP, PC5.5, PerCP-Cy5.5*, 7-AAD, Qdot655*
	FL5	PC7, PE-Alexa Fluor 750, Qdot705*
405 nm	FL6	Pacific Blue, Cascade Blue, DAPI, CFP*
	FL7	Cascade Yellow, Pacific Orange, AmCyan*, Qdot565*
642 nm	FL8	APC
	FL9	APC-Cy7, APC-Alexa Fluor 700*, APC-Cy5.5*, Alexa Fluor 700*

[20] source CyAn ADP High Speed Analyzer Instructions for Use, Beckman Coulter, January 2009, Fullerton CA

Figure 5-2 Spectral overlap of common fluorochromes for each CyAn laser[21]



The brightness of the fluorochromes can be compared using the stain index values (see Figure 5-3 for a comparison). Brightly staining fluorochromes should be paired with antibodies against cell markers that are less commonly expressed to enable detection and dimly staining fluorochromes should be paired with antibodies against cell markers that are more commonly expressed to minimise spillover.

[21] adapted from Fluorochrome/Laser Reference Poster, bdbioseciences.com/colours, BD biosciences

Figure 5-3 Relative brightness of common fluorochromes[22]

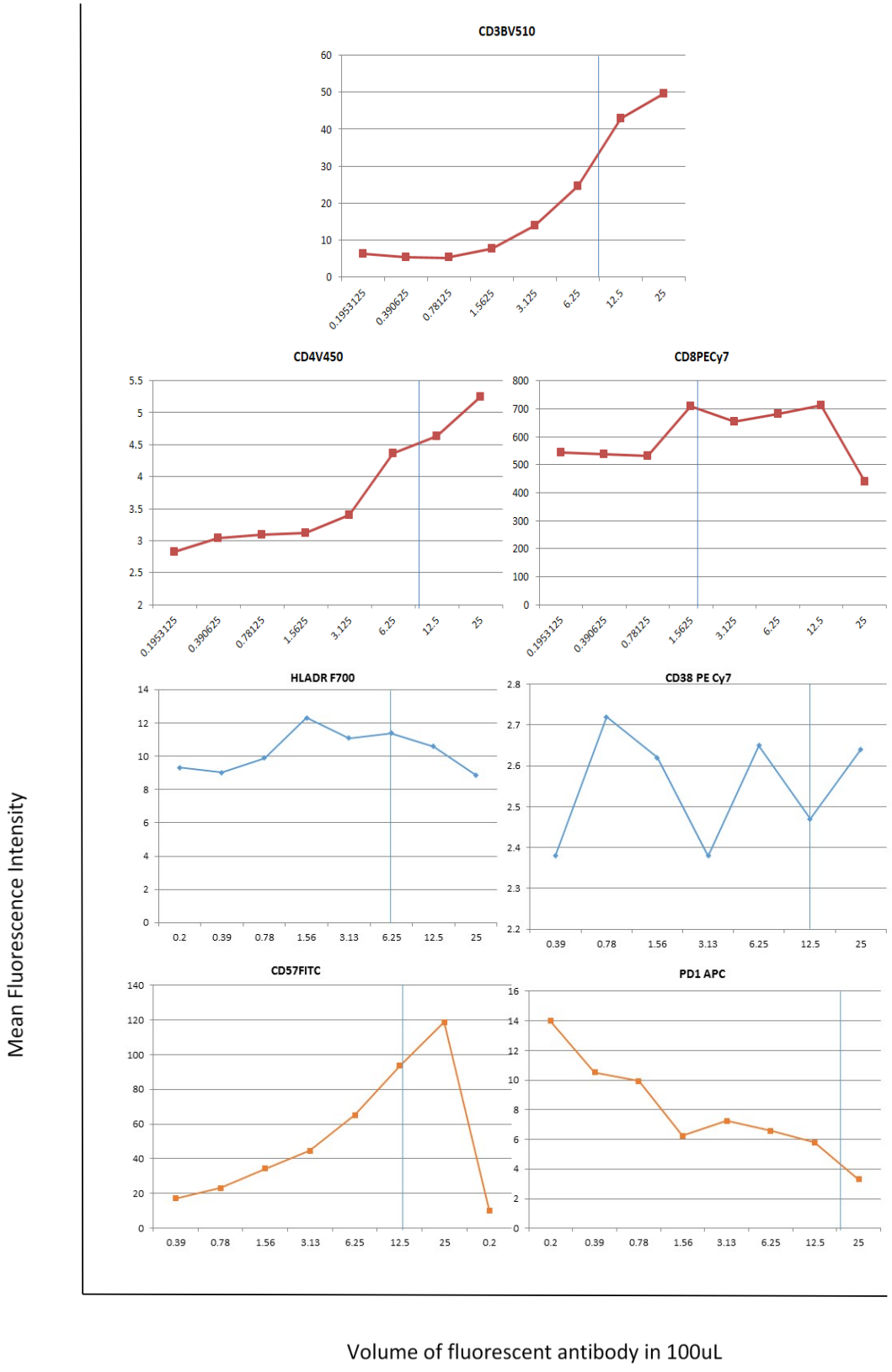
Relative Brightness	Reagent	Filter
BRIGHTEST	Brilliant Violet™ 421	450/50
	PE	575/26
	Brilliant Violet 605	610/20
	BD Horizon PE-CF594	610/20
	PE-Cy5	670/14
	APC	660/20
BRIGHT	PE-Cy7	780/60
	Alexa Fluor® 647	660/20
	PerCP-Cy5.5	695/40
MODERATE	Alexa Fluor® 488	530/30
	FITC	530/30
	BD Horizon V450	450/50
	Pacific Blue™	450/50
DIM	Alexa Fluor® 700	730/45
	PerCP	695/40
	APC-Cy7	780/60
	AmCyan	525/20
	BD Horizon V500	525/20
	BD APC-H7	780/60

The cell surface markers stained for in the T cell panel were CD3 (T cell marker), CD4 and CD8 (T cells of interest), CD38 and HLA-DR (activation markers), PD1 (exhaustion marker) and CD57 (senescence marker). Initially the cell markers were paired with fluorochromes as follows: CD3 BV510 (BD horizon), CD4 V450 (BD horizon), CD8 PE (Biolegend), CD38 PE Cy7 (BD Pharmingen), HLA-DR AF700

[22] source Fluorochrome Reference Chart, bdbiosciences.com/colors, BD biosciences

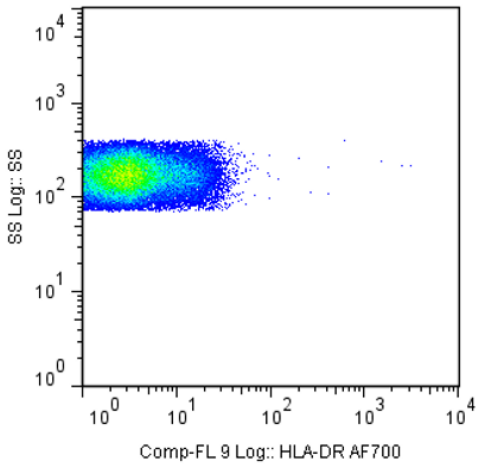
(BD Pharmingen), PD1 APC (BD Pharmingen) and CD57 FITC (BD Pharmingen). The volume of fluorescent antibody to be used was first assessed individually and then tested as part of the panel. Higher volumes of antibody can produce a higher intensity signal up to an optimal point, past which the intensity plateaus and can even decrease. Titrations for each antibody initially tested for our T cell panel are shown in Figure 5-4 with the final volume of antibody used represented by the blue line. For antibodies that did not demonstrate a clear plateau (CD38 PECy7 and PD1 APC), higher volumes were chosen to be sure that sufficient antibody was being used.

Figure 5-4 Antibody titrations for T cell panel

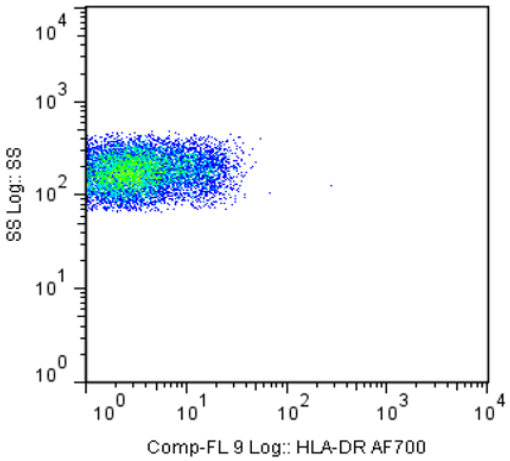


When the full panel was then tested together, two issues were identified. Firstly, HLA-DR was not staining as brightly as would be expected. Figure 5-5 part A shows HLA-DR mean fluorescence intensity (MFI) on PBMCs without HLA-DR AF700 staining and part B shows the same patient sample with HLA-DR AF700 staining. Good separation of the HLA-DR population is not achieved when stained with AF700. Increased volumes of antibody did not improve the MFI of the HLA-DR staining (see Figure 5-6) and so HLA-DR was tried on various different fluorochromes (Figure 5-7). The best separation between HLA-DR negative and positive populations was found with HLA-DR APC Cy7 (BD Pharmingen) staining.

Figure 5-5 Comparison of HLA-DR expression using AF700 fluorochrome compared to APC Cy7



A. Cells stained with all antibodies in the T cell panel apart from HLA-DR (HLA-DR FMO)



B. Cells stained with all antibodies in the T cell panel including HLA-DR

Figure 5-6 Comparison of different volumes of HLA-DR AF700 on HLA-DR separation

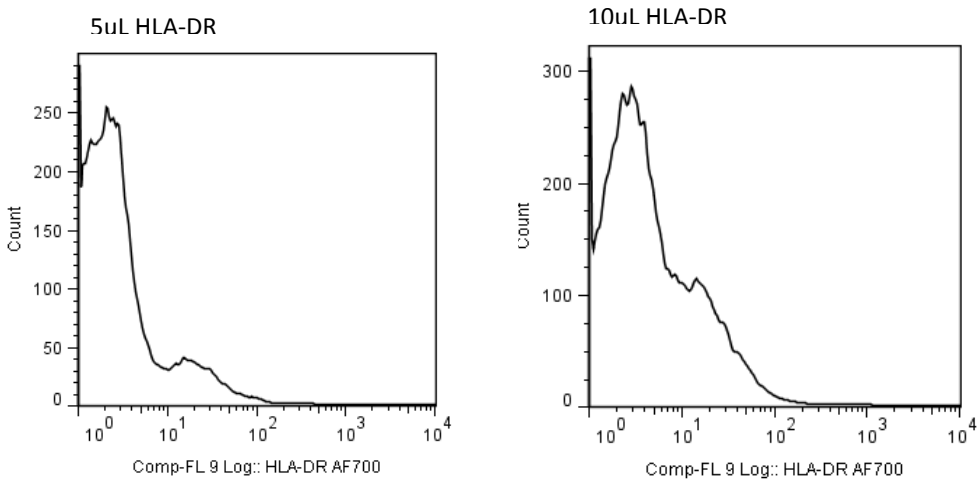
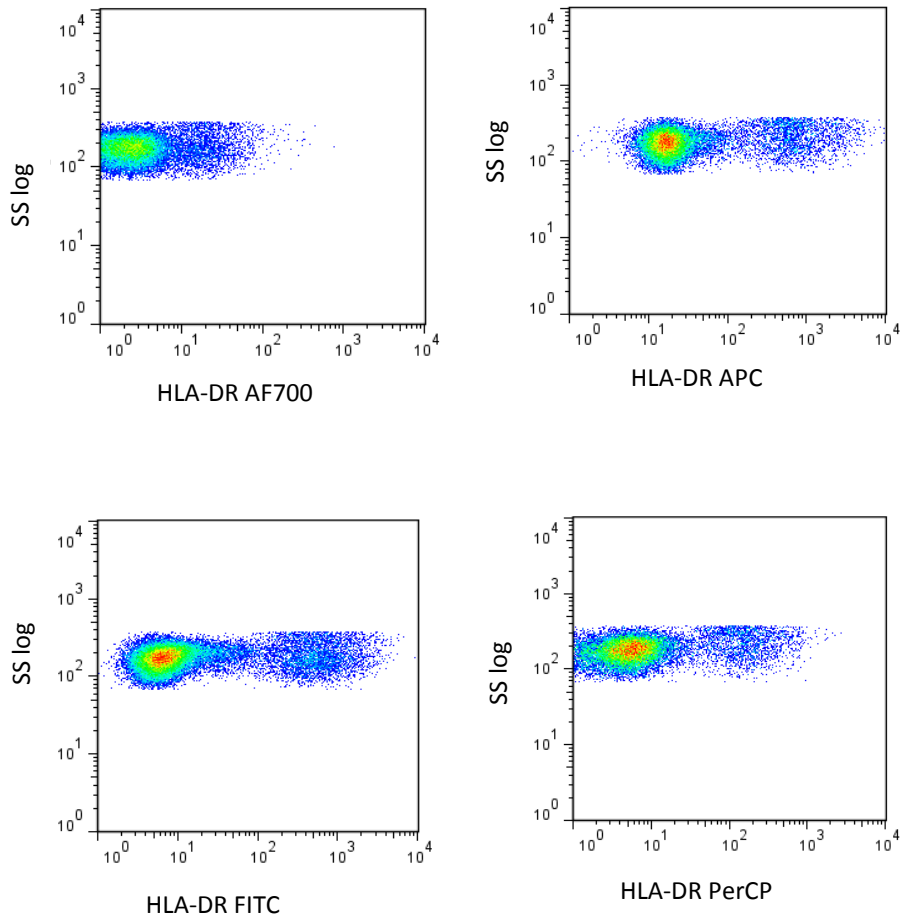
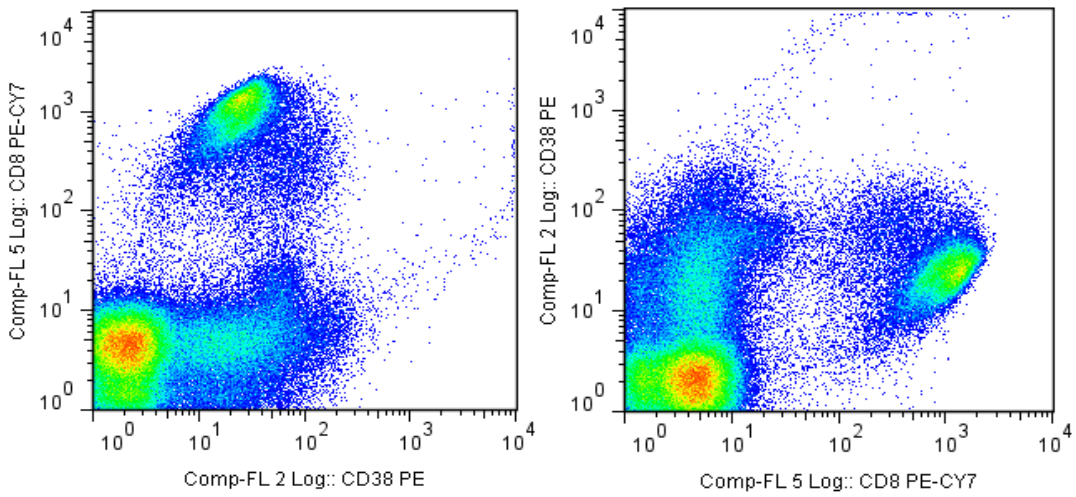


Figure 5-7 Comparison of different fluorochromes for staining HLA-DR



Secondly CD8 PE was spreading into the CD38 PE Cy7 channel making compensation difficult. Figure 5-8 shows post compensation plots for CD38 PE versus CD8 PE Cy7 and vice versa. These plots show staining on all PBMCs following maximum compensation. The populations remain misaligned. The use of a different clone and different volumes of CD38 PE did not resolve the overspill.

Figure 5-8 Compensation plots for CD8 PE-Cy7 and CD38 PE



Therefore, the fluorochrome for staining CD8 was changed from CD8 PE to CD8 PerCP (Biolegend) and the fluorochrome for CD38 was changed from CD38 PE Cy7 to CD38 PE (BD Pharmingen).

During panel optimisation, the voltage levels for each filter channel were also reviewed. The channel voltage can also modify the MFI detected for a population – lower voltages can reduce separation of populations, but voltages that are too high can lead to the loss of events from the plots. During panel optimisation, a range of voltages were tried for each channel until the optimum separation was achieved.

5.3.2.2.3 Monocyte panel staining and optimisation

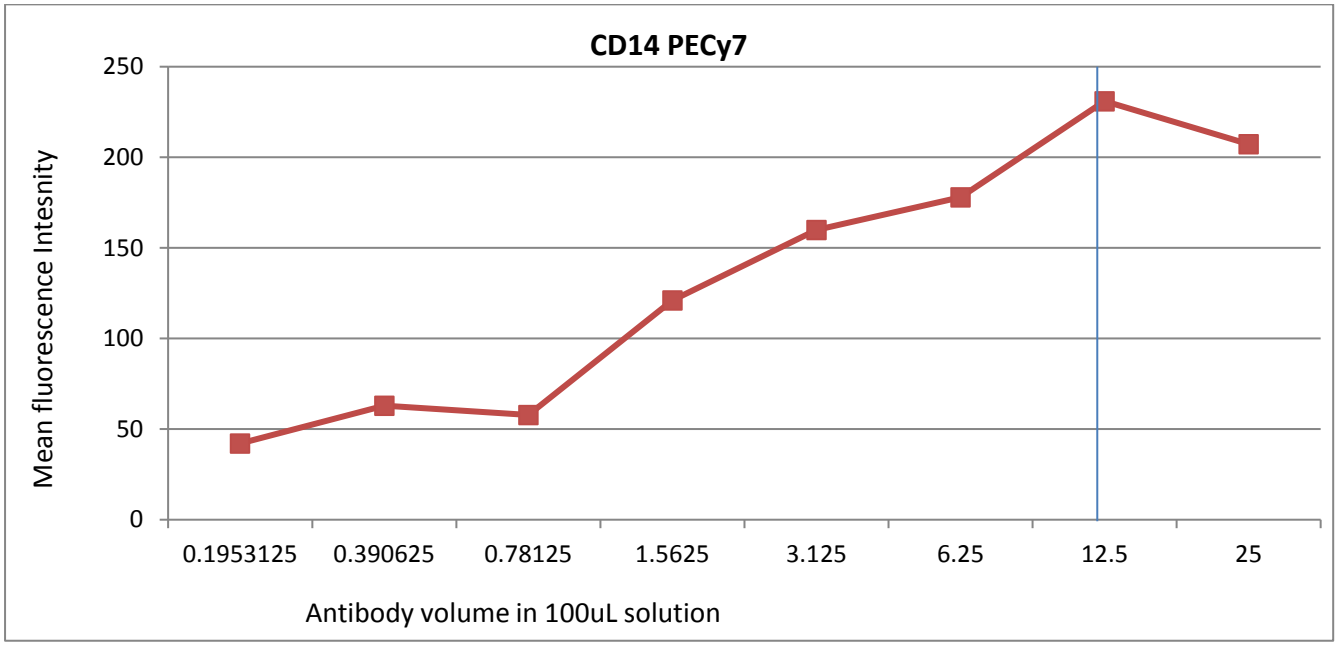
For monocytes, a staining concentration of $1x10^6$ cells in 50uL was initially tried, but too few monocytes were yielded. Because monocytes only make up approximately 3% of PBMCs, for a yield to produce reliable immunophenotyping results of monocyte subsets, a higher initial concentration was necessary. So for monocytes a final concentration of $3x10^6$ cells in 50uL was used. The final monocyte panel is shown in Table 5-3. Because the total volume of antibodies for the monocyte panel was 20uL, 30uL PBS was added to make a total staining volume of 100uL.

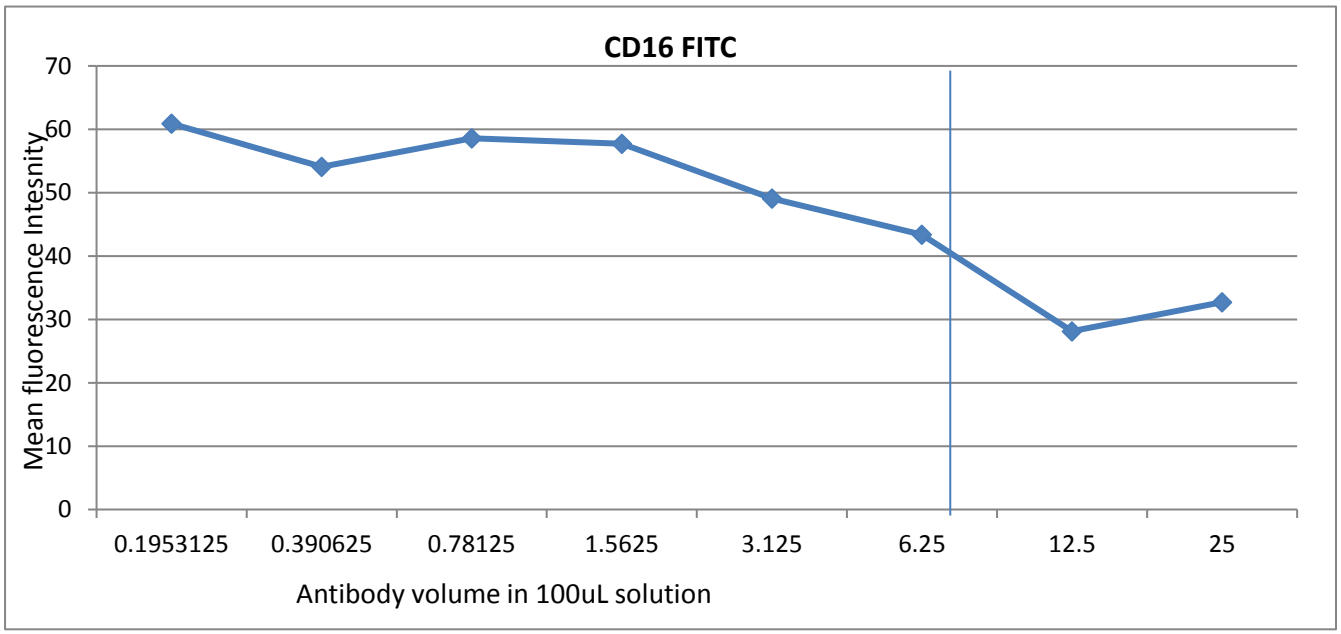
Table 5-3 Final Monocyte Panel

Target cell surface marker	Fluorochrome	Antibody volume (uL)	Flow cytometer Channel	Voltage
CD16	PE	5	2	630
CD14	PE Cy7	10	5	800
HLA-DR	APC Cy7	5	9	850

The cell surface markers stained for in the monocyte panel were HLA-DR (used as a monocyte marker to identify monocyte population), CD14 (ubiquitous monocyte marker) and CD16 (to identify monocyte subsets). Optimal antibody volumes were also tested for the CD14 PE Cy7 (BD Pharmingen) and CD16 PE (BD Pharmingen) markers and titration results are shown in Figure 5-9 (final antibody volume used represented by blue line). Because a clear plateau wasn't seen with CD16 FITC and it is part of a small panel, 5uL was chosen as per manufacturers recommendations and led to a good separation of CD16 FITC populations when the full panel was tested.

Figure 5-9 Antibody titrations for monocyte panel





Apart from switching HLA-DR from AF700 to APC Cy7 for the same reasons described in the T cell panel, no further adjustments were made to the initial monocyte panel.

5.3.2.3 Flow cytometry

5.3.2.3.1 Overall method

CyAn maintenance schedules including cleaning and monitoring of coefficient of variation thresholds were adhered to according to manufacturer guidance. A system clean was performed on machine

start up every day and a sample clean was performed prior to acquiring each new set of samples. Standard protocols for both the T cell panel and monocyte panels were designed and stored on the SUMMIT software (Beckman Coulter) and used consistently for sample acquisition. Compensation tubes were acquired before stained tubes and the acquisition rate was set to slow to minimise doublets. Resulting flow plots were saved and then exported to FlowJo software (Treestar, Inc.) for analysis.

5.3.2.3.2 Compensation

Unstained and single stained compensation beads were used as a reference to automatically generate compensation matrices for both T cell and monocyte panels which were then manually verified. The first 100 samples were individually compensated using this method. Compensation matrices generated from these data were then used to program SUMMIT software to automatically compensate stained samples following acquisition. The final compensation matrices for the T cell panel and the monocyte panel are shown in Figure 5-10 and Figure 5-11 respectively. The grid at the top of each figure gives the percentage of compensation needed for each pairing of fluorescent antibodies. The dot diagrams show staining of all PBMCs for each pairing of fluorescent antibodies – the blue dots represent uncompensated cells and the black dots represent compensated cells.

Figure 5-10 Compensation matrix for T cell panel

	FL 2 Log :: CD1...	FL 4 Log :: Perc...	FL 1 Log :: FITC...	FL 8 Log :: APC,...	FL 7 Log :: BV51...	FL 9 Log :: HLA-...	FL 6 Log :: v450,...
FL 2 Log	100	8.364	1.0571	0	0	0	0.0215
FL 4 Log	2.1227	100	1.626	0	0.0923	0	6.2263
FL 1 Log	27.905	1.9345	100	0	0.1007	0	0.0302
FL 8 Log	0.0195	0.7483	0.02	100	0	1.6141	0.0392
FL 7 Log	0.8876	0.0405	1.4145	0	100	0	22.3968
FL 9 Log	0.1511	0.1143	0.0277	3.7059	0	100	0.0464
FL 6 Log	0.1595	0	0.0335	0	7.5312	0	100

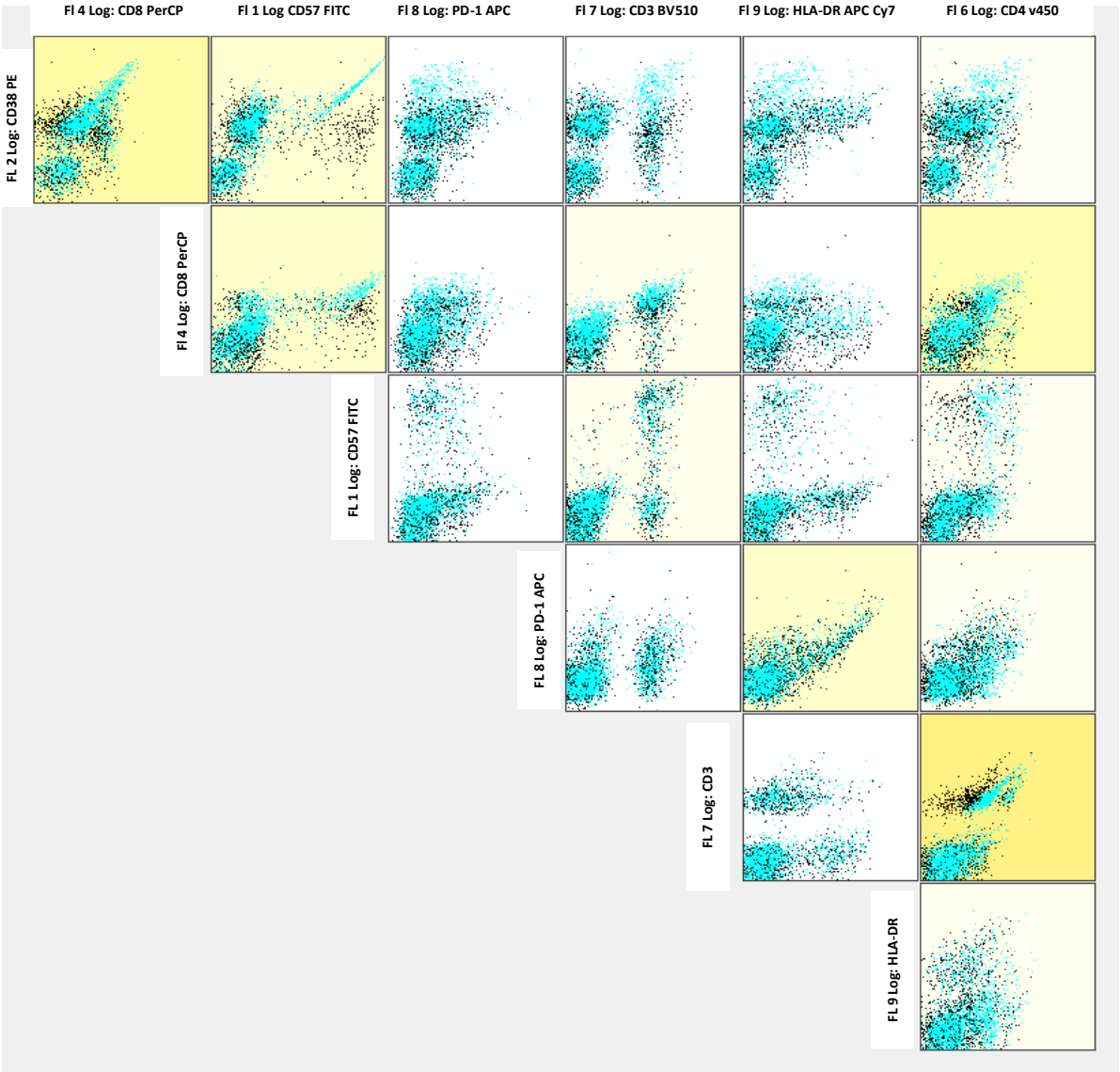
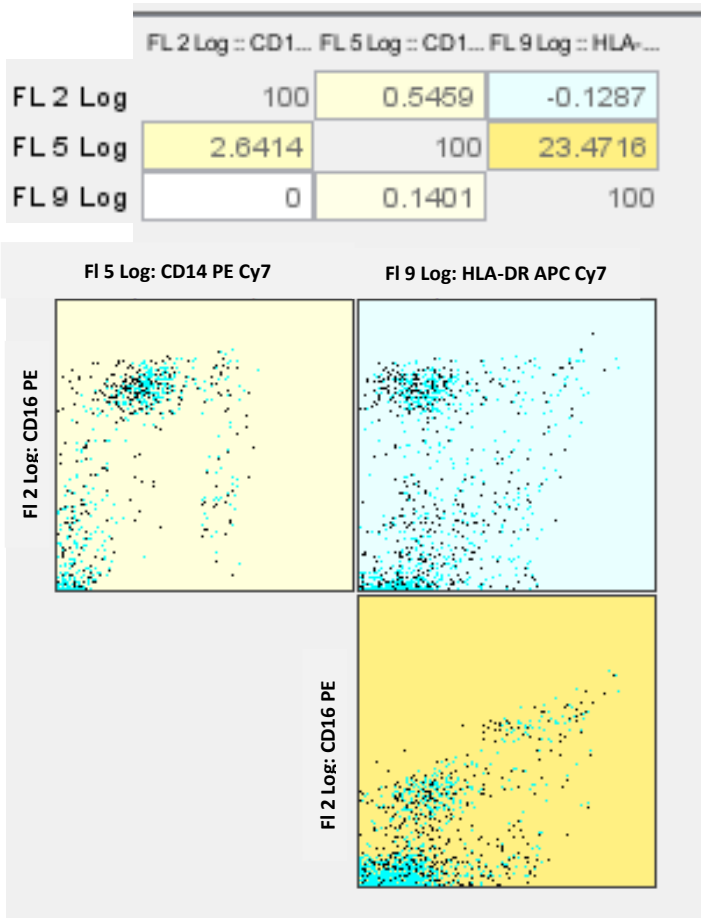


Figure 5-11 Compensation matrix for monocyte panel



5.3.2.3.3 Gating strategy

A standardised gating strategy was developed for each panel. The gating strategy for the T cell panel is presented in Figure 5-12. From top left of the figure, singlets were identified on forward scatter linear versus area. The lymphocyte population was then identified on forward and side scatter light properties and was split into CD3+ and CD3- cells. CD3+ cells were divided into CD8+ and CD4+ cells. CD8 and CD4 cells were then individually gated to look for activation (HLA-DR versus CD38) and then to look for senescence / exhaustion (CD57 versus PD1). Expression of HLA-DR and PD1 on CD8 and CD4 T cells is continuous (as opposed to either being present or not present) and so positive and negative populations are not clearly separated. However, CD3- cells show distinct populations of HLA-DR and PD-1 positive cells and therefore can be used as a standardised method of defining positive and negative populations. This approach was validated using FMOs (fluorescence minus one samples) meaning that cells were stained with all fluorescent antibodies in the panel apart from the antibody of interest (either HLA-DR or PD-1). This provides a negative cut off value for that marker. In Figure 5-13, panel A shows where the gates are placed to identify the HLA-DR positive population on CD4 and CD8 T cells using the gate provided by identifying the negative HLA-DR population for

CD3- cells. Panel B shows where these gates would be placed if we had used FMOs to identify the HLA-DR negative population for that same sample. Similar percentages of HLA-DR positive cells are identified using the two methods. The same process was carried out with a PD-1 FMO and is shown in Figure 5-14.

Figure 5-12 T cell panel gating strategy

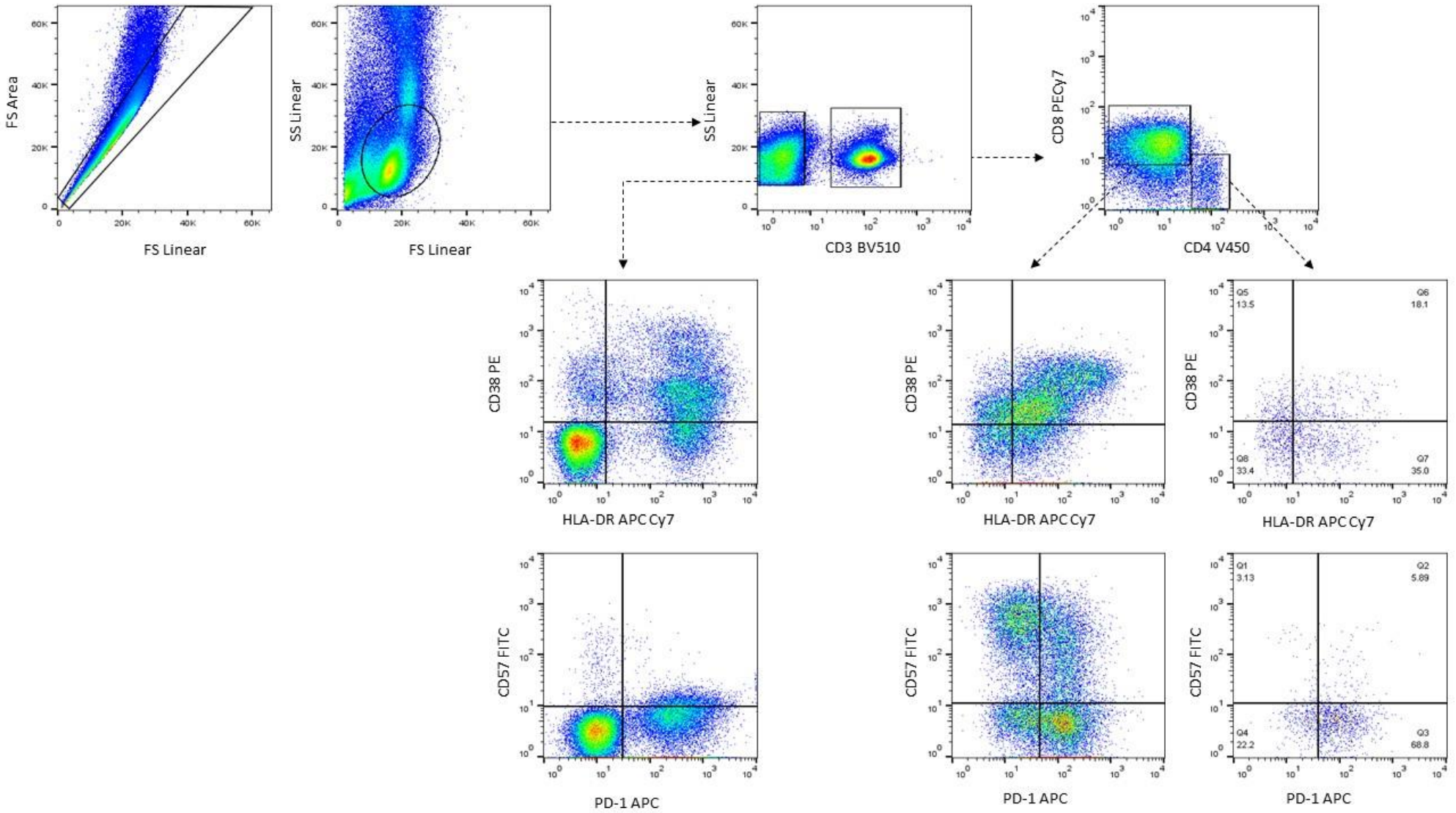


Figure 5-13 Comparison of CD3- and FMO gating strategy to gate HLA-DR positive CD4 and CD8 T cells

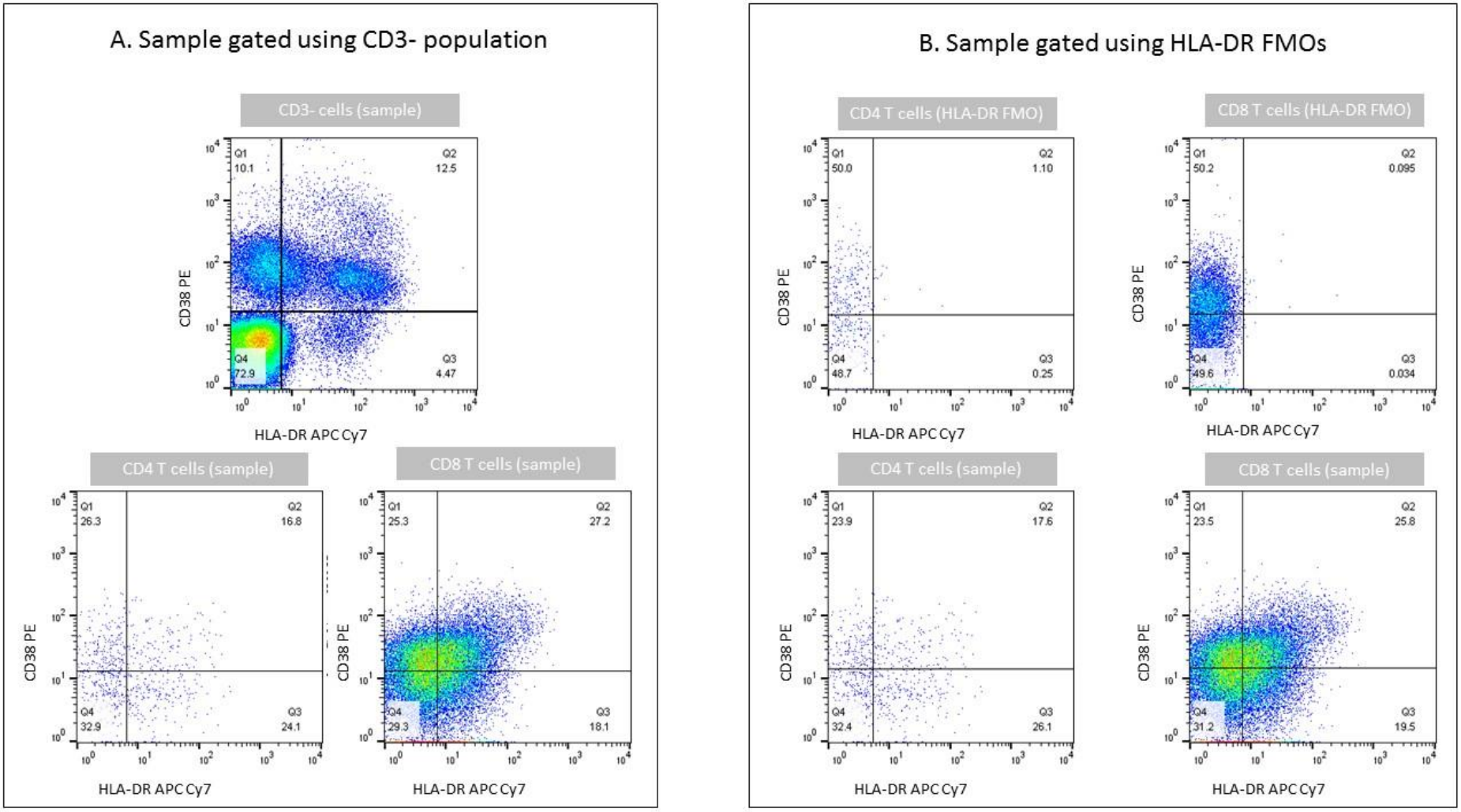
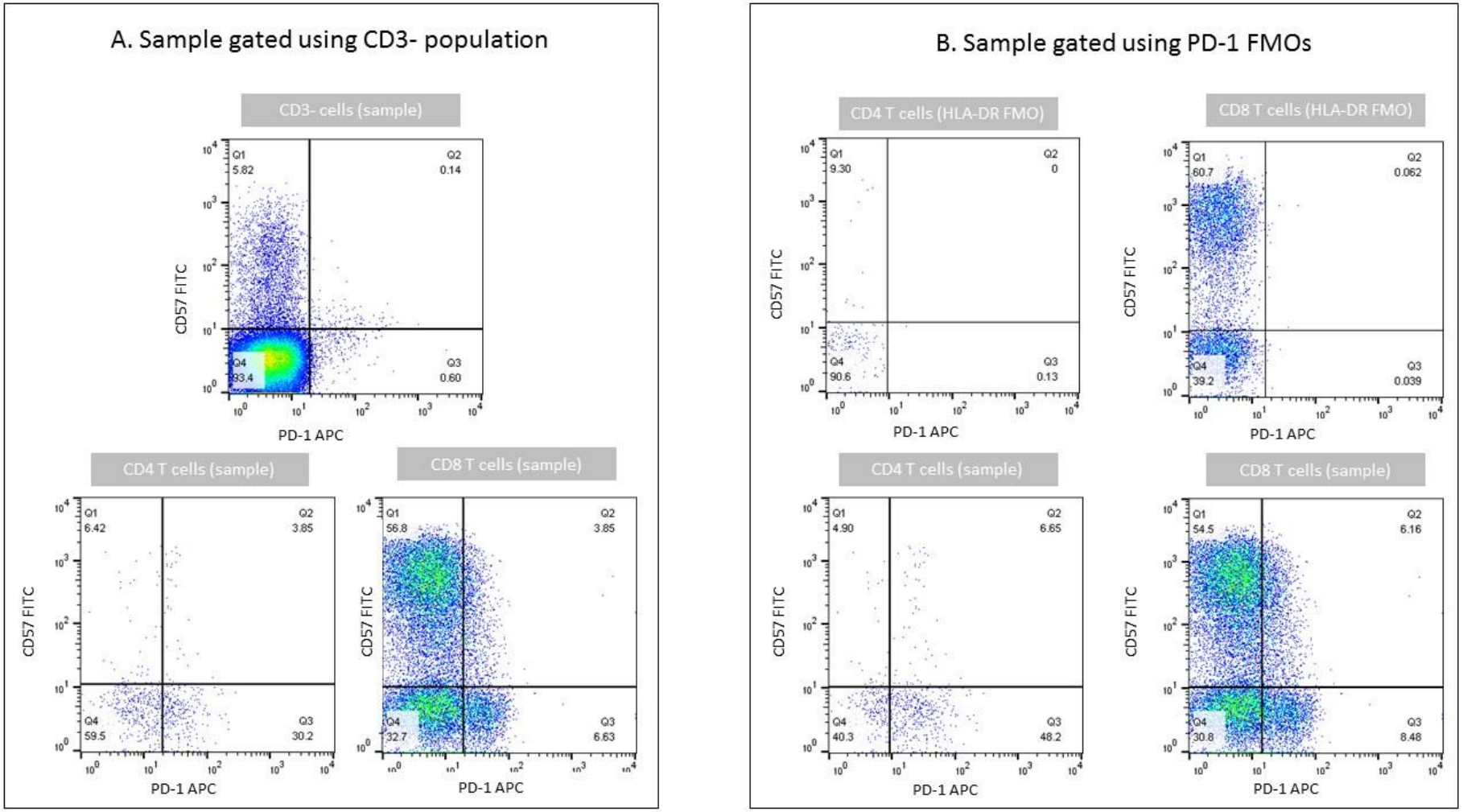


Figure 5-14 Comparison of CD3- and FMO gating strategy to gate PD-1 positive CD4 and CD8 T cells



The gating strategy for the monocyte panel is shown in Figure 5-15. Singlets were again identified on forward scatter linear versus area. A HLA-DR positive population was then identified based on forward scatter light properties (representing cell size). This population was then used to find CD14 and CD16 monocyte subsets. The method used to identify monocyte subsets in this work is based on accepted monocyte subset gating strategy [83]. To provide further evidence differentiating between monocyte and NK cell populations (both express HLA-DR and can express CD16), a CD56 marker was added to the monocyte panel. Monocyte and NK cell populations were first identified on the HLA-DR versus side scatter plot (see Figure 5-16). Although both the monocyte and NK cell populations expressed CD56, there were no identifiable monocyte populations when gating for CD14 and CD16 on the NK cell population.

Figure 5-15 Monocyte gating strategy

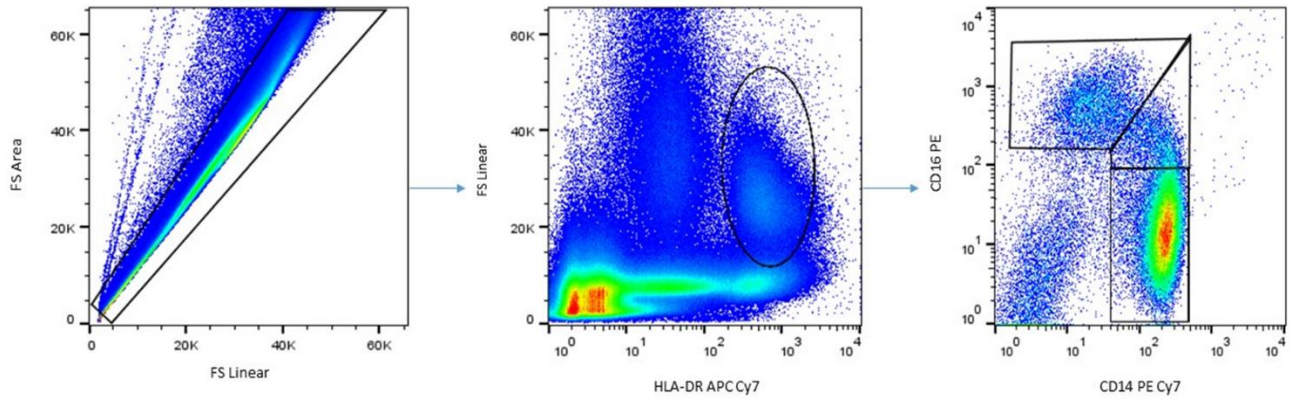
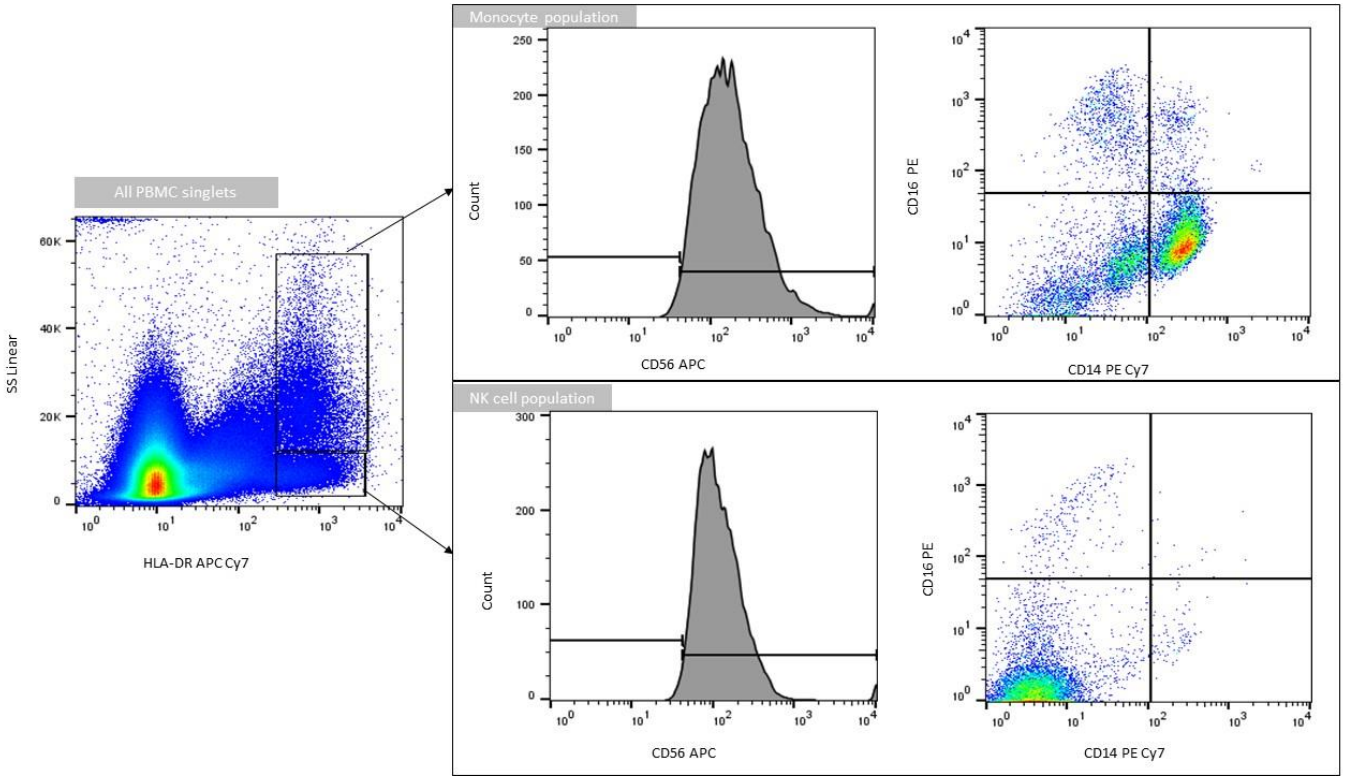


Figure 5-16 Differentiating between monocyte and NK cell populations



If the number of CD4 or CD8 T cells or monocytes available for analysis in any sample was fewer than 1000 cells, the sample was excluded from analysis due to the potential for inaccurate results at low cell counts.

5.3.3 Sample size calculations

The sample size calculation for objective 4 is described in the general methods section (subsection 3.6.1)

5.3.4 Statistical analysis

5.3.4.1 Classification of immune markers

T cell activation was classified as the percentage of T cells (either a CD4 or a CD8 T cell) that expressed HLA-DR or CD38 or both. This is the equivalent to the proportion of cells in the outer three quadrants compared to the percentage of cells in the bottom left quadrant. The percentage of cells expressing HLA-DR irrespective of CD38 was also measured and is referred to as 'HLA-DR+'. Similarly, the percentage of cells expressing CD38 irrespective of HLA-DR was measured and is referred to as 'CD38+'. T cell senescence was defined as the proportion of cells expressing PD1 (they

could be either CD57+ or CD57-). T cell exhaustion was defined as the proportion of cells expressing CD57 (they could be either PD1+ or PD1-).

5.3.4.2 Objective 4: "Establish to what extent immune activation is a risk factor for higher cIMT and arterial stiffness"

Firstly, we performed univariate analysis of all immune markers according to HIV status and, then according to PWV and cIMT values. Wilcoxon Ranksum was used to analyse associations for categorical data and Spearman rho for continuous data.

Next, we asked the question: "Does immune activation, exhaustion or senescence explain the effect of HIV on PWV?" Immune markers were added one at a time to the model constructed to assess the effect of HIV on PWV in subsection 4.4.3.

Lastly, we asked the question: "Does the addition of immune markers to traditional cardiovascular risk factor models improve the risk prediction for arterial stiffness or intima-media thickness?" Again, immune markers were added one at a time to the final model of traditional risk factors associated with both PWV and cIMT.

5.3.4.3 Identification of immune activation categories by principal components analysis

A Principal Components Analysis of immune markers was performed to group patients according to immune marker profiles. A principal components correlation and cluster dendogram were both constructed using Stata (version 13.1) to identify the most appropriate number of groups. The corresponding number groups were used to generate clusters of data which were then examined in a scatter graph and according to HIV status.

5.4 Results

5.4.1 Description of T cell surface immune phenotypes according to HIV status

The percentage of CD4 and CD8 T cells expressing markers of immune activation, exhaustion and senescence are shown in Table 5-4. The 'CD4 activated' phenotype represents the total percentage of cells expressing either CD38 or HLA-DR. 'CD4 Exhaustion' represents all cells expressing PD1 (whether CD57 positive or not) and 'CD4 Senescence' represents all cells expressing CD57 (whether PD1 positive or not). The same classifications have been used for CD8 T cells.

The percentage of CD4 T cells expressing all activation, exhaustion and senescence markers is higher in HIV infected compared to uninfected participants. The CD4+CD38+HLA-DR- phenotype is lower in HIV infected participants, which may represent the fact that HLA-DR is the more important marker

of activation. For CD8 T cells, double expression of CD38+HLA-DR+ is significantly higher in HIV infected participants compared to uninfected. Immune markers measuring PD1+ are significantly higher in HIV infected than uninfected groups. The percentage expression of senescent cells increased with age for CD8 T lymphocytes, but not for CD4 T lymphocytes, and this was true for HIV infected and uninfected participants (see Figure 5-17 and Figure 5-18).

Table 5-4 T cell expression of immune markers according to HIV status

		HIV infected n=202	HIV uninfected n=94	P value
Absolute CD4 count (cells/uL)		42 (18 – 62)	NA	NA
HIV viral load (copies/ml)		113,560 (41,747 – 293,663)	NA	NA
CD4 T cells	**CD4/CD8 ratio**	0.09 (0.04 – 0.20)	1.39 (1.02 – 2.15)	<0.00001
	CD4 %	8.2 (4.1 – 16.5)	58.1 (50.5 -68.3)	<0.0001
	CD4 CD38+HLA-DR-	26.7 (17.6 – 39.6)	35.7 (28.1 – 42.7)	<0.001
	CD4 CD38+HLA-DR+	22.25 (11.4 – 33.8)	5.0 (2.6 – 8.7)	<0.0001
	CD4 CD38-HLA-DR+	17 (10.5 – 23.3)	6.7 (4.1 – 11.8)	<0.0001
	CD4 Activated	74.5 (61.5 – 85.7)	53.5 (40.5 - 65.0)	<0.0001
	CD4 CD57+PD1-	5.3 (2.6 – 8.7)	3.2 (1.7 – 5.7)	<0.001
	CD4 CD57+PD1+	7.8 (4.6 – 14.4)	2.2 (1.4 – 4.0)	<0.0001
	CD4 CD57-PD1+	40.9 (24.8 – 52.8)	12.8 (7.4 – 19.2)	<0.0001
	CD4 Exhausted	54.0 (31.3 – 66.9)	15.0 (9.3 – 23.6)	<0.0001
	CD4 Senescent	14.8 (9.0- 23.9)	6.7 (3.6 – 9.03)	<0.0001
CD8 T cells	**CD8%**	81.8 (83.5 – 95. 9)	41.1 (31.7 – 50.0)	<0.0001
	CD8 CD38+HLA-DR-	24.2 (16.8 – 37.0)	23.9 (11.8 – 41.8)	0.85
	CD8 CD38+HLA-DR+	34.1 (21.4 – 48.5)	11.2 (6.1 – 19.1)	<0.0001
	CD8 CD38-HLA-DR+	9.9 (5.6 – 17.1)	9.1 (5.4 – 22.5)	0.63
	CD8 Activated	78.6 (64.5 – 87.6)	59.6 (39.8 – 72.7)	<0.0001
	CD8 CD57+PD1-	31.8 (22.8 – 42.2)	28.5 (18.6 0-37.6)	0.05
	CD8 CD57+PD1+	20.3 (12.2 – 26.8)	10.3 (5.9 – 15.2)	<0.0001
	CD8 CD57-PD1+	17 (11.8 – 25.2)	9.6 (5.8 – 13.9)	<0.0001
	CD8 Exhausted	37.7 (28.6 – 49.5)	20.9 (12.2 – 29.4)	<0.0001

	CD8 Senescent	53.6 (43.9 – 64.1)	39.7 (26.5 – 53.4)	<0.0001

Figure 5-17 Median % Senescent CD8 T cells according to age group and HIV status

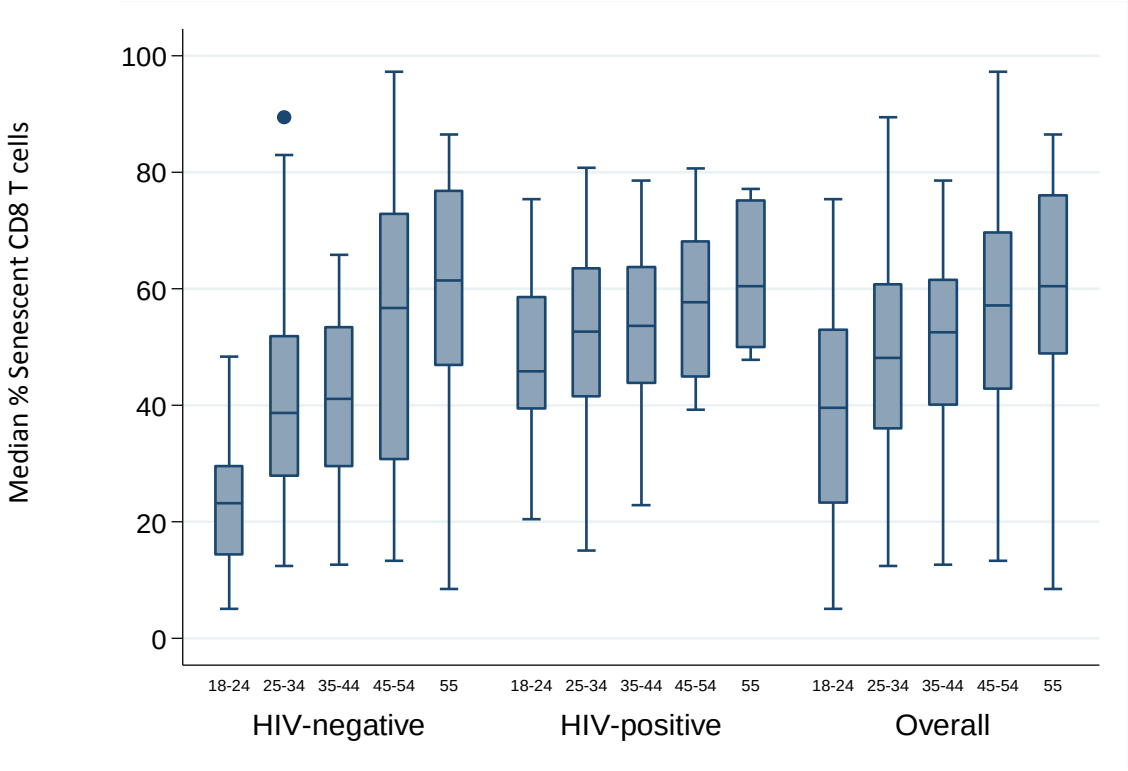
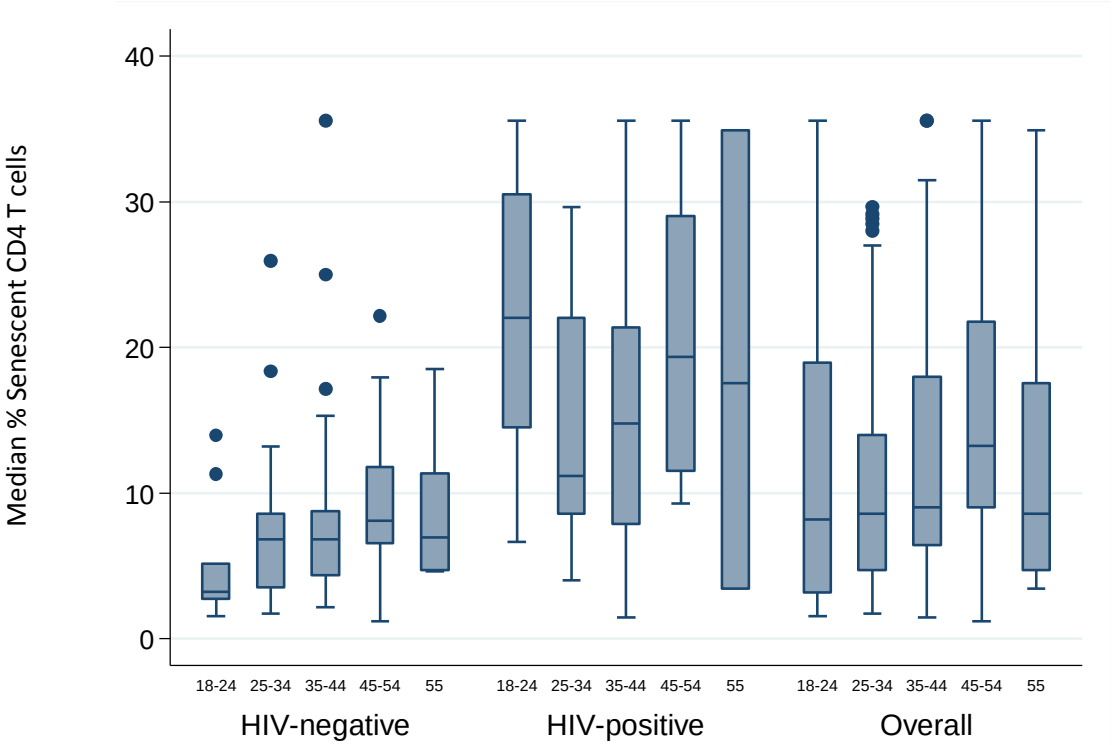


Figure 5-18 Median % Senescent CD4 T cells according to age group and HIV status



5.4.2 Description of monocyte cell surface subtypes according to HIV status

There was no difference in distribution of monocyte subset according to HIV status as shown in Table 5-5. It had been hypothesised that in HIV infection, intermediate monocytes would be expanded due to their pro-inflammatory properties. However, instead it was noted that there was a marked expansion of the nonclassical monocyte subset in both HIV infected and uninfected participants. From this it was hypothesised that Malawian adults might have an expanded population of nonclassical monocytes due to repeated malarial or bacterial infections.

Table 5-5 Distribution of monocyte subsets according to HIV status

		HIV infected n=202	HIV uninfected n=94	P value
Monocytes	**Classical**	75.5 (66.3 – 82.5)	75.4 (64.9 – 80.7)	0.79
	Intermediate	9.9 (6.0 – 13.1)	9.4 (6.7 – 14.4)	0.99
	Nonclassical	9.0 (20.6 – 1.6)	13.1 (9.8 – 22.0)	0.59

5.4.3 Description of immune phenotypes in Non-Malawian participants

To begin to test this hypothesis, we also performed immunophenotyping on 6 non-Malawian adults who were born in the UK and lived there until at least the age of 18 years. Volunteers were recruited from the local research institution or hospital. They all had HIV tests carried out to confirm HIV negative status and underwent a symptom screen to ensure there were no signs of current infection. Bloods were then drawn and processed according to the same protocol as the SHIELD participants. There were no differences between CD T cell expression of activation, exhaustion or senescence markers in the HIV uninfected Malawian group as compared to the non-Malawian group (Figure 5-19). Expression of these markers in CD8 T cells tended to be higher in HIV uninfected Malawians as compared to non-Malawians, but these differences did not reach significance (Figure 5-20). However, when assessing monocyte subsets, there was a clear decrease in classical monocytes and increase in nonclassical monocytes in both HIV infected and uninfected Malawians compared to non-Malawians (Figure 5-21). There was no difference between either of the three groups in intermediate monocytes.

Figure 5-19 Comparison of CD4 T cell phenotypes in non-Malawian adults

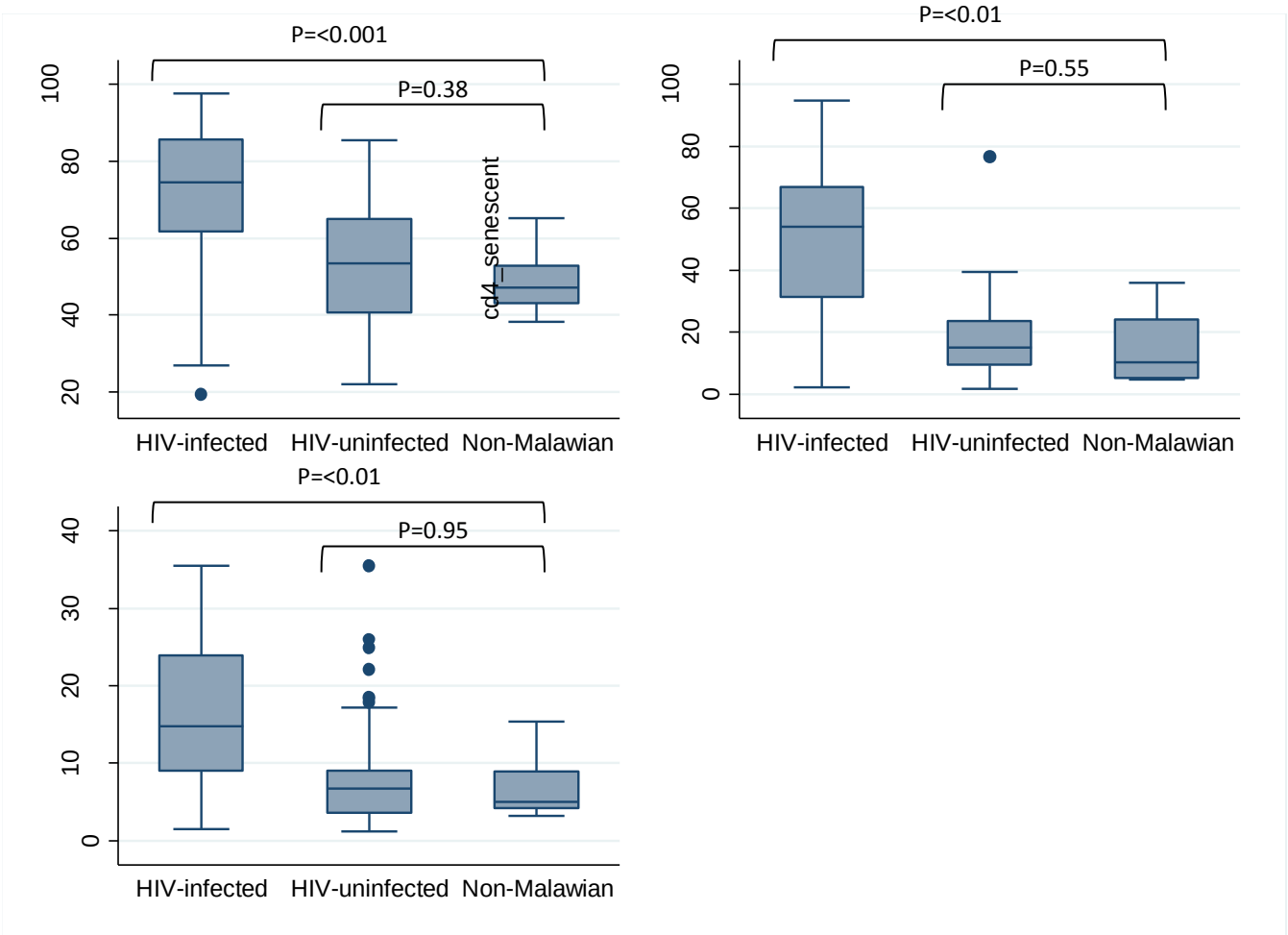


Figure 5-20 Comparison of CD8 T cell phenotypes in non-Malawian adults

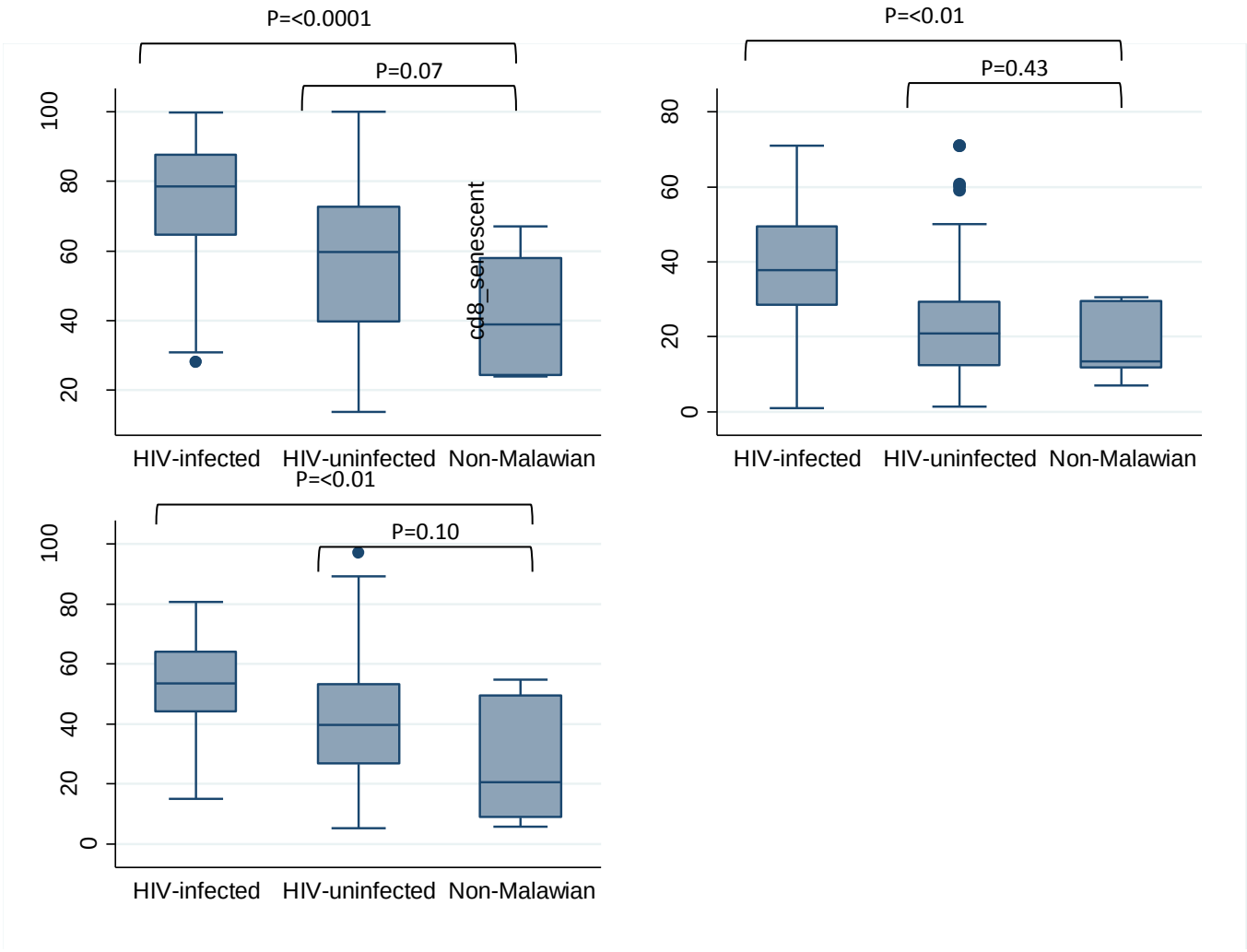
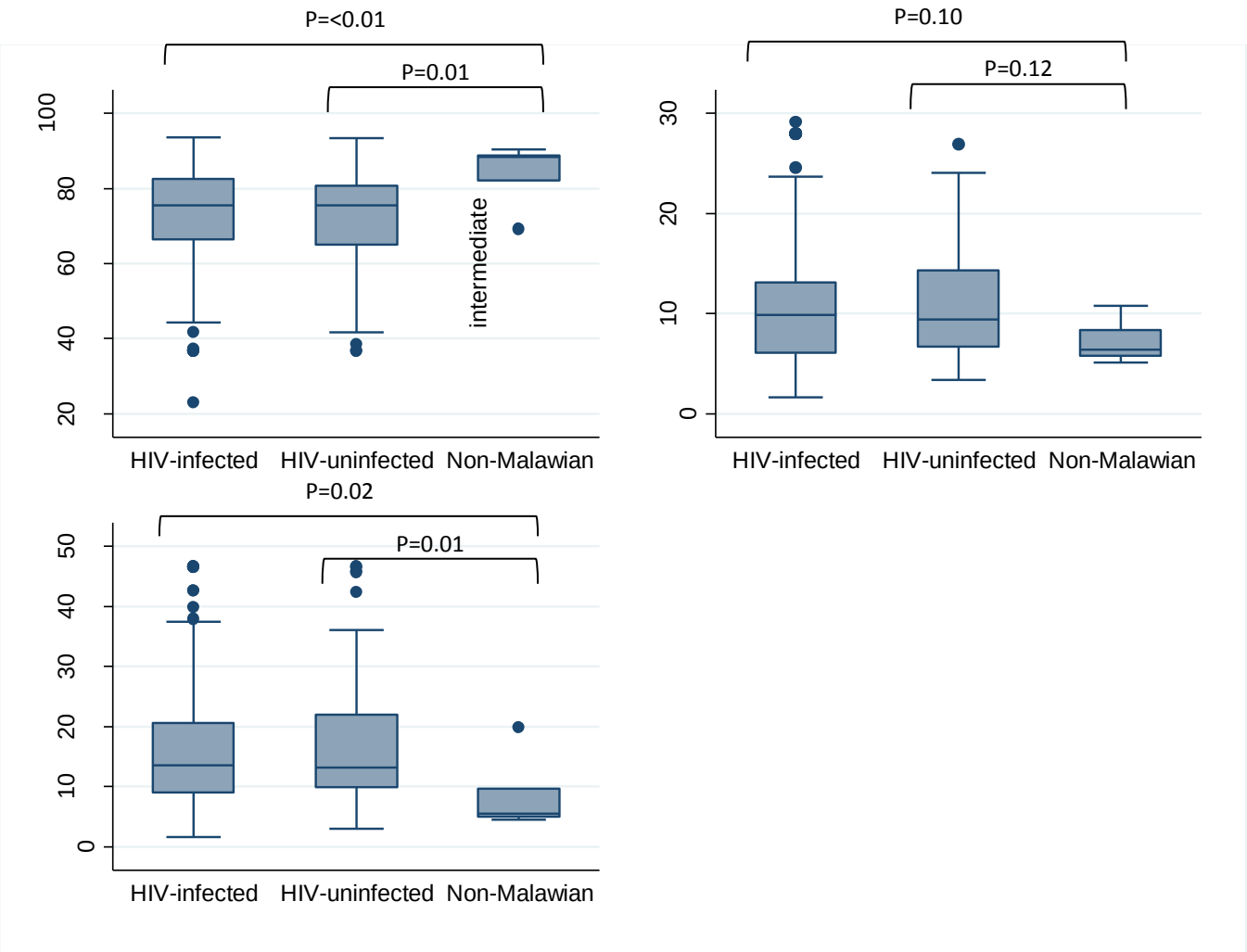


Figure 5-21 Comparison of Monocyte subsets in non-Malawian adults



5.4.4 Association between immune parameters and endothelial damage in adult Malawians

5.4.4.1 Univariate analysis of association between immune parameters and measures of endothelial damage

The percentage expression for each immune marker was assessed for a univariate correlation with the outcome measures as continuous variables (Table 5-6). Both CD4 and CD8 T cell exhaustion and senescence showed borderline significant positive correlations with PWV. There was also a non-significant trend towards a positive correlation between classical monocytes and PWV. Although associations between CD4/CD8% or CD8 activation and cIMT reached significance, the absolute values of the associations were small.

Table 5-6 Univariate correlations between immune markers and PWV or cIMT

		PWV		cIMT	
		Spearman rho n=295	p value	Spearman rho n=259	p value
Absolute CD4 count (cells/uL)		0.002	0.98	0.07	0.34
HIV viral load (copies/ml)		0.006	0.92	0.06	0.44
CD4 T cells	**CD4/CD8 ratio**	0.01	0.86	0.15	0.02
	CD4 %	0.01	0.85	0.15	0.02
	CD4 CD38+HLA-DR-	-0.21	<0.01	0.13	0.13
	CD4 CD38+HLA-DR+	0.05	0.45	-0.09	0.26
	CD4 CD38-HLA-DR+	0.18	0.13	-0.08	0.32
	CD4 Activated	-0.03	0.65	-0.07	0.40
	CD4 CD57+PD1-	0.13	0.07	0.10	0.22
	CD4 CD57+PD1+	0.12	0.09	-0.05	0.55
	CD4 CD57-PD1+	0.11	0.11	-0.09	0.28
	CD4 Exhausted	0.13	0.07	-0.08	0.35
	CD4 Senescent	0.15	0.04	0.06	0.51
CD8 T cells	**CD8%**	-0.01	0.85	-0.15	0.02
	CD8 CD38+HLA-DR-	-0.03	0.59	-0.05	0.48
	CD8 CD38+HLA-DR+	0.04	0.50	-0.18	<0.01
	CD8 CD38-HLA-DR+	0.05	0.37	0.13	0.05
	CD8 Activated	0.03	0.61	-0.14	0.03
	CD8 CD57+PD1-	0.04	0.11	-0.03	0.65
	CD8 CD57+PD1+	0.11	0.05	-0.02	0.81
	CD8 CD57-PD1+	0.01	0.91	-0.13	0.05
	CD8 Exhausted	0.10	0.09	-0.05	0.46
	CD8 Senescent	0.11	0.052	-0.01	0.87
Monocytes	**Classical**	0.17	0.09	-0.05	0.52
	Intermediate	0.09	0.17	0.01	0.86
	Nonclassical	0.04	0.57	0.06	0.42

5.4.4.2 Does immune activation, exhaustion or senescence explain the effect of HIV on PWV?

Because HIV was found to be a risk factor for only PWV in the HIV models (see table 4-12), the role of immune activation markers in mediating an effect on outcomes was only assessed for PWV. Each immune marker with a univariate association with PWV and with a p value of less than 0.1 was added one at a time to the initial model examining HIV as a risk factor for PWV as outlined in section 4.4.3.1. Models adding the percentage of CD4 and CD8 Exhaustion and Senescence are outlined below (Table 5-7, Table 5-8, Table 5-9, Table 5-10). When the percentage of either CD4 or CD8 Exhausted T cells are added to the model, the effect of HIV is lost and the immune markers are retained in the model. This suggests that T cell exhaustion may be responsible for the effect of HIV on PWV. When the percentage of either CD4 or CD8 Senescent T cells are added, the effect of HIV is retained and the immune markers are lost from the model. The percentage of classical monocytes did not alter the effect of HIV on PWV either (Table 5-11).

Table 5-7 Effect of CD4 Exhaustion on HIV as a risk factor for PWV in a multivariate model adjusting for confounders

Variable	Fold change in PWV	P value	95% CI (min)	95% CI (max)
CD4 Exhaustion (per 10% increase)	1.02	0.02	1.00	1.04
HIV		>0.1		
Age (per 10-year increase)	1.16	<0.0001	1.10	1.21
Female sex	0.83	<0.0001	0.76	0.92
Systolic BP (per 10 mmHg increase)	1.05	<0.01	1.01	1.08
Haemoglobin		>0.1		

Table 5-8 Effect of CD4 Senescence on HIV as a risk factor for PWV in a multivariate model adjusting for confounders

Variable	Fold change in PWV	P value	95% CI (min)	95% CI (max)

CD4 Senescence		>0.1		
HIV	1.09	0.09	0.99	1.21
Age (per 10-year increase)	1.16	<0.0001	1.10	1.23
Female sex	0.85	<0.01	0.77	0.94
Systolic BP (per 10 mmHg increase)	1.05	<0.01	1.01	1.08
Haemoglobin		>0.1		

Table 5-9 Effect of CD8 Exhaustion on HIV as a risk factor for PWV in a multivariate model adjusting for confounders

Variable	Fold change in PWV	P value	95% CI (min)	95% CI (max)
CD8 Exhaustion (per 10% increase)	1.03	0.03	1.00	1.05
HIV		>0.1		
Age (per 10-year increase)	1.16	<0.0001	1.11	1.21
Female sex	0.88	<0.01	0.81	0.95
Systolic BP (per 10 mmHg increase)	1.04	<0.01	1.02	1.07
Haemoglobin		>0.1		

Table 5-10 Effect of CD8 Senescence on HIV as a risk factor for PWV in a multivariate model adjusting for confounders

Variable	Fold change in PWV	P value	95% CI (min)	95% CI (max)
CD8 Senescence		>0.1		
HIV	1.11	0.06	1.00	1.23
Age (per 10-year increase)	1.16	<0.0001	1.11	1.21
Female sex	0.92	0.08	0.84	1.01
Systolic BP (per 10 mmHg increase)	1.04	<0.01	1.01	1.07
Haemoglobin	1.22	0.09	0.97	1.53

Table 5-11 Effect of Classical Monocyte subset on HIV as a risk factor for PWV in a multivariate model adjusting for confounders

Variable	Fold change in PWV	P value	95% CI (min)	95% CI (max)
Classical monocytes		>0.1		
HIV	1.15	<0.01	1.04	1.28
Age (per 10-year increase)	1.19	<0.0001	1.13	1.25
Female sex		>0.1		
Systolic BP (per 10 mmHg increase)	1.03	0.07	1.00	1.06
Haemoglobin	1.03	<0.01	1.01	1.05

5.4.4.3 Does the addition of immune markers to traditional cardiovascular risk factor models improve the risk prediction for arterial stiffness or intima-media thickness?

Next we assessed whether substituting HIV status with immune markers associated with PWV or cIMT could improve the traditional cardiovascular risk factor model constructed in section 4.4.4.Table 5-12 shows three models using traditional cardiovascular risk factors to predict PWV but with the addition of either HIV, CD4 T cell exhaustion or CD8 T cell exhaustion. Both CD4 and CD8 Exhaustion are retained in the traditional cardiovascular risk factor model, with a weaker correlation than HIV status. The percentage of CD4 and CD8 T cells as well as the percentage of Activated CD8 T cells were added into the cIMT traditional risk factor model but none were retained.

Table 5-12 Addition of immune markers to traditional cardiovascular risk factors PWV models.

Variable	Traditional risk factors model with HIV status added				Traditional risk factors model with CD4 Exhaustion added				Traditional risk factors model with CD8 Exhaustion added			
	Fold change in PWV	P value	95% CI (min)	95% CI (max)	Fold change in PWV	P value	95% CI (min)	95% CI (max)	Fold change in PWV	P value	95% CI (min)	95% CI (max)
Age (per 10-year increase)	1.19	<0.0001	1.15	1.24	1.16	<0.0001	1.10	1.22	1.16	<0.0001	1.11	1.21
Female sex	0.92	0.02	0.85	0.99	0.83	<0.0001	0.77	0.92	0.88	<0.01	0.81	0.95
Systolic BP (per 10mmHg increase)	1.04	<0.001	1.02	1.07	1.05	<0.01	1.01	1.08	1.04	<0.01	1.02	1.07
HIV infection	1.09	0.045	1.00	1.18	NA	NA	NA	NA	NA	NA	NA	NA
CD4 T cell exhaustion (per 10% increase)	NA	NA	NA	NA	1.02	0.02	1.00	1.04	NA	NA	NA	NA
CD8 T cell exhaustion (per 10% increase)	NA	NA	NA	NA	NA	NA	NA	NA	1.02	0.049	1.00	1.05

5.4.5 Principal components analysis of immune activation

Principal components analysis of all immune markers was conducted to assess whether immune markers could be divided into common groups (the list of all markers included can be found in Table 5-14). The dendogram for the cluster analysis is shown in Figure 5-22 and the various different cluster groups are shown in Table 5-13. The number of groups was arrived at by identifying the biggest jump in the pseudo-F statistic. When three groups were taken this also correlated visually with three main groups on the dendogram.

Figure 5-22 Dendogram for cluster analysis of immunophenotyping markers

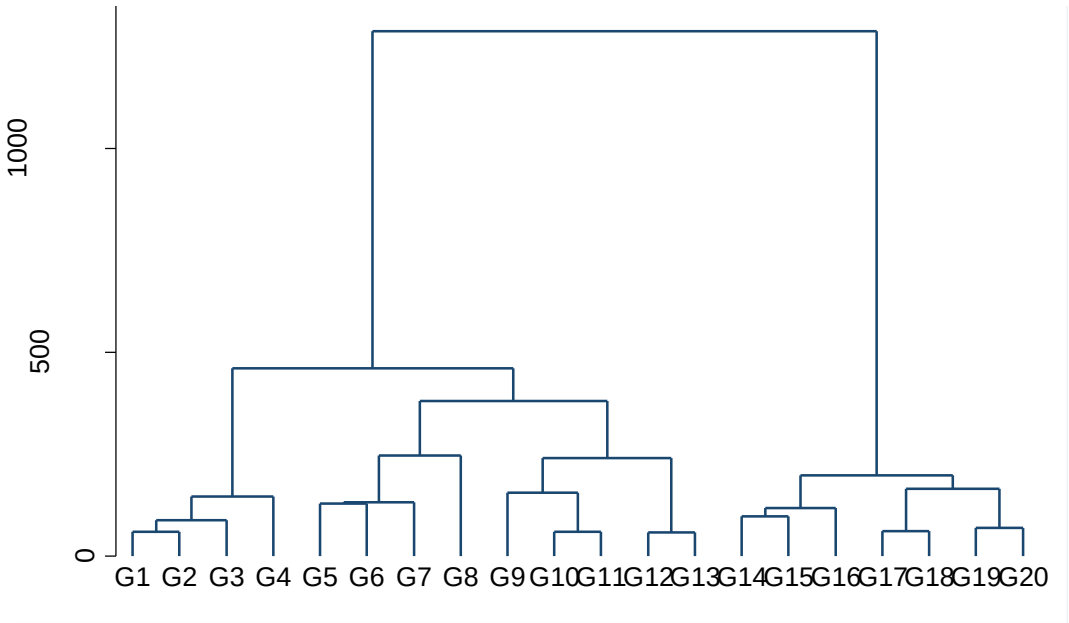
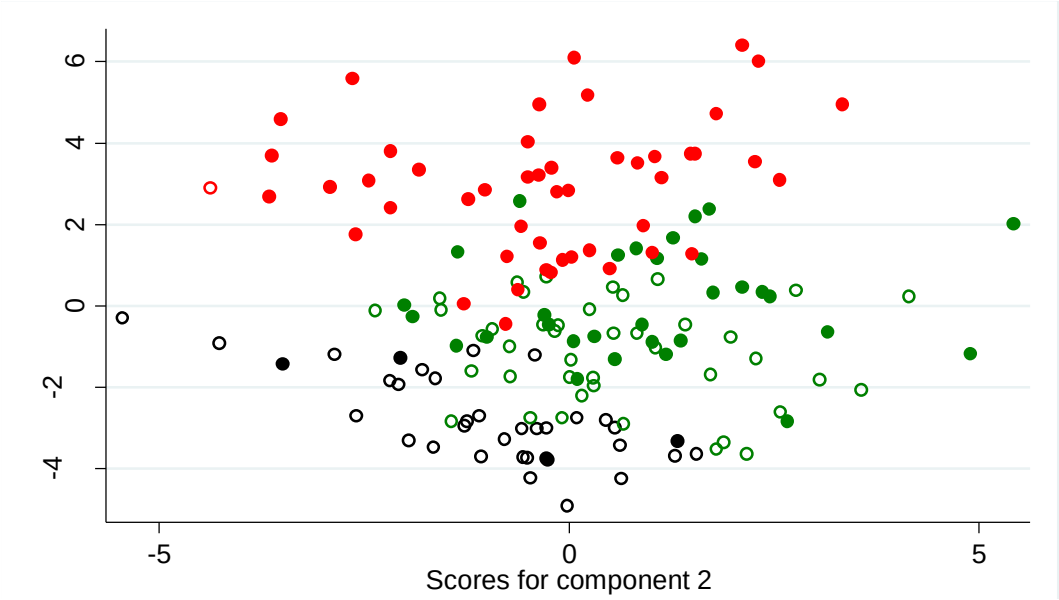


Table 5-13 Immune marker cluster group analysis

Number of clusters	Calinski/ Harabasz pseudo-F
2	44.22
3	33.19
4	29.50
5	26.27
6	24.71
7	23.45
8	22.34
9	21.60
10	21.08
11	20.67
12	20.45
13	20.26
14	19.94
15	19.63

The make-up of the three identified clusters according to HIV status is shown in Figure 5-23. Group 1 was largely made up of HIV uninfected participants and Group 3 HIV infected participants. Group 2 showed a mix of both HIV infected and uninfected participants. To further identify the immune marker composition of each group the median for each immune marker was calculated and presented in Table 5-14. Group 1 showed the lowest values of markers associated with immune activation, exhaustion or senescence and was termed the non-immune activated group. Group 3 showed the highest percentages of expression of activation, exhaustion and senescence markers and was termed the high immune activation group. Group 2 values fell between these two groups and was termed the moderate immune activation group. These trends were seen, in particular with the T cell exhaustion and senescence markers. Interestingly the proportion of classical monocytes was highest and intermediate monocyte lowest in the high immune activation group, with no differences in non-classical monocytes across the three groups. This could imply that markers such as PD-1 expression, or possibly soluble markers of immune activation might act as biomarkers in both HIV infected as well as uninfected adults.

Figure 5-23 Cluster analysis of immune marker groups according to HIV status



Group	HIV uninfected (hollow circles)	HIV infected (filled circles)	Total
1 (black dots)	32	4	36
2 (green dots)	42	31	73
3 (red dots)	1	48	49
Total	75	83	158

Table 5-14 Median values of immune markers for each cluster analysis group

		Group 1: Non-immune activated	Group 2: Moderately immune activated	Group 3: Highly immune activated	Kruskal-Wallis p value
T cells	CD4 CD38+HLA-DR-	28 (23 – 39)	38 (29 – 46)	18 (15 – 23)	<0.001

	CD4 CD38+HLA-DR+	19 (10 – 25)	6 (3 – 9)	33 (26 – 47)	<0.001
	CD4 CD38-HLA-DR+	16 (11 – 23)	7 (4 – 10)	23 (18 – 31)	<0.001
	CD4 Activated	68 (57 – 76)	50 (40 – 67)	81 (75 – 89)	<0.001
	CD4 CD57+PD1-	5 (3 – 11)	3 (2 – 6)	3 (2 – 7)	<0.01
	CD4 CD57+PD1+	7 (4 – 12)	2 (1 – 3)	12 (7 – 20)	<0.001
	CD4 CD57-PD1+	31 (22 – 41)	12 (7 – 19)	53(47 – 62)	<0.001
	CD4 Exhausted	13 (9 - 22)	24 (15 – 35)	63 (53 – 72)	<0.001
	CD4 Senescent	4 (3 – 7)	9 (5 – 17)	18 (9 – 22)	<0.001
	CD8 CD38+HLA-DR-	20 (12 – 32)	28 (18 – 44)	19 (9 – 23)	<0.001
	CD8 CD38+HLA-DR+	22 (13 – 35)	13 (6 – 24)	46 (35 – 55)	<0.001
	CD8 CD38-HLA-DR+	15 (9 – 29)	8 (5 – 17)	17 (9 – 23)	<0.001
	CD8 Activated	68 (58 – 80)	60 (42 – 75)	85 (75 – 90)	<0.001
	CD8 CD57+PD1-	32 (26 – 42)	29 (18 – 38)	27 (18 – 38)	0.13
	CD8 CD57+PD1+	15 (12 – 20)	11 (6 – 19)	22 (18 – 27)	<0.001
	CD8 CD57-PD1+	16 (11 – 23)	11 (6 – 16)	29 (18 – 37)	<0.001

	CD8 Exhausted	17 (10 – 23)	30 (21 – 41)	43 (37 – 56)	<0.001
	CD8 Senescent	31 (23 – 38)	53 (42 – 64)	50 (40 – 58)	<0.001
Monocytes	Classical	74 (63 – 78)	76 (68 – 82)	80 (70 – 86)	0.01
	Intermediate	11 (9 – 15)	8 (6 – 13)	7 (5 – 12)	<0.01
	Nonclassical	14 (11 – 23)	13 (10 – 19)	14 (9 – 19)	0.51

When comparing participants in the highly immune activated group to HIV infected participants in the moderately immune activated group, there were signs of more advanced HIV disease (weight 54 versus 59kg, p<0.01; haemoglobin 11.6 versus 13g/dL, p<0.00001; lymphocytes 1.3 versus 1.75 $x10^3$/uL, p<0.001; HIV viral load 1.2 $x10^6$ versus 0.9 $x10^6$, p=0.03) and more evidence of acute infection (systolic BP 120 versus 128mmHg, p=0.09; heart rate 84 versus 72bpm, p<0.0001), but there was no difference in age (40 versus 36 years, p=0.62) or baseline PWV (7.3 versus 7.4 m/s, p=0.96). The HIV uninfected participants from the moderately immune activated group had a lower total lymphocyte count (2.0 versus 2.5 $x10^3$cells/uL, p=0.04) and were older (38 versus 32 years, p<0.01) than the HIV uninfected participants from the non-immune activated group.

Differences were also noted in baseline PWV measures. Participants from the non-immune activated group had a lower PWV than both the moderately immune activated group (6.7 versus 7.4 m/s, p=0.01) and the highly immune activated group (6.7 versus 7.3 m/s, p=0.02). Although the moderately and highly immune activated groups had a higher median [interquartile range (IQR)] age [36 (30 – 39) and 40 (29 – 45) years respectively] than the non-activated group [33 years (30 – 39], the fold change in PWV associated with being in either the moderately or highly immune activated group compared to the non-activated group was 1.15 (CI 1.02 – 1.31, p=0.02) when adjusted for age. Figure 5-25 shows higher PWV values for age in the moderately immune activated group. Although the non-immune activated group had a lower median PWV than the uninfected participants in the moderately immune activated group (6.7 versus 7.7 m/s, p=0.01), the moderately immune activated group were older and there was no longer an effect of immune group amongst HIV uninfected participants after adjusting for age. However, there was a stronger correlation between PWV and age in the HIV uninfected moderately immune activated group (Figure 5-25).

Figure 5-24 PWV according to age for each immune marker cluster analysis group

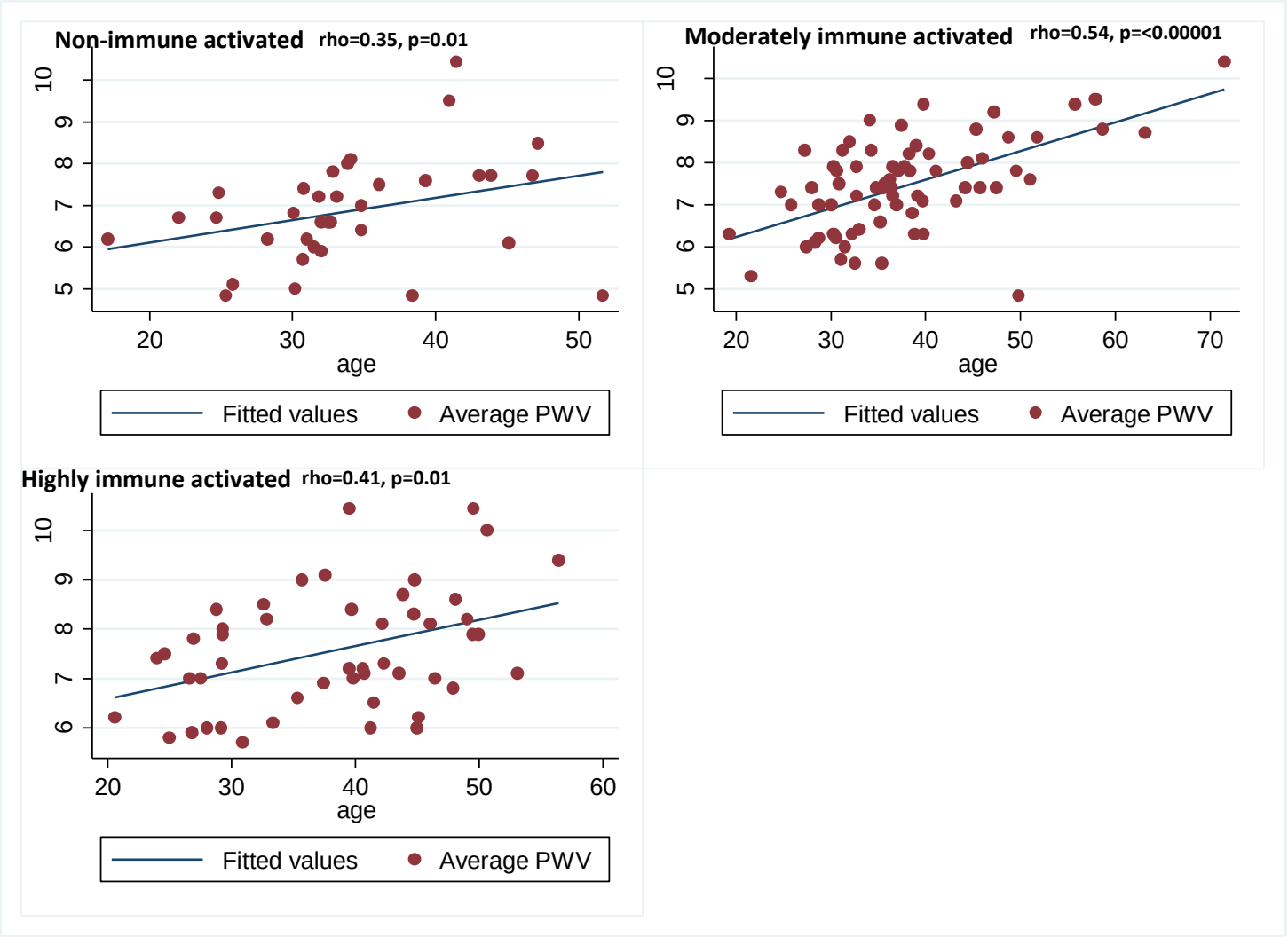
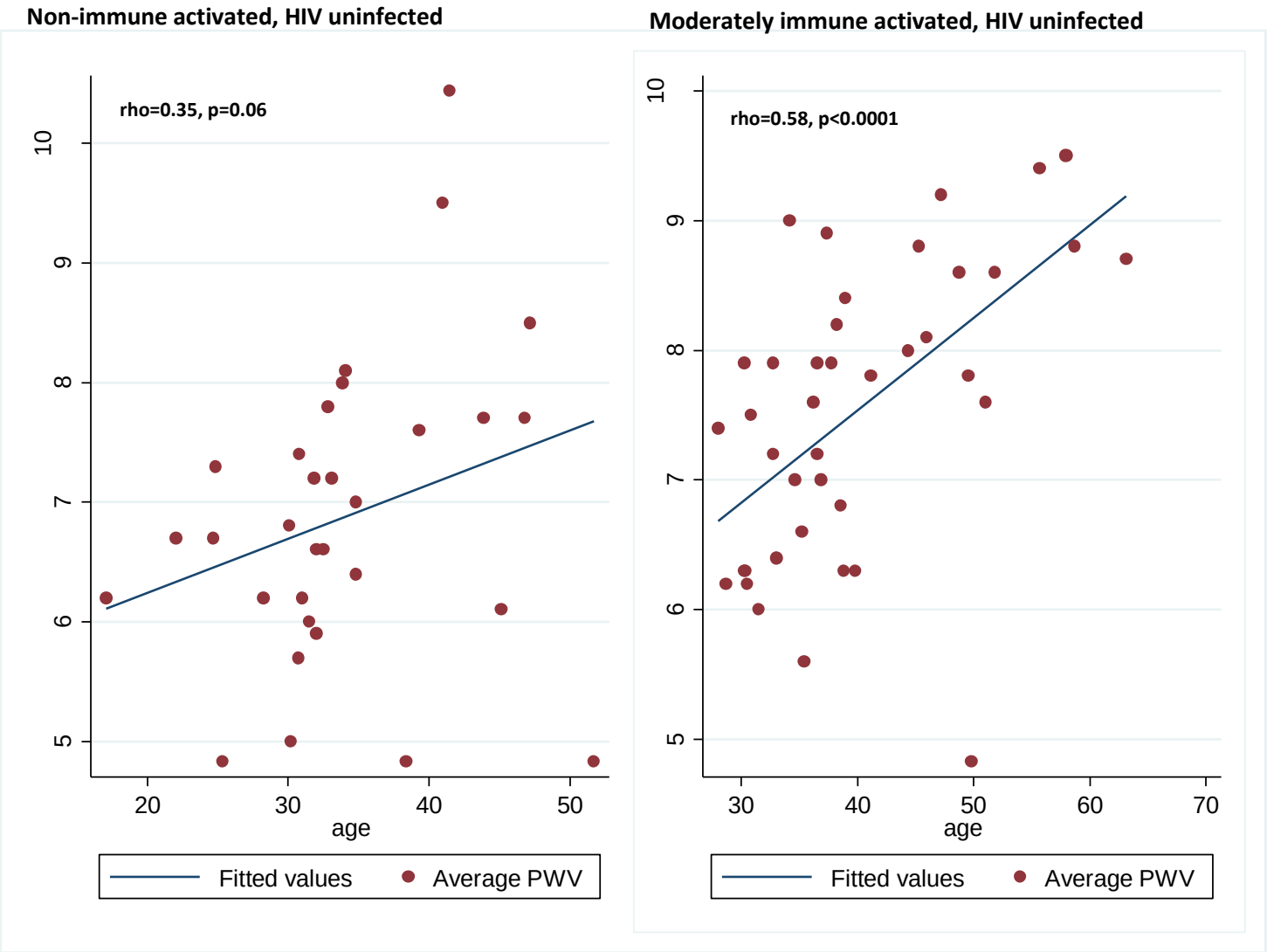


Figure 5-25 PWV according to age for HIV uninfected participants according to immune marker cluster analysis group



5.5 Discussion

T cell activation, exhaustion and senescence were higher in HIV infected participants than uninfected. However, the extent of immune activation in HIV uninfected participants was also higher when compared to literature from high income settings [426]. Two aspects of the flow cytometry protocol may limit the generalisability of this finding. Firstly, FMOs were not performed for every run. Although FMOs were initially performed and then validated with a standardised method (meaning that samples within this study were comparable), the lack of FMOs limits the generalisability of these results to other published literature. FMOs were not included with every run because resources were not available to provide the amount of antibody that would have been required to do this on such a large number of cells. Secondly, a dead cell stain was not used for every run of cells. Although this should not have influenced the comparison of patients within this study (as all cells followed the same protocol), some dead cells may have accounted for seemingly activated cells giving falsely high activation results. However, cells were prepared from fresh and so dead cells would have been relatively few. Therefore, non-Malawian volunteers were recruited to ascertain whether the high activation rates were due to the study protocol or whether there was a higher level of immune activation in HIV uninfected Malawian controls. Although only a few non-Malawian volunteers were recruited, they demonstrated lower activation levels when compared to Malawian controls.

Further, studies from other African countries have confirmed high proportions of activated CD4 and CD8 T cells in HIV uninfected adults [427]. However, this difference was less pronounced in CD4 compared to CD8 T cells, suggesting that there is a higher background CD8 T cell activation in Malawian adults. Activated CD4 and CD8 T cells in a Ugandan cohort of HIV infected and uninfected were functionally more inflammatory than those of Italian HIV uninfected individuals [428]. HIV uninfected Ethiopian adults have been shown to have higher immune activation (at comparable proportions to our results) and lower CD4 T cell counts than those reported for Caucasians [429]. This difference was not seen in neonates, suggesting that it develops as a result of environmental triggers during early childhood [430]. High CD8 T cell activation in HIV uninfected Malawians may have implications for HIV transmission – even an increase from 0.8% to 1.3 % was associated with increased risk of HIV transmission during the iPrEx study [431]. Low CD4 T cell activation in HIV uninfected Africans may also be a marker of a cellular phenotype that is protective against acquisition of HIV infection in highly exposed adults [432].

Unexpectedly, there was no difference in proportion of monocyte subsets between HIV infected and uninfected participants [172]. Furthermore, this lack of difference was due to an expanded population of nonclassical monocytes which was not present in non-Malawian controls. It is important to consider that the proportion of nonclassical monocytes identified will be dependent on gating strategy. However, our gating strategy was standardised and independently verified. We also performed controls to ensure that we were not gating on NK cells which may also express CD16 and are of similar size. Only one study has previously described monocyte subsets in low income sub Saharan Africa populations. It reported an expanded population of CD16+ monocytes in healthy pregnant Malawian women to the same extent as we have shown in this study, which is 2 to 3 fold higher than the 10% expected in healthy individuals from high income settings [433].

The role of nonclassical monocytes in disease has not been extensively described. Transcriptome characterisation of monocyte subsets has shown that nonclassical monocytes have diverse immunological functions, somewhat separate from the two other monocyte populations, that include antigen processing, monocyte activation, angiogenesis and potent induction of T cell proliferation [434, 435]. Although they have been shown to expand during acute infection, their primary function is thought to involve 'patrolling' of the endothelium. Pro-inflammatory responses are thought to be relatively less important in these cells when compared to intermediate monocytes and they act preferentially via TLR 7 pathways, meaning that they may activate in response to virus more than the typical TLR3/4 bacterial product activation [84, 436]. The expansion of nonclassical monocytes in this setting could be due to repeated exposure to acute infections such as malaria, pneumonia or gastroenteritis, possibly even from childhood. This, in turn, may be related to socio-economic factors. Or, a genetic component to proportions of monocyte subsets may have evolved in a population that has historically been exposed to a high burden of malaria and bacterial diseases. It should also be noted that the period of stay in the Malawian setting amongst the non-Malawian comparison group was not accounted for. Although the number of non-Malawian comparison adults here in this study is small, the low proportion of activated T cells and monocytes is comparable to proportions reported in other studies from high resources settings. The proportion of nonclassical monocytes is likely to have clinical implications for patients with HIV in SSA. Associations have been made with an increased risk of TB IRIS as well as cryptococcal meningitis [88, 437]. Additionally, it is known that CD16 monocytes are more permissive to HIV and it may be that the high proportion of nonclassical

monocytes in HIV uninfected adults increases the risk of transmission and progression of HIV following infection [87] [438].

Both CD4 and CD8 T cell exhaustion and senescence were associated with PWV in univariate analysis. However, in adjusted analysis only CD4 and CD8 T cell exhaustion (PD-1+) were retained. Although the effect size was small, this would suggest that PD-1+ T cells either play a role in the effect exerted by HIV on PWV or signify a process that is important for vascular injury. This is in keeping with previous reports linking T cell activation and low CD4 counts (which share overlapping pathophysiological pathways) with arterial stiffness [163, 413]. It is possible that this is a bystander effect and that PD-1 expression on T cells could be triggered by the same mechanism that leads to arterial stiffness. Or, T cell activation and exhaustion could play a direct role in the pathogenesis of arterial stiffness [439]. Whether expression of PD-1 on CD8 T cells is associated with increased function can depend on the stage of differentiation and also the length of time exposed to ART. The exhausted CD8 T cell phenotype with chronic expression of PD-1 seems to persist in the context of established ART, whereas the acute phenotype is more associated with a pro-inflammatory hyper-functioning state [414, 440, 441]. Lastly, consideration should be given, in particular, to the possible contribution of CMV. Good evidence exists to implicate CMV reactivation in both endothelial damage and T cell exhaustion and this could be of particular relevance in this highly immune suppressed cohort [167] .

In this study, HIV viral load was not an important factor for PWV or cIMT. Although Eller et al in Uganda found progression in patients with HIV seroconversion to be independently associated with clinical disease rather than T cell activation, they were looking at a group with much higher CD4 counts [427]. It may be that at lower CD4 counts, viral load becomes relatively less important. An analysis of a cohort of Malawian adults on long term ART was assessed using the same protocols and showed that arterial stiffness was independently associated with HIV viral load and default from treatment [442]. T cell activation and senescence have previously been associated with subclinical carotid artery disease in high income settings [183, 443], but these factors have not been studied in sub-Saharan Africa to date. At baseline, there was no clear association between monocyte subsets and PWV or cIMT. Nonclassical monocytes have recently been implicated in progression of subclinical atherosclerosis [444] and coronary artery calcification[445], but have been shown not to be associated when studied with T cells [446]. Single nucleotide polymorphisms of monocyte surface marker expression have recently been implicated in the pathogenesis of atherosclerosis [447], suggesting that genetics could influence the contribution of monocytes to CVD in an African population.

Principal components analysis identified three immune activation groups based on the extent of immune activation. HIV infected participants almost exclusively fell into the highly immune activated group or the moderately immune activated group. HIV uninfected participants almost exclusively fell into the non-immune activated or moderately immune activated group. Moderately activated HIV uninfected participants were older, had lower lymphocyte counts and had higher PWV values. The lower lymphocyte values found in moderately immune activated HIV uninfected Malawians suggests a role for exhaustion of lymphopoiesis even in this HIV uninfected cohort [448]. This finding, that a subset of HIV uninfected adults has chronic immune activation and higher arterial stiffness, has not been previously reported in the literature and requires further investigation.

Of note, the proportion of both CD4 and CD8 T cells with the CD38+HLA-DR- phenotype was inversely correlated with higher immune activation levels (as seen especially with the principal components analysis) and arterial stiffness. A Chinese group have recently published the same observation and suggested that during untreated HIV infection, CD38 and HLA-DR expression on T cells may represent different processes [449]. Increased HIV-specific responses, in particular, have been demonstrated in CD38-HLA-DR+ T cells and may be associated with better long term control of the virus [450]. Previous work by Ramzaoui et al at the beginning of the AIDS epidemic demonstrated that progressive increase in HLA-DR expression on CD38 positive cells was associated with disease progression [451]. The original work looking at the importance of CD38 and HLA-DR identified the absolute number of molecules of CD38 per CD8 T cell as being highly predictive of development of AIDS [452] and it should be noted that here we have divided CD38 expression into a binary state of either expressed or not expressed and the number of cells with that proportion, rather than the average intensity of expression of markers on cell surface. Together, it is likely that expression of CD38 but not HLA-DR is associated with better immune outcomes but further work would be required to identify whether this cell type conferred a functional benefit or whether it is decreased in immune activation as a consequence of compartmental dynamics and increases in other cell types.

This is the first study to demonstrate a relationship between T cell activation and arterial stiffness in low income sub-Saharan Africa. We have shown, for the first time, that it is T cell activation rather than expansion of inflammatory monocytes that is important during ART initiation in this setting. Further we have demonstrated high background immune activation in HIV uninfected individuals characterised, in particular, by expansion of nonclassical monocytes and immune activated CD8 T cells. It should be noted that these associations are based only on

cell surface expression of activation markers. More work is required to characterise the function of both T cell and monocyte subsets in this setting and identify the mechanisms which lead to arterial stiffness.

These data suggest that immune activation plays a different role in endothelial damage in low-income sub Saharan Africa where frequent acute infections from an early age, poverty and malnutrition are common. Although they also implicate immune activation in the pathogenesis of endothelial damage, immune activation does not seem to be solely related to HIV infection in this setting and other factors should be examined.

6 CHARACTERISATION OF CIRCULATING MICROPARTICLES

6.1 Introduction

Microparticles (MPs) are released into the circulation following activation or apoptosis of the affected cells [453]. Through a process of blebbing, microparticles are formed from the originating cell's outer membrane. During this process, Annexin V molecules, which are normally located on the inner membrane of a cell, are flipped round to become expressed on the microparticle surface [454]. Therefore, molecules less than 3µm in size and expressing Annexin V are classified as microparticles. These microparticles also express the markers expressed on the surface of the cell of origin and so microparticle subsets are an indication of which cells are undergoing stress.

MPs are involved in intercellular communication and play an important role in the pathogenesis of cardiovascular disease [455, 456]. In particular, endothelial microparticles (EMPs) act as a marker of damage either by exacerbating disease progression through attenuation of pro-atherogenic activity, or by triggering a repair response through induction of differentiation of endothelial progenitor cells (EPCs)[457]. In the Framingham Offspring cohort each tertile increase in Framingham risk score corresponded to a 9% log increase in EMPs[458].

As well as EMPs, microparticles of cells from compartments affected by systemic inflammation can be measured, including leucocyte, platelet and smooth muscle microparticles [459]. Tissue factor (TF) is mainly expressed on leucocytes and is upregulated during the coagulation cascade leading to increased cell to cell adherence [460]. Some evidence suggests that an upregulation of TF on monocytes is associated with an increased risk of coronary events in patients with HIV infection [169].

We aimed to quantify the total number of circulating MPs as well as characterise MP subsets in adult Malawians and to help understand potential mechanisms underlying the effect of HIV on immune activation and endothelial damage.

6.2 Specific objectives

This chapter aims to use quantification and characterisation of microparticles to help address overall objective number 5: "Explore the mechanisms involved in endothelial dysfunction according to different HIV related immune phenotypes". From this, two main specific objectives have been developed:

1. In Malawian adults, is arterial stiffness associated with:
    a. the total number of circulating microparticles
    b. particular subsets of circulating microparticles
2. In Malawian adults, is HIV infection associated with:
    a. the total number of circulating microparticles
    b. particular subsets of circulating microparticles

6.3 Methods

6.3.1 Study cohort

SHIELD patients were first divided into HIV infected and HIV uninfected and then ordered according to baseline PWV values. Participants with a PWV in the highest quartile (>9 m/s) were chosen randomly in a 2:1 ratio to patients with a PWV below the highest quartile from the HIV infected cohort. This was to enrich the number of potential microparticles for subset analysis (we hypothesised they would be higher at higher ranges of PWV) whilst also capturing a range of values to analyse associations with total microparticle count. HIV uninfected participants were then matched as closely as possible on age, systolic BP and diastolic BP. Because we were comparing microparticles in HIV infected compared to HIV uninfected and there was an equal number of those identified from high and low PWV values this was not thought to introduce any risk of bias.

6.3.2 Sample size calculations

A convenience sample of 36 HIV infected participants and 36 HIV uninfected matched controls were chosen from across the spectrum of PWV values.

6.3.3 Statistical analysis

Microparticle data was heavily left skewed and so Wilcoxon Ranksum and Chi^2 were used to evaluate continuous and categorical variables respectively. Microparticles were analysed as continuous data.

6.3.4 Study procedures

6.3.4.1 Microparticle analysis training

Onsite training was provided to the study PI and the study lab technician by Rijan Gurung, PhD student at ICH, UCL who also assisted in post-acquisition analysis of microparticle data.

6.3.4.2 Isolation of microparticles

Plasma samples frozen at -80C were thawed in a 37C water bath for 1 minute. 250uL was centrifuged at 5000g for 5 minutes in order to isolate platelet poor plasma (PPP). PPP was then centrifuged at 16000g for 60 minutes and the PPP was decanted to leave 20uL of microparticle pellet. Distilled water was filtered through 0.22 um syringe filter under a flow hood. Distilled filtered water was then added to Annexin V 10x buffer at a 1:10 dilution. Sufficient Annexin V 1x buffer was then added to the microparticle pellet to a volume sufficient to allow 35uL of microparticle/Annexin V buffer solution for each antibody combination being tested and controls.

6.3.4.3 Microparticle staining panel

In addition to a total microparticle count, further characterisation identified origins of microparticle subsets using cell surface markers indicative of the cell of origin. Endothelial, leucocyte, monocyte, platelet and smooth muscle microparticles were assessed and the staining panel is summarised in Table 6-1. Microparticles were identified by size and by the expression of Annexin V (AnV). Endothelial microparticles were those expressing either VCAM or E-selectin. PECAM was also taken to be an endothelial marker on particles that were not expressing CD41a (platelet marker). Given the high number of nonclassical monocytes identified in the PBMC analysis, CD14/CD16 double positive microparticles were also included to help assess the potential significance of this subset.

Table 6-1 Microparticle staining panel

Microparticle origin	**AnV stain**	**MP origin stain**
Endothelial	AnV+ FITC (BD Pharmingen)	$VCAM^+$ PE (BD Pharmingen)

	AnV$^+$ FITC	E-selectin$^+$ PE (BD Pharmingen)	
	AnV$^+$ FITC	PECAM$^+$ APC Cy7 (BD Pharmingen)	CD42a$^-$ PE (BD Pharmingen)
Leukocyte	AnV$^+$ FITC	CD66b$^+$ PE (BD Pharmingen)	
	AnV$^+$ FITC	CD16$^+$ PE (BD Pharmingen)	
Monocyte	AnV$^+$ PE (BD Pharmingen)	CD14$^+$ APC Cy7 (Biolegend)	TF$^+$ FITC (Sekisui Diagnostics)
	AnV$^+$ FITC	CD14$^+$ APC Cy7	CD16$^+$ PE
Platelet	AnV$^+$ FITC	CD42a$^+$ PE (BD Pharmingen)	
Smooth Muscle	AnV$^+$ FITC	PDGFβ$^+$ PE (R&D Systems)	PECAM$^-$ APC Cy7
	AnV$^+$ FITC	Endoglin$^+$ PE (BD Pharmingen)	PECAM$^-$ APC Cy7
	AnV$^+$ FITC	NG2$^+$ PE (R&D Systems)	PECAM$^-$ APC Cy7
	AnV$^+$ FITC	ICAM1$^+$ PE	PECAM$^-$ APC Cy7

Each antibody was diluted to a 1:100 concentration in either AnV buffer for AnV antibodies or in PBS for all remaining antibodies. 5uL of AnV antibody was added to each well containing 35uL of microparticle AnV buffer solution. The remaining origin stains were then added at a volume of 10uL for those tubes that only had one origin stain and 5uL for those tubes that had two origin stains. This was to ensure a total staining volume of 50uL for all samples. Single stain samples were also acquired for the purposes of compensation and isotype controls were analysed for the purposes of gating. For the isotype controls, 10uL of 1:40 isotype control antibody was added to the 35uL microparticle AnV buffer solution along with 5uL of AnV antibody (IgG1 PE, R&D Systems; IgG1k PE, IgG1 FITC, R&D Systems; IgG1k APC Cy7, BD Pharmingen; IgGMk PE, BD Pharmingen).

Following staining plates were covered with foil and agitated at room temperature for 20 minutes. 200uL AnV buffer was added to every well and then transferred to FACS tubes. A further 400uL AnV buffer was then added to every tube. Finally, 6uL of 3um latex beads (SIGMA) were added to 2ml of distilled filtered water and 10uL of that was added to 650uL distilled filtered water.

6.3.4.4 Flow cytometer acquisition

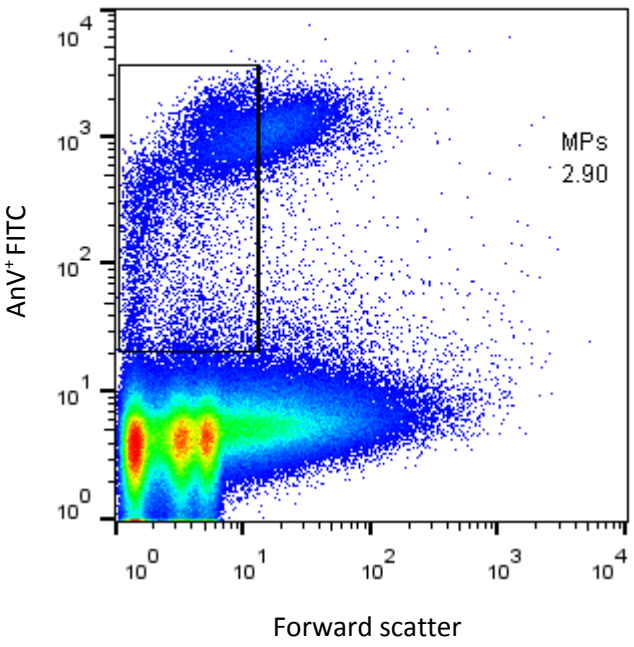
A microparticle protocol was created on the CyAn flow cytometer with the same voltage settings as the T cell and monocyte panels but with a lower capture threshold of 0.01% instead of 2%. This was

to ensure that microparticles were not excluded as debris. 350uL of each FACS tube was acquired and the plots were then transferred to Flow Jo (Tree star Inc.) for analysis.

6.3.4.5 Microparticle gating

After identification of singlets, the microparticle pellet was gated on forward scatter and AnV (FITC) to identify the microparticle population which was less than 3um in size and expressing AnV (see Figure 6-1). The microparticle population was then used to characterise further surface markers indicating the origin of the cells. Those tubes with two stains in addition to AnV staining were gated using crosshairs to identify 3 populations and those tubes with only one additional population was gated into two populations. Gates were applied using thresholds provided by isotype controls.

Figure 6-1 Identification of the microparticle population



6.3.5 Analysis of TF expression on monocyte subsets

High TF expression was found on microparticle subsets and therefore to help further characterise the link between nonclassical monocytes, CD16+ microparticles and TF expression, a small subset of monocyte cells was also stained for TF (TF FITC, Sekisui Diagnostics) in addition to the existing protocol. Each batch of cells was compensated individually with compensation beads and the existing monocyte gating strategy was used to identify monocyte subsets. The mean fluorescence intensity of TF expression was then calculated for each monocyte subset, and the median value was used to compare groups.

6.4 Results

6.4.1 Summary of characteristics of patients included in microparticle analysis

Microparticle data were available for 36 HIV uninfected and 33 HIV infected participants. Sufficient plasma samples were not available for 3 HIV uninfected participants. The baseline clinical characteristics of the 69 participants with available microparticle data is given in Table 6-2. Median PWV was 1 m/s higher in the HIV infected participants with microparticle data and haemoglobin was 2 units less.

Table 6-2 Clinical characteristics of 67 SHIELD participants with microparticle data

	HIV uninfected n=36	**HIV infected n=33**
Age (years, median IQR)	40 (34 – 48)	40 (35 – 50)
Waist: height ratio	0.48 (0.45 – 0.53)	0.46 (0.42 – 0.50)
Systolic BP	128 (118 – 134)	130 (118 – 135)
Diastolic BP	76 (69 – 79)	79 (74 – 88)
Haemoglobin	14 (13 – 15)	12 (11 – 12)
Cholesterol	4.2 (3.7 – 4.9)	4.2 (3.6 – 4.7)
Glucose	4.6 (4.2 – 5.4)	4.6 (4.2 – 5.0)
Creatinine	66 (57 – 80)	69 (58 – 84)
PWV	9.0 (8.1 – 10.3)	8.0 (6.7 – 8.7)
Mean CCA	0.56 (0.55 – 0.64)	0.59 (0.54 – 0.66)
Female (f, %)	15 (42%)	12 (39%)
Primary school education or less	20 (57%)	17 (55%)
Ever smoked	5 (14%)	6 (19%)
Ever drank alcohol	12 (33%)	14 (45%)
History of CVD	4 (11%)	3 (10%)
Current infection	1 (3%)	3 (10%)

6.4.2 Relationship between microparticles and clinical variables

Total microparticle counts ($x10^3$) are given for categorical variables in Table 6-3 and associations between total microparticle count and continuous variables are given in Table 6-4. Microparticles

were significantly higher in the HIV infected participants compared to uninfected. Diastolic BP, heart rate, creatinine and PWV were all positively correlated with microparticles; lymphocytes and haemoglobin were negatively associated.

Table 6-3 Comparison of total microparticle counts for categorical variables

	Median microparticle count x10^3	**P value**
HIV infected	5100 (2000 – 18000)	
HIV uninfected	410 (200 – 6000)	<0.00001
Male	1000 (401 – 5100)	
Female	480 (300 – 3400)	0.17
Primary school education or less	900 (260 – 5100)	
Greater than primary school education	730 (420 – 4700)	0.66
Smoker or ex-smoker	900 (400 – 6700)	
Never smoked	680 (320 – 4700)	0.61
Drinks alcohol or past alcohol	2700 (400 – 5100)	
Never drank alcohol	530 (290 – 4700)	0.12
Pre-existing cardiovascular diagnosis	980 (400 – 1800)	
No previous cardiovascular diagnosis	790 (320 – 4100)	0.29
Prescribed CV drugs	530 (400 – 980)	
Never prescribed CV drugs	870 (320 – 4900)	0.87
Clinically hypertensive	1700 (320 – 17000)	
Not clinically hypertensive	600 (340 – 275)	0.16
Current infection	2550 (1300 – 4100)	
No current infection	730 (320 – 4700)	0.42

Table 6-4 Correlation between continuous variables and total microparticle count

	Spearman's rho	**P value**

Age	0.20	0.10
Waist: height ratio	-0.17	0.17
Heart rate	0.30	0.01
Systolic BP	0.14	0.26
Diastolic BP	0.23	0.07
Haemoglobin	-0.23	0.07
Fasting Cholesterol	0.11	0.40
Fasting Glucose	-0.14	0.28
Creatinine	0.31	0.01
Lymphocytes	-0.26	0.04
Monocytes	0.06	0.67
PWV	0.42	<0.001
Mean CCA	0.16	0.22

When assessing correlations between microparticle subsets and PWV, PECAM+ and Eselectin+ microparticles (endothelial in origin) were closely associated (see Table 6-5). Leucocyte and monocyte particles also correlated significantly with PWV, except the non-classical monocyte phenotype. Platelet microparticles were closely associated with PWV as well as most smooth muscle markers.

Table 6-5 Correlations between microparticle subsets and PWV

		Spearman's rho	**P value**
Total MPs		0.42	<0.001
Endothelial	PECAM+CD42a-	0.58	<0.00001
	PECAM+Eselectin-	-0.31	0.01
	PECAM+Eselectin+	0.52	<0.00001
	PECAM-Eselectin+	0.49	<0.00001
	ICAM+PECAM+	0.25	0.09
	VCAM+PECAM-	0.41	<0.01
	VCAM+PECAM+	0.20	0.19
	Eselectin+	0.57	<0.00001
Leucocyte	CD66b	0.44	<0.01
	CD16+TF-	0.43	<0.01

	CD16+TF+	0.47	<0.01
	CD16-TF+	0.52	<0.001
	CD14-CD16+	0.69	<0.00001
Monocyte	CD14+CD16+	0.003	0.99
	CD14+TF-	0.52	<0.001
	CD14+TF+	0.42	<0.001
Platelet	CD42a+	0.56	<0.00001
Smooth muscle	NG2+PECAM-	0.52	<0.001
	PDGFRβ+PECAM-	0.47	<0.01
	Endoglin+PECAM-	0.43	<0.01
	Endoglin+PECAM+	0.12	0.23
	ICAM+PECAM-	0.53	<0.001

6.4.3 Effect of HIV on microparticles

The total microparticle count was significantly higher amongst HIV infected participants compared to HIV uninfected Malawian and HIV uninfected non-Malawian controls (see Figure 6-2). The breakdown of the origin of these microparticles is shown in Figure 6-4. Axes for the HIV uninfected and infected groups have different ranges in order to accommodate the large difference in values for both groups. Platelet and Eselectin+ endothelial microparticles are greatly increased in HIV infected participants compared to HIV uninfected. Microparticles originating from both TF cells that were not CD16+ and CD16+ cells that were not monocytes were higher in HIV infected participants.

Figure 6-2 Total microparticle frequency according to HIV status

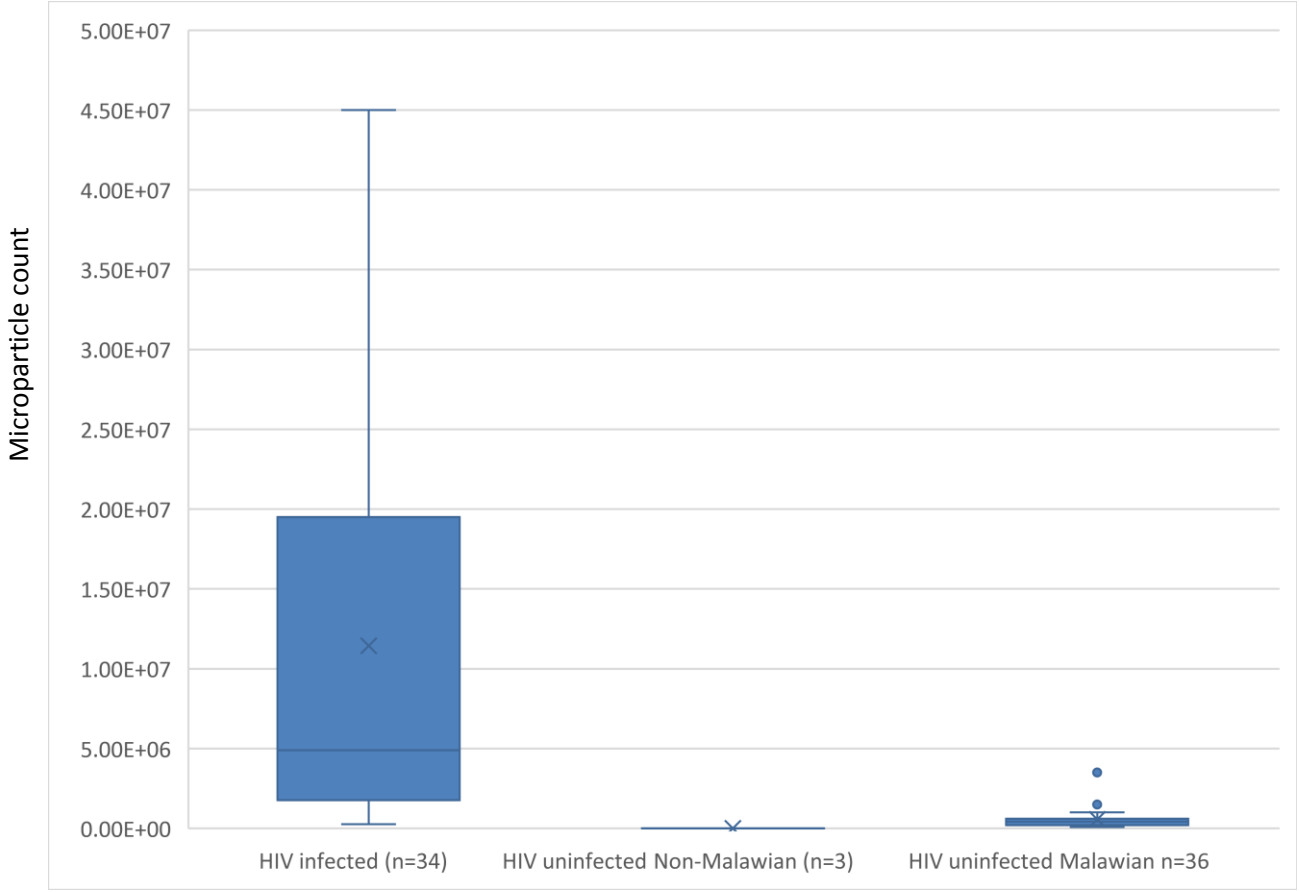


Figure 6-3 Frequency of microparticle subsets for HIV uninfected and infected participants

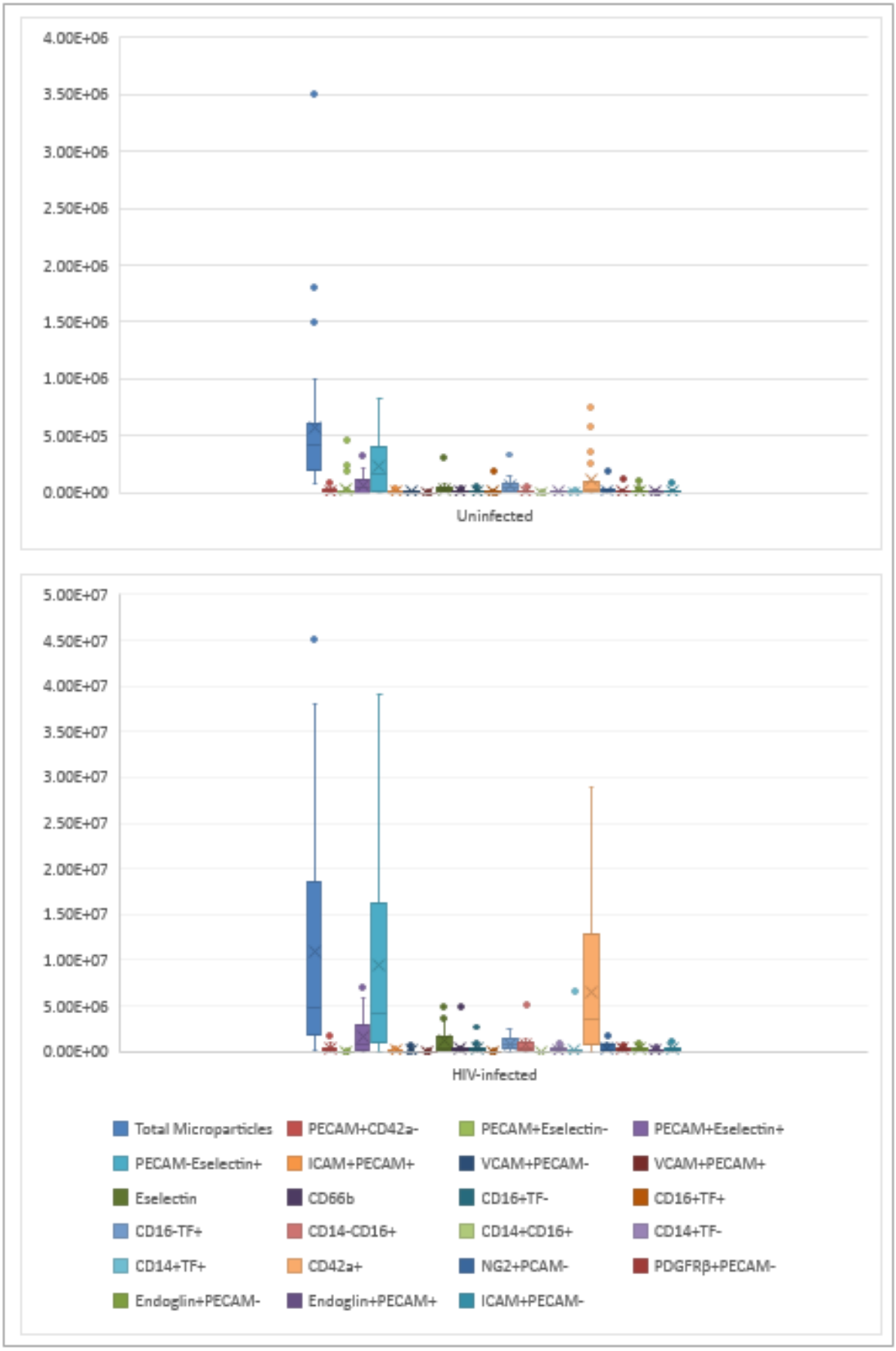
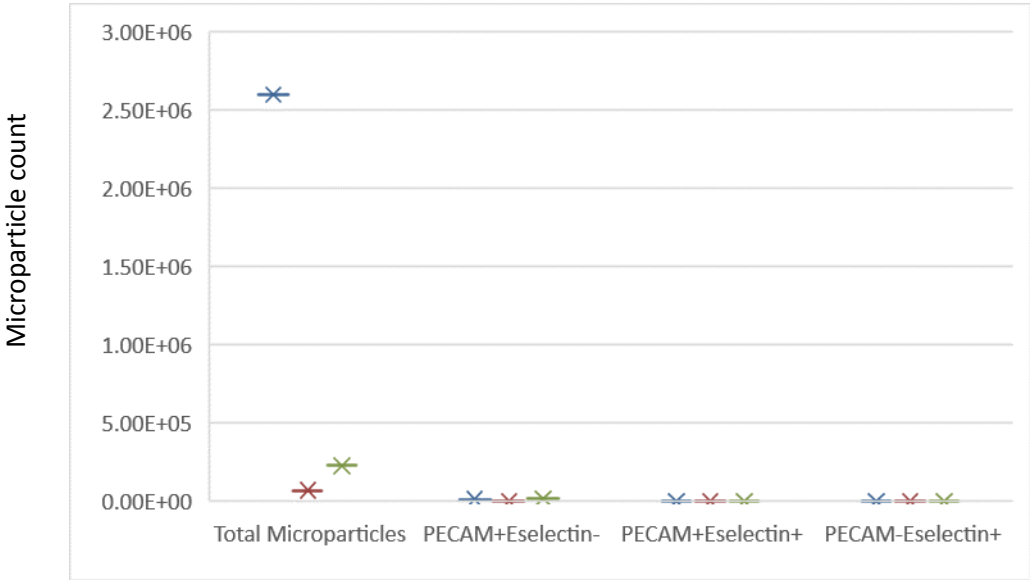


Figure 6-4 Endothelial microparticle frequencies for 3 HIV uninfected non-Malawian controls



6.4.1 TF expression on monocyte subsets

An example of monocyte gating of TF expression for each of the three monocyte subsets is presented in Figure 6-5. Figure 6-6 shows the mean MFI for each monocyte subset for 15 HIV infected and 9 HIV uninfected participants. Although TF expression was highest on nonclassical monocytes (in keeping with their hypothesised endothelial 'rolling' function), there was no significant difference in TF expression on monocytes from HIV infected and HIV uninfected participants and the frequency of CD16+ or CD14+ microparticles also staining for TF was not raised.

Figure 6-5 Tissue factor Mean Fluorescence Intensity on monocyte subsets

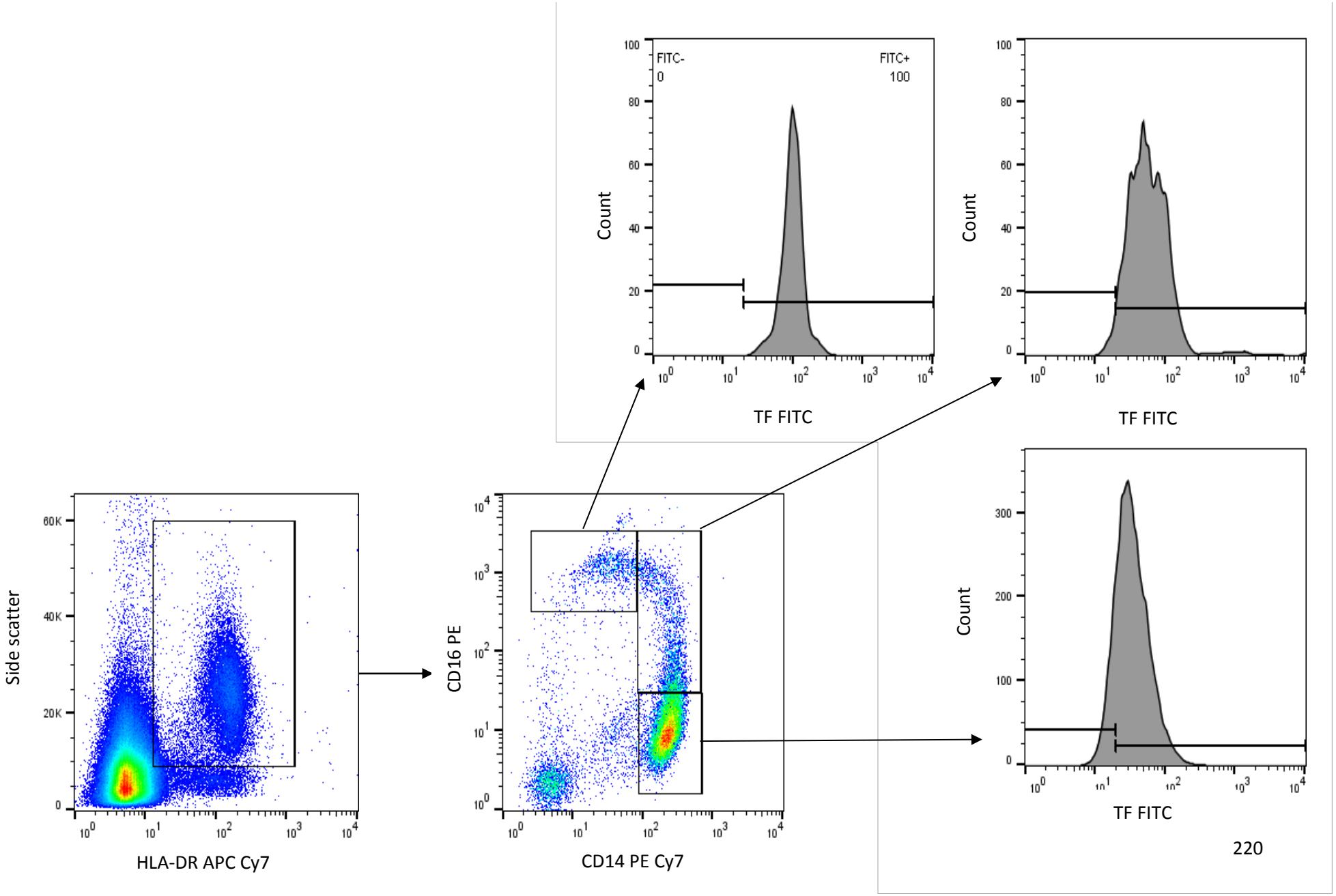
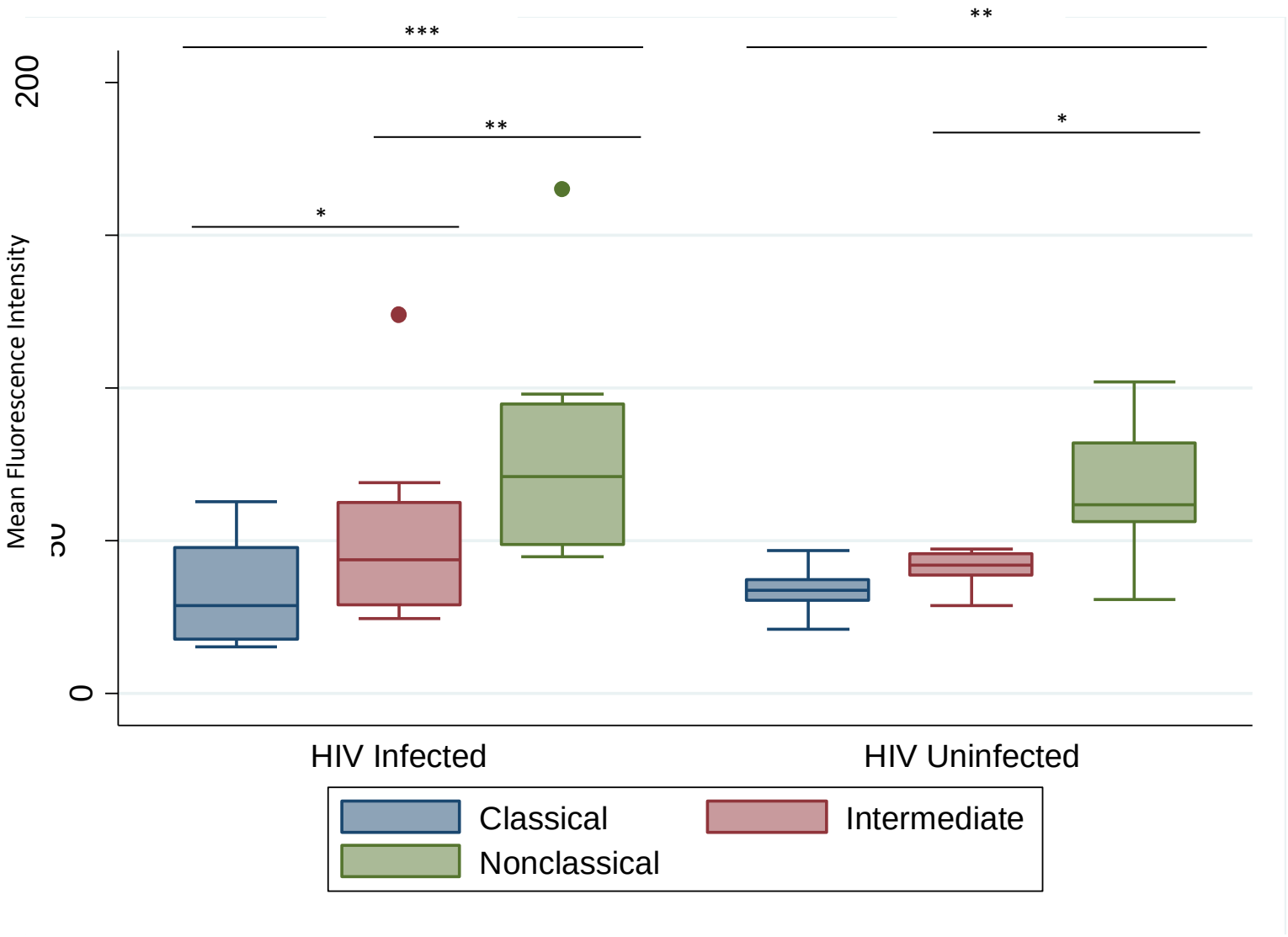


Figure 6-6 Mean Fluorescence intensity of Tissue Factor on monocyte subsets according to HIV status



6.4.2 Relationship between microparticles and immune markers including principal components analysis groups

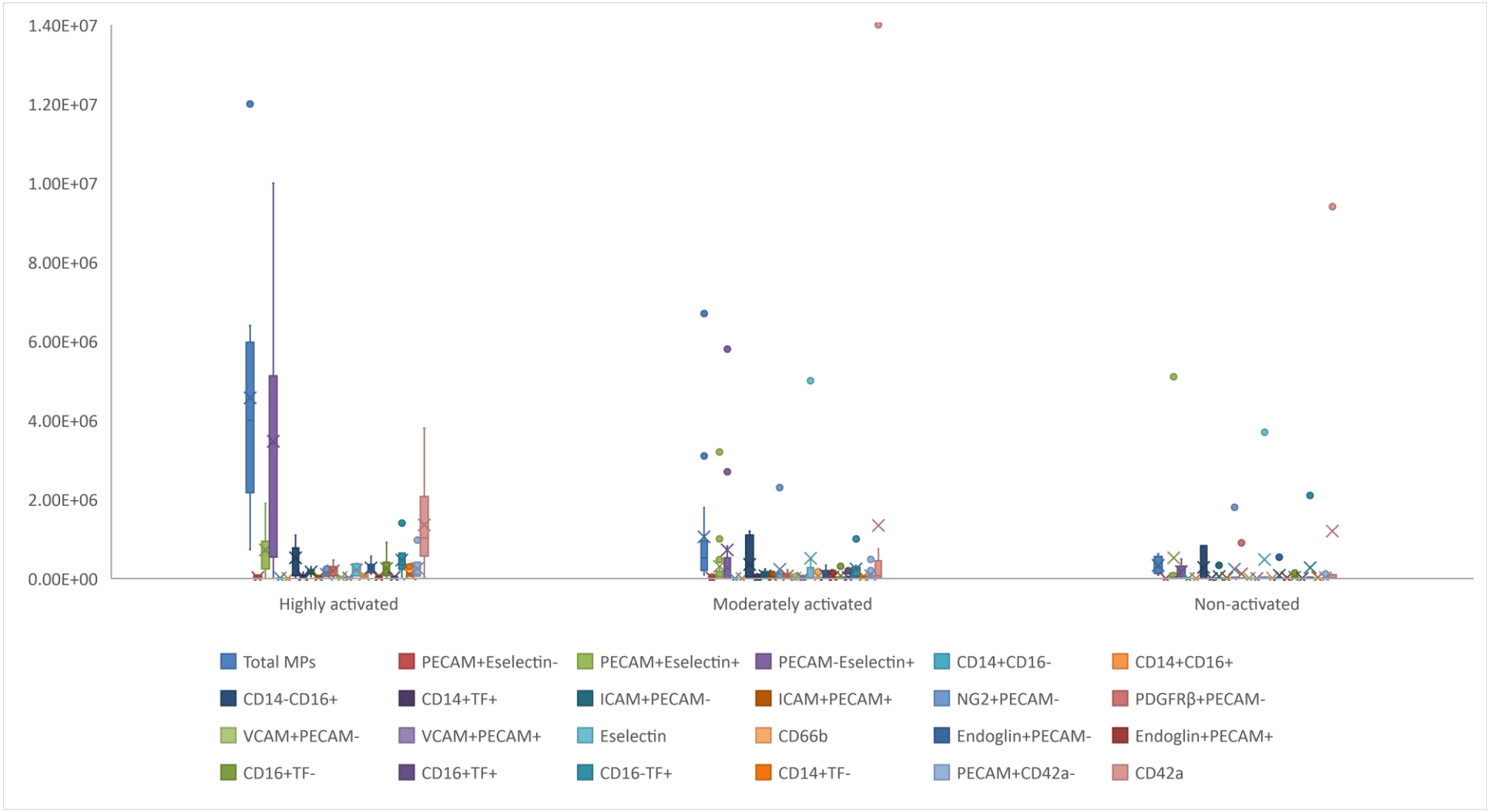
The relationship between the total number of circulating microparticles and their subsets was compared to immune activation markers using the three immune activation groups generated in the principal components analysis in section 5.4.5. The total number of microparticles was significantly raised in the highly immune activated group compared to the moderately immune activated group which in turn were higher than the non-immune activated group, as analysed by Wilcoxon ranksum (Table 6-6). As was the case when comparing HIV infected and uninfected individuals, the type of microparticles raised in the highly immune activated group compared to the moderately activated group were E selectin positive endothelial microparticles and platelet microparticles. Both median (IQR) platelet and non-monocyte TF positive microparticles were raised in HIV infected participants in the moderate group compared to those in the non-immune activated group [59 $x10^3$ (36 $x10^3$ – 75 $x10^3$) versus 16 $x10^3$ (7 $x10^3$ – 22 $x10^3$), $p<0.01$ and 2 $x10^3$ (0.5 $x10^3$ – 10 $x10^3$) versus 0.02 x 10^3 (0 – 0.08 $x10^3$), $p<0.01$ respectively]. Absolute CD4 and HIV viral load indices were not included in the PCA analysis because they were only available for HIV infected participants. Although there was no association between CD4 count and microparticles, TF positive monocyte microparticles were significantly associated with HIV viral load (spearman rho= 0.40, p=0.03).

Table 6-6 Comparison of median microparticle counts for each subset according to immune activation group

		Highly activated [median (IQR) count x10^3] n=8	Moderately activated [median (IQR) count x10^3] n=27	P value (Highly activated compared to moderately activated)	Non-activated [median (IQR) count x10^3] n=10	P value (Non-activated compared to moderately activated)
Total MPs		4000 (2400 – 5600)	440 (170 – 890)	<0.001	270 (130 – 530)	0.10
Endothelial	PECAM+CD42a-	110 (64 – 220)	5.9 (1.9 – 43)	<0.01	2.2 (1.9 – 11)	0.08
	PECAM+Eselectin-	0 (0 – 610)	0.12 (0 – 2.4)	0.69	0.02 (0 – 0.8)	0.07
	PECAM+Eselectin+	670 (370 – 890)	52 (13 – 130)	0.01	47 (18 – 110)	0.74
	PECAM-Eselectin+	3400 (730 – 48000)	250 (64 – 470)	0.02	120 (69 – 280)	0.22
	ICAM+PECAM+	30 (4 – 52)	1.4 (0.31 – 4)	0.11	1.4 (0.94 – 33)	0.93
	VCAM+PECAM-	22 (16 – 32)	3.3 (1.3 – 8.0)	<0.01	2.3 (1.3 – 3.4)	0.09
	VCAM+PECAM+	2.0 (0 - 24)	0.16 (0 – 0.47)	0.48	0.31 (0 – 0.94)	0.39
	Eselectin+	220 (68 – 370)	21 (6.4 – 43)	0.01	9.8 (5.9 – 34)	0.18
Leucocyte	CD66b	86 (64 – 96)	14 (5.6 – 3.2)	0.07	3.4 (1.1 – 6.9)	0.18
	CD16+TF-	130 (96 – 25)	8.7 (5.5 – 20)	<0.01	8.8 (5.5 – 18.6)	0.83
	CD16+TF+	34 (4.0 – 44)	1.1 – (0.47 – 5.3)	0.10	0.94 (0.31 – 3.9)	0.58
	CD16-TF+	130 (96 – 25)	8.8 (5.5 – 20)	<0.01	16 (6.6 – 22)	<0.01
	CD14-CD16+	490 (76 – 780)	33 (4.7 – 610)	0.18	6.0 (1.6 – 10)	0.32
Monocyte	CD14+TF-	90 (64 – 92)	6.4 (3.1 – 12)	<0.01	3.6 (3.1 – 7.3)	0.19

	CD14+TF+	40 (4.0 – 88)	1.4 (0.37 – 4.0)	<0.01	0.47 (0.27 – 4.1)	0.72
Platelet	CD42a+	1000 (750 – 1500)	35 (10 – 260)	0.01	17 (0.94 – 41)	0.06
Smooth muscle	NG2+PECAM-	8.6E4 (6.4E4 – 9.6E4)	1.4E4 (5.6E3 – 3.2E4)	0.02	7.8 (5.6 – 18.8)	0.24
	PDGFRβ+PECAM-	180 (80 – 240)	10 (5.0 – 28)	<0.01	7.2 (4.7 – 9.5)	0.02
	Endoglin+PECAM-	230 (170 – 280)	14 (10 – 21)	<0.01	12 (10 – 14)	0.61
	Endoglin+PECAM+	18 (8.0 – 32)	0.94 (0.47 – 6.4)	0.11	0.94 (0.47 – 2.3)	0.77
	ICAM+PECAM-	160 (130 – 200)	11 (5.3 – 25)	<0.01	7.3 (5.3 – 11)	0.24

Figure 6-7 Microparticle subsets according to Immune Activation PCA groups



6.5 Discussion

By examining circulating microparticles we have provided further evidence that HIV infection and immune activation are associated with adverse pathophysiological consequences at a cellular level. Circulating microparticles are induced in response to cellular stress in the form of activation, apoptosis or physical shear stress [461]. The total proportion of circulating microparticles is a marker of the extent of apoptosis in response to this ongoing stress. We showed that the quantity of circulating microparticles is strongly associated with HIV infection, immune activation and PWV. Given that patients with and without HIV were not matched for heart rate, lymphocyte count or creatinine the finding that these measures were associated with the number of microparticles may simply reflect their association with HIV infection.

The total number of circulating microparticles was over 40 fold higher in HIV infection than in those without HIV infection. For patients in the high immune activation group, the number of total circulating microparticles was 10 fold higher than the moderate immune activation group which was, in turn, twice as high as the non-immune activated group. Using the same protocols looking at adults from the UK, the total number of circulating microparticles was 10 fold higher in myocardial infarction and 20 fold higher in pulmonary artery hypertension as compared to Malawian adults with HIV infection (personal communication, Dr Rijan Gurung, UCL). The rise in total microparticles for both the HIV infected group as well as the higher immune activation groups consisted mainly of an increase in endothelial microparticles (PECAM-Eselectin+) and platelet microparticles (CD42a). Consistent with this finding, platelet microparticles have previously been shown to be more frequent and more activated in HIV infection as defined by upregulated expression of TF [462]. Additionally, da Silva et al showed that endothelial microparticles were 20 times higher in HIV infected compared to HIV uninfected participants (although endothelial microparticles were defined as CD51+ which limits comparison with our current results) [463]. Non-monocyte CD16 expressing cells were raised across all three immune activation groups and are likely to represent neutrophil microparticles (which make up a higher number of circulating white blood cells than NK cells). It should be remembered that the circulating microparticles were characterised from plasma from whole blood samples, not from PBMCs and therefore may have contained neutrophil microparticles even though we were unable to quantify neutrophil whole cells from the PBMC analysis in this study. It is therefore possible that neutrophil microparticles are frequent circulating microparticles in healthy HIV uninfected Malawian adults, even without immune activation.

Total circulating microparticles were higher in HIV uninfected participants than in non-Malawian controls and were also mainly made up of endothelial and platelet microparticles. Although there

were only 3 non-Malawian controls and one had a higher number of microparticles than the other two, these were still significantly lower than the HIV uninfected Malawian participants. When comparing to data from healthy UK controls, the total number of circulating microparticles in HIV uninfected adult Malawians was approximately twice as high (personal communication, Rijan Gurung). There are no published data on circulating microparticles from a sub-Saharan Africa cohort either in HIV infected or uninfected individuals. Platelet microparticles and TF positive microparticles were elevated in HIV negative participants with moderate immune activation. The microparticles identified as expressing TF were CD14 negative and therefore unlikely to have been monocyte microparticles. Activation of platelets and TF expression in general would be in keeping with activation of pro-thrombotic pathways which have been previously identified in HIV infection [464], myocardial ischaemia [465] and stroke [466] and have been shown to improve with ART in HIV infected adults [467]. Studies from cancer models have shown that TF positive microparticles can induce platelet activation, leading to perpetuation of the pro-thrombotic state [468]. Similarly, platelet microparticles have been found to be key mediators of pathological thrombotic responses [469] and interestingly, can differentiate between severity of Dengue Virus infection in plasma from patients with acute infection [470]. The significance of the discovery of a high level of pro-thrombotic microparticles in an otherwise healthy population is unknown.

The number of circulating microparticles as well as the majority of subtypes of microparticles were closely associated with PWV including endothelial, leucocyte, smooth muscle and platelet microparticles. Classical monocytes with and without expression of TF were associated with PWV, but not monocytes expressing CD16. An analysis of EMPs in the Framingham Cohort demonstrated a 9% increase in EMPs for each tertile of the Framingham risk score [458]. EMPs have been directly correlated with arterial stiffness [471, 472] and endothelial damage [456]. Further, EMPs are reduced following use of aspirin in patients with diabetes [473] and have been associated with a reduction in arterial stiffness in patients being treatment for hypertension and hyperlipidaemia [474].

We also assessed whether microparticle data could help shed light on whether the highly expanded group of nonclassical monocytes identified in Malawian adults is of functional significance. Firstly, we studied TF expression on monocyte cells, which has been previously implicated in atherosclerosis and is also related to pro-inflammatory coagulation pathways [169]. We found that in a small number of patients, nonclassical monocytes had the highest expression of TF and there was no difference found in the TF expression of monocyte subsets according to HIV status. When looking at microparticles, classical monocyte microparticles expressing TF and those not expressing TF were

both positively associated with PWV, but there was no difference in these microparticle subtypes in those with and without HIV. Because TF is also a soluble factor, it is possible that some of the TF results identified in microparticle analysis may be explained by adherence of soluble TF to circulating microparticle membranes. Even if this was the case, this would still represent increased circulating levels of TF which would contribute to a pro-inflammatory state. Monocyte-platelet aggregates are also common in inflammatory states and lead to upregulation of expression of CD16 on the monocyte cell surface as well as increased adhesion to endothelial cells [475]. It is also therefore possible that an increase in nonclassical monocyte microparticles is not seen due to a consumption rather than underproduction. Interestingly, the only type of microparticle that was associated with HIV viral load were TF expressing monocytes, suggesting that the production of TF may be more closely related to viral stimulus, possibly explaining the upregulation of nonclassical subtypes which have been shown to respond via viral pathways.

So far we have largely discussed circulating microparticles in reference to their role as biomarkers of endothelial damage. However, a growing body of research suggests that microparticles play an important functional role in cellular communication and disease pathogenesis. For example, TF expression on microparticles has been associated with high rates of deep venous thromboembolism (DVT) [476, 477]. Endothelial microparticles may have different actions depending on the environment under which they were produced: microparticles produced under apoptosis can lead to increases in nitric oxide and bystander apoptosis, whereas those produced under activation can propagate inflammation, recruit cells and promote angiogenesis [478]. Microparticles may also transfer important material to other cells. EMPs have been shown to transfer TF to monocytes [475] and the CCR5 receptors may be transferred to endothelial cells from leucocyte derived microparticles potentially rendering them permissible to direct HIV infection [479].More recently, the clinical utility of microparticle quantification and characterisation as biomarkers and potential therapeutic targets is being recognised [480].

This is the first characterisation of circulating microparticles in sub-Saharan Africa. The characterisation of microparticles in this study lends weight to a model where active and significant immune activation is strongly related to endothelial damage. Although this finding is more pronounced in people with HIV, perturbations in the inflammatory axis have been shown in HIV uninfected adults. Further research into the relationship between immune activation and endothelial damage in adult Malawians is therefore warranted.

7 CHAPTER 7: CHANGES IN ENDOTHELIAL DAMAGE AND IMMUNE ACTIVATION FOLLOWING 46 WEEKS OF ART

7.1 Introduction

Significant improvements in immune activation on effective ART are well documented [481, 482]. This is closely related to CD4 reconstitution, viral suppression and improvement in inflammatory markers [483]. However, for a significant proportion of patients, a state of chronic immune activation persists despite suppressive therapy [484]. Chronic immune activation is closely associated with low nadir CD4 counts and therefore we chose a cohort of patients with marked immune suppression to enable analysis of chronic immune activation in this vulnerable group of patients [485].

Most longitudinal studies assessing the change in endothelial damage on ART have focussed on soluble biomarkers [486], showing that they resolve early [487] but remain elevated compared to HIV uninfected individuals for up to at least 12 years [488]. The same pattern was observed in a cohort of South African patients during a median follow up of 7 months where coagulation markers improved but did not normalise compared to HIV uninfected controls [489]. Two studies have shown improvement in flow-mediated dilatation (a measurement of endothelial function) sustained at 6 months of ART, starting as early as 4 weeks post ART initiation [490, 491]. No studies have assessed longitudinal changes in endothelial damage on ART in a sub-Saharan Africa setting.

ART itself contributes to endothelial damage, but evidence suggests that the effect of ART is accumulated over time and is less important during early ART treatment [492]. One advantage of studies from a setting like Malawi is that the majority patients are on the same ART (and with few regime changes) and therefore this variable is standardised across patients. The first three months following the initiation of ART is a particularly high risk period in terms of AIDS related morbidity and mortality [493]. Notably, it is associated with a high risk of acute stroke which may be related to the higher risk of Immune Reconstitution Inflammatory Syndrome (IRIS) amongst patients initiating ART with a low CD4 count [494]. The reconstitution of immune cells and unmasking of underlying co-infections may increase the risk of developing endothelial damage during this time.

The REALITY interventions focus on reducing mortality within the first three months of ART treatment. Each intervention may also target an underlying pathway for the development of chronic immune activation. The enhanced prophylaxis arm may treat or prevent co-infections including TB, cryptococcal meningitis and invasive bacterial infection. The addition of an integrase inhibitor may lead to a more rapid decrease in viral load and a lower viral load set point leading to less continued

viral replication and an earlier recovery of immune activation. RUSF may lead to a reduction in the production of oxidised lipids and may increase the potential for immune recovery. These interventions may reduce immune activation and therefore have an effect on endothelial damage, or may reduce endothelial damage through mechanisms independent of the resolution of immune activation.

This chapter aims to detail changes in both immune activation and endothelial damage during the first 46 weeks of ART therapy and to use the REALITY interventions to begin to understand potential pathogenic mechanisms and therapeutic targets for endothelial damage during early ART therapy.

7.2 Specific objectives

This chapter will address specific objectives 6 and 7:

6. Describe the extent to which resolution of immune activation on ART alters endothelial dysfunction as measured by arterial stiffness

7. Investigate whether intensified initial management of HIV confers a larger decrease in endothelial dysfunction as measured by arterial stiffness, compared to standard ART

7.3 Methods

7.3.1 Study procedures

The patient cohort, measurement of arterial stiffness and immunophenotyping methods have been described in 2.33.1, 3.2 and 5.3.2 respectively. An overview of the follow-up schedule for the longitudinal data capture is provided in Figure 3-7.

7.3.2 Sample size calculations

Sample size calculations for objective 6 are provided in section 3.6. Objective 7 is exploratory and as such has no formal sample size calculation.

7.3.3 Statistical analysis

Univariate and multivariate analysis was performed as described in previous chapters.

Two different types of outcomes were assessed within this chapter. Firstly, absolute PWV at all time points measured were individually analysed. In other words, baseline factors predictive of PWV at week 10, 22 and 44 as well as week 44 factors associated with PWV at week 44. This type of outcome is referred to throughout the chapter as ‘absolute PWV’ at any given time point. Secondly, factors that predicted a change in PWV from baseline were measured – i.e. baseline factors that

affected change in PWV from baseline to week 10, baseline to week 22 and baseline to week 44 were assessed. This outcome is referred to as 'change in PWV' from baseline to any given time point.

For the longitudinal analysis of acute co-infections, patients who had an acute infection at the time of recruitment up until the time of exit were counted as having had an infection during the study period. Therefore, this included anyone who had an infection at baseline plus anyone who developed an acute infection during the study but after enrolment. Data reporting CD4 counts and viral loads include only HIV infected participants as these variables were not measured in HIV uninfected participants. HIV viral load at baseline and change in viral load between baseline and week 44 were handled as continuous data, but HIV viral load was handled as a categorical factor (suppressed or not suppressed) at week 44 because the majority of patients had a suppressed viral load and a continuous variable would not have been meaningful at that point. REALITY and SHIELD participants had viral loads measured on different platforms and therefore the lower limit of detection for viral loads for REALITY participants was 50, but for SHIELD participants it was 150. It is important to highlight here that SHIELD viral loads were carried out at 2 weeks and 46 weeks post ART initiation whereas REALITY viral loads were carried out at ART initiation and at 48 weeks after.

Models looking at the effect of individual factors on PWV were adjusted for confounders identified during the baseline analysis (age, sex, haemoglobin and diastolic blood pressure - see section 4.4.3.1). Models assessing the effects of variables recorded at week 44 were adjusted for diastolic blood pressure and haemoglobin at the time of the week 44 visit. Otherwise, baseline variables were used.

If univariate and multivariate analysis of factors contributing to PWV at individual time points was found to be significant, further analysis of those factors was performed using mixed effects models. Spaghetti plots and hierarchical clustering were first used to ensure that change in PWV followed a linear trend and that different groups of trends were not present. Hierarchical clustering was agglomerative average linking clustering as this was felt to be the most accurate approach to differentiating between groups of trends in change in PWV. A mixed effects model was constructed using time as a predictor variable. Additional covariates were added individually as appropriate based on multivariate analysis at individual time points to determine whether these variables influenced the intercept for PWV at baseline. The covariate of interest was then added as an interaction term to assess whether it influenced the slope of change in PWV over time.

7.4 Results

7.4.1 Description of patient follow-up

An overview of the number of patients retained at each study visit and reasons for patient loss is given in Figure 7-1. Fifty-one patients (13%) did not complete the protocol from enrolment to exit visit and 24 of those (6% of the total number recruited) were due to deaths. One HIV uninfected patient died and had hypertension diagnosed during their enrolment visit. Despite being started on anti-hypertensives she died shortly after from a haemorrhagic stroke. There were 23 deaths amongst the HIV infected group which included 6 cases of pulmonary or disseminated TB, 3 cryptococcal meningitis, 3 Kaposi sarcoma, one gastroenteritis and one TB meningitis. The cause of death was unknown for 9 patients. The large difference in mortality between the REALITY and SHIELD cohorts (12.5% versus 8%) is explained by a high mortality in the first two weeks following ART initiation: REALITY patients were recruited to SHIELD 2 weeks following ART initiation whereas non-REALITY HIV-infected patients were recruited directly at ART initiation (although enrolment procedures were carried out 2 weeks post ART initiation on both HIV infected cohorts).

Eight patients withdrew from the SHIELD study. Of the 5 patients who gave a reason for study withdrawal, one could not take part in two studies, one felt the travel to the clinic was too difficult, one could not come due to travelling with work, one could no longer take part because their family disagreed and one moved away from Blantyre. Twenty patients were lost to follow-up after all attempts at getting in contact through telephone or home visits were exhausted. Common reasons for not being able to trace patients included an incorrect map or patients no longer residing in the area and relatives unaware of their new location.

Figure 7-1 Overview of number of patients attending SHIELD study visits according to recruitment group

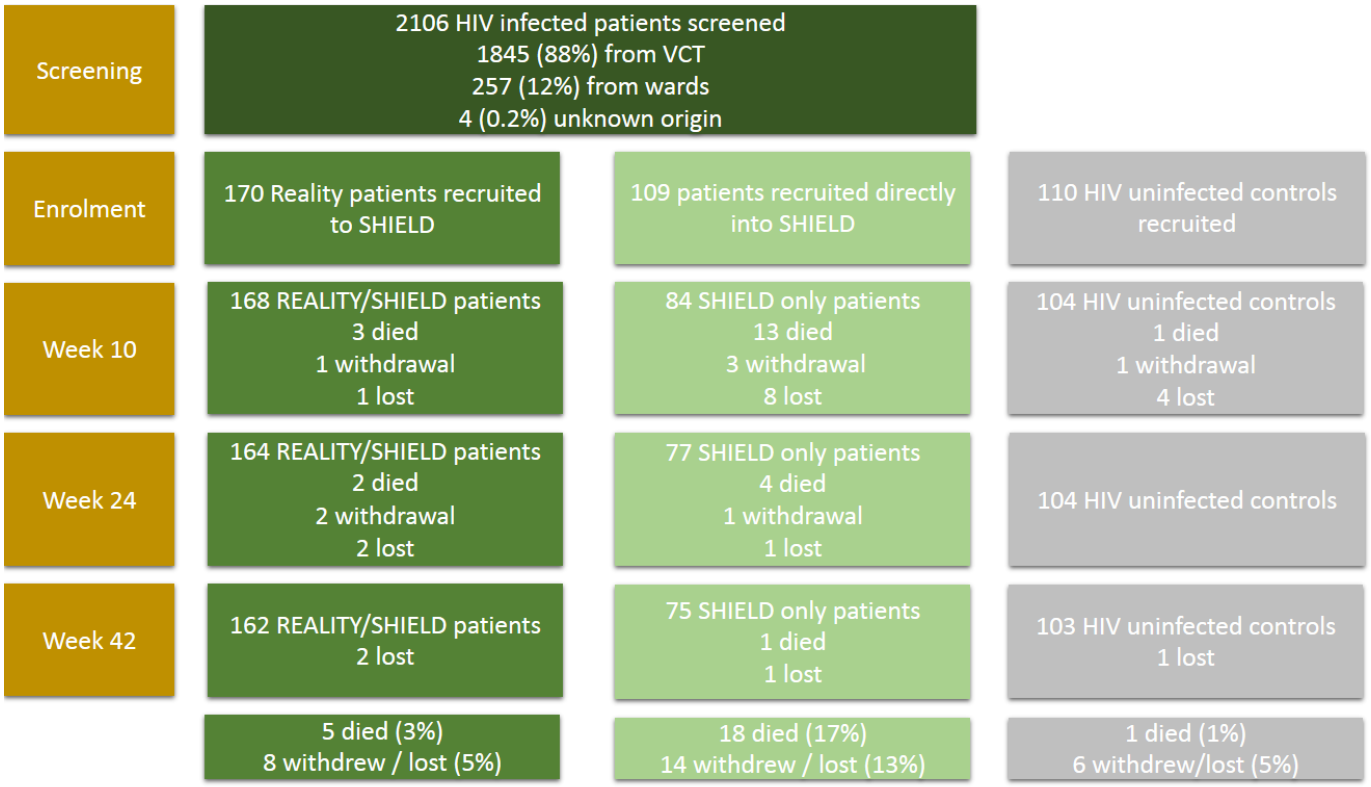


Table 7-1 Compares major characteristics for those patients with HIV infection who died or were lost to follow-up with the overall HIV infected cohort. The proportion of patients with a history of cigarette smoking or alcohol consumption was much greater in both groups of lost patients than in the overall cohort. In addition, the HIV infected patients who died had a lower weight, BMI and haemoglobin compared to the overall HIV infected group (p values <0.01, 0.02 and 0.03 respectively) but had a higher pulse wave velocity, current infection rate and new hypertension diagnoses (p value 0.05, <0.001 and <0.01 respectively). There were no significant differences in mean age or BP and although there was a higher proportion of men in the group of HIV infected patients who died, this did not reach statistical significance (p=0.13).

Table 7-1 Comparison of baseline characteristics for HIV infected patients who died or were lost compared to all HIV infected participants

	HIV infected n=279	**HIV infected participants who**	**HIV infected participants**

			withdrew or were lost n=28	**who died n=23**
Demographic variables	Age	37.5 (+/- 9.8)	36.1 (+/- 10.7)	39.8 (+/- 12.1)
	No. Male	157 (56%)	10 (45%)	16 (70%)
	Primary school education or less	136 (53%)	10 (67%)	8 (57%)
Traditional CV risk factor variables	Weight	54.6 (+/ - 9.8)	50.2 (+/- 7.1)	48.2 (+/- 4.6)
	Waist: height ratio	0.46 (+/- 0.06)	0.44 (+/- 0.04)	0.44 (+/- 0.05)
	BMI	20.6 (+/- 3.5)	19.2 (2.5 +/- 2.5)	18.8 (+/- 2.7)
	Systolic BP	119 (+/- 15)	115 (+/- 19)	116 (+/- 14)
	Diastolic BP	74 (+/- 9)	71 (+/-9)	77 (+/- 12)
	History of smoking	56 (20%)	18 (82%)	18 (78%)
	History of alcohol	119 (43%)	16 (73%)	16 (70%)
	Pre-existing cardiovascular diagnosis	1 (0.4%)	1 (4%)	0
	Prescribed CV drugs	4 (1.5%)	0	0
	Pre-existing diabetes	1 (0.4%)	0	0
	Pre-existing Hypertension	5 (2%)	1 (4%)	0
	New diagnosis of hypertension	88 (32%)	11 (39%)	13 (57%)
	Fasting cholesterol	3.7 (+/- 1.1)	3.6 (+/-0.9)	3.2 (+/- 1.3)
	Fasting glucose	5.0 (+/- 1.1)	4.9 (+/- 0.8)	6.1 (+/- 2.3)
	Creatinine	69.1 (+/- 23.6)	64.3 (+/- 21.9)	77.4 (+/- 31.8)

Infection related variables	Heart rate	86 (+/- 18)	90 (+/- 20)	97 (+/-23)
	Haemoglobin	11.5 (+/- 2.1)	10.8 (+/- 2.1)	10.5 (+/- 1.8)
	Current infection	57 (21%)	2 (7%)	11 (48%)
Immune related variables	Lymphocytes	1.3 (+/- 0.7)	1.3 (+/- 0.6)	1.1 (+/- 0.9)
	Monocytes	0.52 (+/- 0.54)	0.67 (+/- 0.43)	0.67 (+/- 0.60)
	PWV	7.5 (+/-1.3)	7.1 (+/- 1.6)	8.1 (+/- 1.1)
	Mean CCA	0.58 (+/- 0.07)	0.54 (+/- 0.4)	.59 (+/- 0.11)

7.4.2 Description of clinical cohort at 44 weeks

Table 7-2 gives an overview of the clinical characteristics of patients followed up until their exit visit at 44 weeks (46 weeks post ART initiation). For comparison, the same clinical characteristics at baseline are reported in Table 4-1. A diagnosis of an infection at the exit visit includes any acute infection episode diagnosed within the study period (eg malaria, TB, pneumonia, gastroenteritis). At the 44-week visit, patients with HIV infection still had significantly lower weight, haemoglobin and lymphocyte count but significantly higher heart rate and frequency of infection diagnosis (especially TB, pneumonia and gastroenteritis but not malaria). Other traditional cardiovascular risk factors were similar between the two groups, including BP.

Table 7-2 Clinical characteristics of HIV uninfected and infected participants at 44 weeks

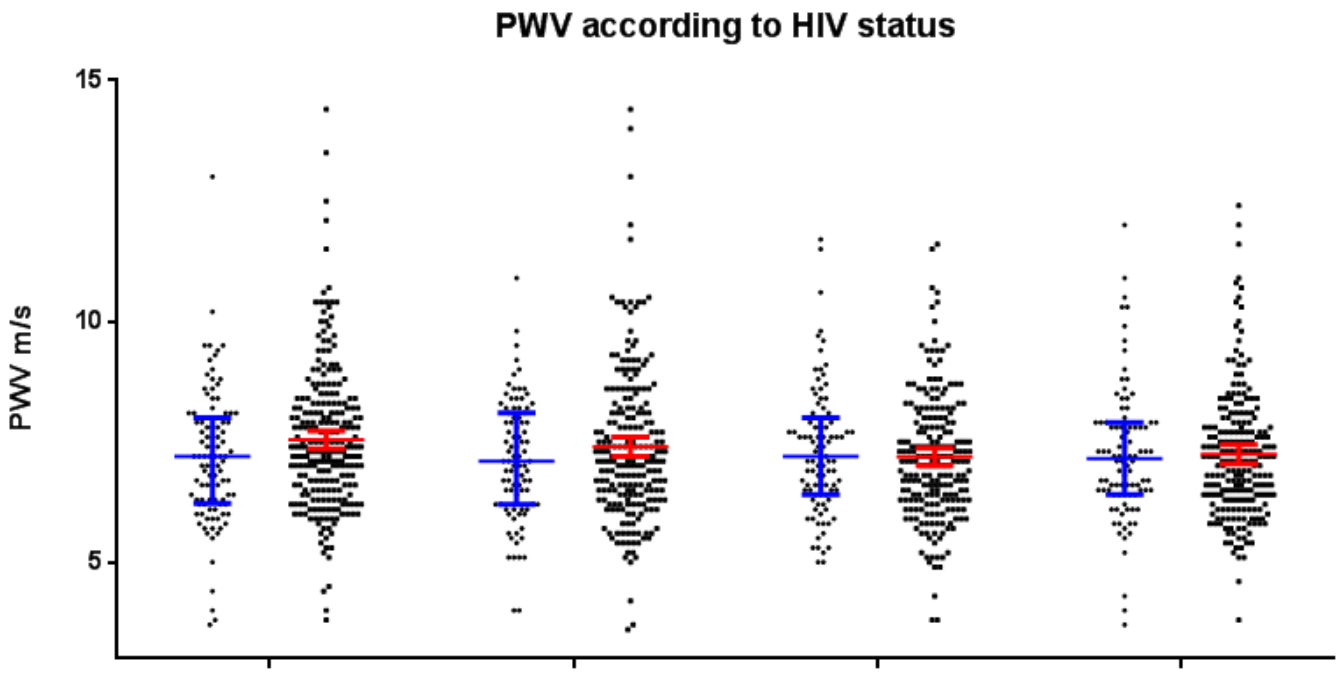
		44 weeks		
		HIV uninfected (n=103)	**HIV infected (n=228)**	**P value**
Traditional CV risk factor variables	Weight	63.7 (+/- 13.4)	60.7 (+/-11.7)	0.08
	Waist: height ratio	0.49 (+/-0.07)	0.49 (+/-0.07)	0.95

	BMI	24.1 (+/-5.5)	22.8 (+/-4.3)	0.14
	Systolic BP	117 (+/-16)	117 (+/-16)	0.11
	Diastolic BP	75 (+/-10)	73 (+/-11)	0.40
	History of smoking	16 (15%)	57 (20%)	0.12
	History of alcohol	28 (25%)	121 (43%)	<0.01
	Pre-existing cardiovascular diagnosis	7 (8%)	17 (8%)	0.60
	Prescribed CV drugs	8 (9%)	8 (4%)	0.06
	Pre-existing diabetes	1 (1%)	1 (0.4%)	0.65
	New diagnosis of hypertension	25 (27%)	41 (18%)	0.11
	Creatinine	60.8 (+/- 13.3)	63.5 (+/-16.7)	0.22
Infection related variables	Heart rate	76 (+/- 13)	81 (+/-14)	<0.001
	Haemoglobin	13.0 (+/-2.1)	12.5 (+/- 2.2)	0.09
	History of infection	15 (16%)	111 (45%)	<0.0001
	TB	0 (0%)	10 (11%)	<0.001
	Cryptococcal meningitis	0 (0%)	5 (2%)	0.19
	Pneumonia	2 (2%)	23 (10%)	<0.01
	Gastroenteritis	3 (3%)	26 (12%)	0.01
	Malaria	6 (6%)	7 (3%)	0.15
Immune related variables	Lymphocytes	2.1 (+/- 0.7)	1.6 (+/-0.6)	<0.00001
	Monocytes	0.34 (+/-0.19)	0.36 (+/-0.40)	0.78

7.4.3 Description of change in PWV on ART

Figure 7-2 gives the PWV values for HIV infected and uninfected participants at each SHIELD time point. SHIELD participants were recruited 2 weeks following the initiation of ART. Although there is an unadjusted increase in PWV in the HIV infected cohort at the SHIELD baseline visit (2 weeks after ART initiation), this difference is lost at the week 10, 22 and 24 time points.

Figure 7-2 Pulse Wave Velocity (PWV) for HIV infected and uninfected participants during four SHIELD study visits



	Baseline	Week 10	Week 22	Week 44
HIV infected (red)	7.3 (6.6 – 8.2)	7.2 (6.4 – 8.1)	7.1 (6.3 – 7.9)	7.15 (6.4 – 7.9)
HIV uninfected (blue)	7.2 (6.2 – 8)	7.1 (6.2 – 8.1)	7.2 (6.4 – 8)	7.0 (6.3 – 7.8)
P value	0.05	0.47	0.37	0.57

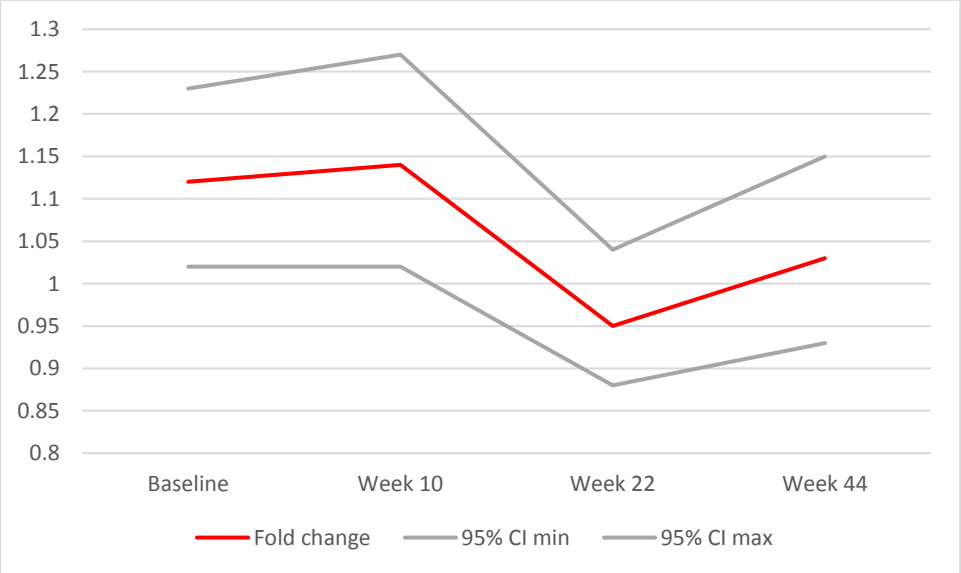
However, when the PWV values were adjusted using the model developed for the baseline assessment (see Table 4-12), PWV was significantly higher in patients with HIV infection. This model is shown in Figure 7-3. The adjusted effect size was similar at week 10 and baseline. HIV was not associated with PWV after adjusting for confounders at week 22 and 42. Figure 7-4 shows the fold change in PWV for patients with HIV infection compared to those without, adjusted for confounders at each SHIELD visit. To assess survivor bias a sensitivity analysis was carried out only on patients

alive at the exit visit which did not affect the effect size or statistical significance of these differences at enrolment and week 10.

Figure 7-3 Multivariate model for effect of HIV status on absolute Week 10 PWV after adjustment for confounders

Variable	Fold change in PWV	P value	95% CI (min)	95% CI (max)
HIV	1.14	0.02	1.02	1.27
Age (per 10-year increase)	1.23	<0.0001	1.17	1.29
Female sex	0.94	>0.2	0.85	1.04
Diastolic BP (per 10 mmHg increase)	1.08	<0.01	1.03	1.13
Haemoglobin	1.01	0.12	1.00	1.04

Figure 7-4 Adjusted fold change in PWV for participants with HIV infection compared to those without adjusted for confounders over study visits



In order to further assess whether change in PWV over time was dependent on HIV status, a mixed effects model was constructed. Spaghetti plots were assessed for the presence of different patterns

of change in PWV over time. This was done for HIV infected (Figure 7-5) and uninfected (Figure 7-6) participants. A minority of HIV infected participants started off with very high PWV values at enrolment and week 10 which then resolved. No other distinct groups were visualised. Furthermore, no distinct groups were identified when using a hierarchical clustering approach as demonstrated in Figure 7-7. Therefore, it was assumed that change in PWV over time was largely linear.

Figure 7-5 Spaghetti plot of changes in PWV over time for HIV infected participants

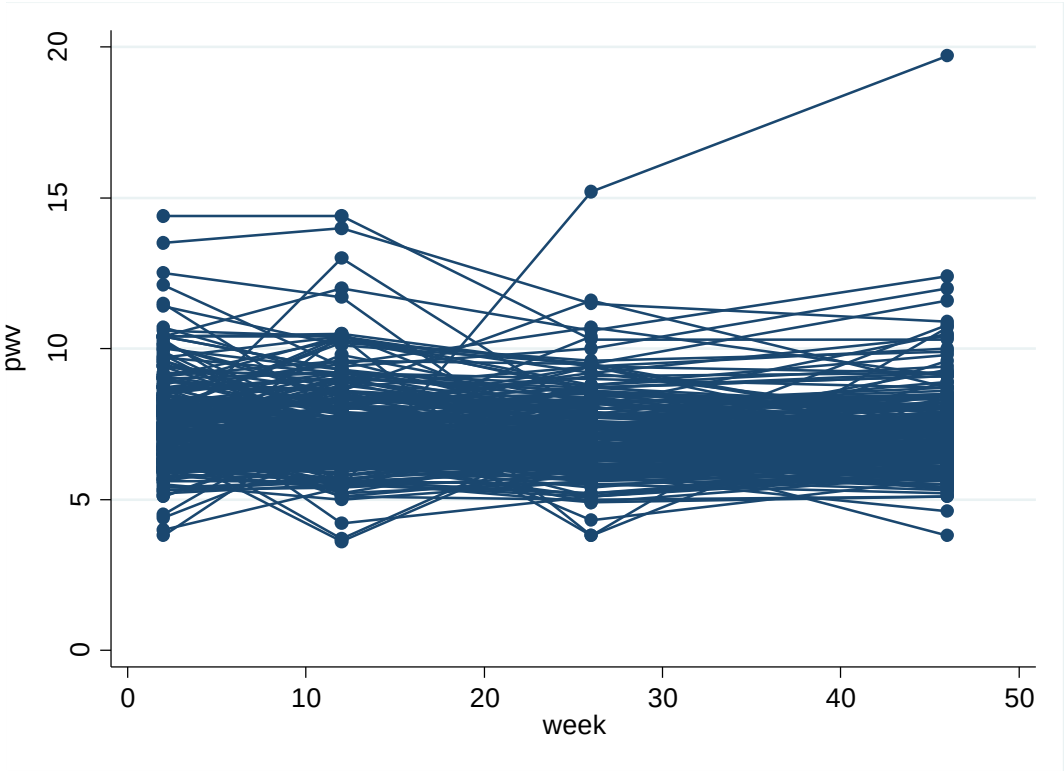


Figure 7-6 Spaghetti plot of changes in PWV over time for HIV uninfected participants

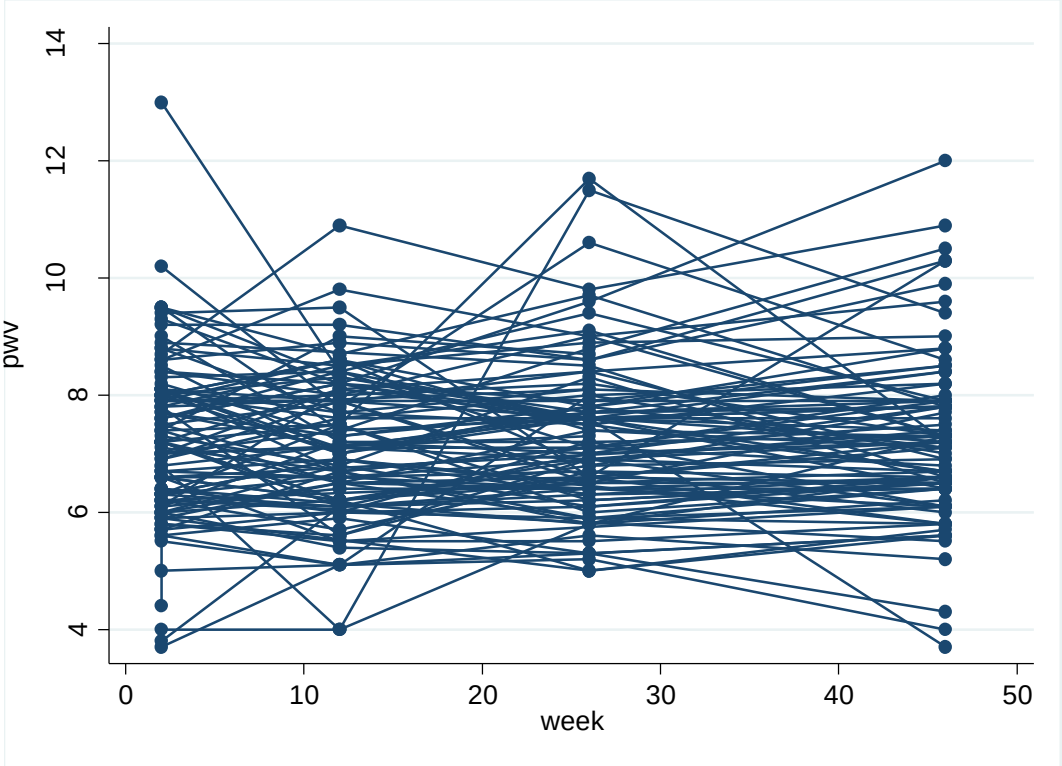
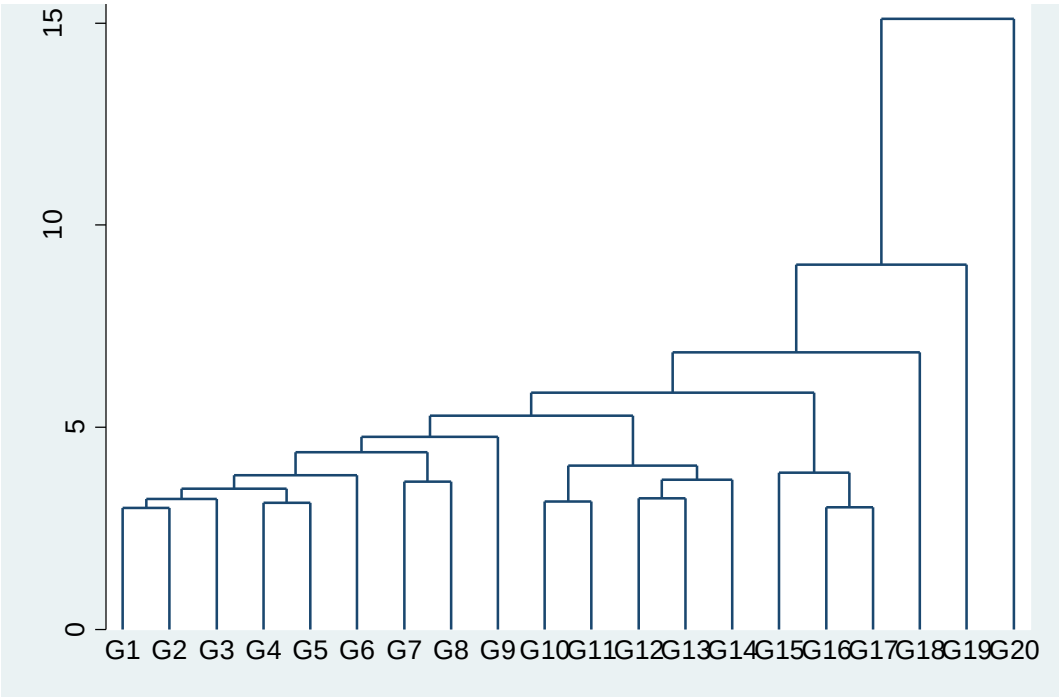


Figure 7-7 Hierarchical clustering dendogram of patterns of change in PWV over time



A mixed effects model approach showed a significant effect of time on change in PWV [fold change in PWV per visit -0.004 (-0.008 - -0.0004) p=0.03]. When HIV was added as a covariate to the model

looking at the effect of time, it was significantly associated with change in PWV [fold change in PWV in HIV infected participants 0.35 (0.04 – 0.66), p=0.03]. When HIV was added as an interaction term, the slope was reduced in HIV infection by a coefficient of -0.008 (-0.02 – 0.00, p=0.06). Other potential confounders were not adjusted for in this model – it only assessed the risk of HIV and time on PWV.

7.4.4 Clinical predictors of absolute PWV at week 10 visit

On univariate analysis, the only baseline variables associated with a change in PWV between baseline and 10 weeks were waist height ratio, systolic BP, haemoglobin and CD4% [spearman rho (p value) 0.07 (0.18), -0.09 (0.10), -0.09 (0.13) and -0.16 (0.07) respectively]. Entering these variables into a multivariate analysis, the fold change in PWV difference (CI min – CI max, p value) for waist height ratio, systolic BP, haemoglobin and CD4% was 0.25 per 0.1 cm^{-1} (-26.4 - 26.9, 0.06) -0.10 per 10mmHg (-0.21 - 0.01, 0.07), -0.01 g/dL (-0.9 - 0.07, 0.83) and -0.05 per 10% increase (-0.12 - 0.02, 0.13).

7.4.5 Clinical predictors of absolute PWV at week 22 visit

In univariate analysis HIV infection and co-infection at enrolment were associated with a fold decrease in change of PWV of -0.42 (CI -0.89 – 0.04, p=0.07) and -0.14 (CI -0.31 – 0.04, p=0.07) respectively, but were not significantly associated on multivariate analysis.

7.4.6 Clinical predictors of absolute PWV at week 44 visit

Firstly, clinical variables measured at week 44 were checked for univariate associations with PWV at week 44 and are shown in Table 7-3 and Table 7-4. The final model for clinical variables measured at 44 weeks and associated with PWV at 44 weeks, retained age [fold change 1.27 (CI 1.20 – 1.34) per 10 years, p<0.00001), diastolic BP [1.11 (1.07 – 1.16) per 10 mmHg, p<0.01] and female sex [0.92 (0.84 – 1.01), p=0.07].

Table 7-3 Univariate associations between clinical variables measured at 44 weeks and absolute PWV at 44 weeks for continuous data

	Spearman's rho	**P value**	**Included in model**
Age	0.16	<0.00001	X
Weight	0.16	<0.01	
Waist: height ratio	0.17	<0.01	X
BMI	0.09	0.12	
Heart rate	0.03	0.65	

Systolic BP	0.34	<0.00001	X
Diastolic BP	0.38	<0.00001	
Haemoglobin	-0.08	0.19	X
Creatinine	0.07	0.28	
Lymphocytes	0.01	0.84	
Monocytes	0.03	0.64	

Table 7-4 Univariate associations between clinical variables measured at 44 weeks and absolute PWV at 44 weeks for categorical data

	Median PWV (m/s)	**P value**	**Included in model**
HIV infected	7.0 (6.4 – 7.8)		
HIV uninfected	7.2 (6.4 – 7.9)	0.70	
Male	7.2 (6.5 – 7.8)		
Female	6.7 (6.1 – 7.8)	<0.01	X
Primary school education or less	7.0 (6.4 – 7.9)		
Greater than primary school education	7.1 (6.4 – 7.8)	0.85	
Smoker or ex-smoker	7 (6.4- 7.8)		
Never smoked	7.2 (6.4 – 7.9)	0.80	
Drinks alcohol or past alcohol	7.1 (6.4 – 7.8)		
Never drank alcohol	7.1 (6.2 – 7.9)	0.73	
Cardiovascular diagnosis	7.5 (6.9 – 8.4)		
No previous cardiovascular diagnosis	7 (6.4- 7.8)	0.08	
Prescribed CV drugs	8.1 (7.4 – 8.9)		
Never prescribed CV drugs	7 (6.4 – 7.8)	<0.01	X
Clinically hypertensive	7.6 (6.9 – 8.7)		
Not clinically hypertensive	6.8 (6.3 – 7.7)	<0.00001	
Infection during study period	7.1 (6.3 – 7.8)		
No infection during study period	7.1 (6.4- 7.9)	0.48	

Next, clinical variables measured at baseline were assessed for associations with PWV at week 44. Table 7-5 and Table 7-6 show univariate associations and factors included in the final multivariate

model. Table 7-7 gives the final model with the baseline clinical characteristics retained for predicting week 44 PWV. In addition to age and male sex, diastolic BP and creatinine at baseline are associated with a higher PWV at week 44.

Table 7-5 Univariate associations between clinical variables measured at baseline and absolute PWV at 44 weeks for continuous data

	Spearman's rho	**P value**	**Included in model**
Age	0.58	<0.0001	X
Weight	0.29	<0.0001	X
Waist: height ratio	0.18	<0.01	
BMI	0.21	<0.001	
Heart rate	-0.02	0.69	
Systolic BP	0.26	<0.00001	
Diastolic BP	0.32	<0.00001	x
Haemoglobin	0.14	0.01	x
Creatinine	0.19	<0.001	x
Fasting cholesterol	0.17	<0.01	x
Fasting glucose	-0.02	0.76	
Lymphocytes	0.05	0.34	
Monocytes	0.03	0.64	

Table 7-6 Univariate associations between clinical variables measured at baseline and absolute PWV at 44 weeks for categorical data

	Median PWV (m/s)	**P value**	**Included in model**
HIV infected	7.0 (6.3 – 7.8)		
HIV uninfected	7.2 (6.4 – 7.9)	0.70	
Male	7.2 (6.5- 7.8)		
Female	6.7 (6.1 – 7.8)	<0.01	X
Primary school education or less	7.0 (6.4 – 7.9)		
Greater than primary school education	7.1 (6.4 – 7.8)	0.85	
Smoker or ex-smoker	7.2 (6.4 – 7.8)		

Never smoked	7.0 (6.4 – 7.9)	0.95	
Drinks alcohol or past alcohol	7.1 (6.4 – 7.8)		
Never drank alcohol	7.1 (6.2 – 7.9)	0.78	
Cardiovascular diagnosis	7.7 (7.0 – 8.4)		
No previous cardiovascular diagnosis	7.0 (6.4 – 7.8)	0.04	x
Prescribed CV drugs	7.8 (7.1 – 8.6)		
Never prescribed CV drugs	7.1 (6.4 – 7.8)	0.07	
Clinically hypertensive	7.8 (7.1 – 9.1)		
Not clinically hypertensive	7.0 (6.4 – 7.8)	0.04	
Current infection	7.1 (6.3 – 7.4)		
No current infection	7.1 (6.4 – 7.9)	0.75	

Table 7-7 Clinical baseline variables predictive of absolute Week 44 PWV

Variable	**Fold change in PWV**	**P value**	**95% CI (min)**	**95% CI (max)**
Age (per 10-year increase)	1.23	<0.00001	1.17	1.29
Female sex	0.92	0.08	0.84	1.01
Diastolic BP (per 10 mmHg increase)	1.03	<0.00001	1.00	1.05
Creatinine (per 10 umol/L)	1.11	0.02	1.05	1.16

Finally, clinical variables measured at baseline were assessed for univariate associations with the change in PWV between baseline and week 44 (Table 7-8 and Table 7-9) and those with p value<0.2 were entered into the model. The final model using clinical variables collected at baseline to predict change in PWV at week 44 retained only current infection [fold change 0.43 (CI 0.15 – 1.25), p=0.12]. When the model was run without current infection, only HIV was retained [fold change 0.56 (CI 0.24 – 1.30), p=0.18].

Table 7-8 Univariate associations between clinical variables measured at baseline and change in PWV between baseline and 44 weeks for continuous data

	Spearman's rho	**P value**	**Included in model**
Age	0.05	0.35	x

Weight	0.10	0.07	X
Waist: height ratio	0.09	0.11	
BMI	0.10	0.08	
Heart rate	-0.07	0.26	
Systolic BP	-0.03	0.59	
Diastolic BP	-0.05	0.35	
Haemoglobin	-0.003	0.96	
Creatinine	-0.01	0.83	
Fasting cholesterol	0.05	0.38	
Fasting glucose	-0.11	0.06	X
Lymphocytes	0.05	0.43	
Monocytes	0.04	0.54	

Table 7-9 Univariate associations between clinical variables measured at baseline and change in PWV between baseline and 44 weeks for categorical data

	Median PWV (m/s)	**P value**	**Included in model**
HIV infected	-0.40 (-1.00 – 0.20)		
HIV uninfected	-0.10 (-0.79 – 0.70)	0.02	x
Male	-0.30 (-0.90 – 0.20)		
Female	-0.20 (-0.90 – 0.40)	0.33	
Primary school education or less	-0.40 (-1.00 – 0.20)		
Greater than primary school education	-0.20 (-0.90 – 0.50)	0.18	X
Smoker or ex-smoker	-0.50 (-1.20 – 0.30)		
Never smoked	-0.20 (-0.90 – 0.40)	0.18	X
Drinks alcohol or past alcohol	-0.25 (-0.90 – 0.40)		
Never drank alcohol	-0.30 (-0.90 – 0.20)	0.58	
Cardiovascular diagnosis	-0.35 (-0.95 – 0.00)		
No previous cardiovascular diagnosis	-0.20 (-0.90 – 0.40)	0.23	
Prescribed CV drugs	-0.20 (-0.40 – 0.00)		
Never prescribed CV drugs	-0.30 (-0.90 – 0.40)	0.79	
Clinically hypertensive	-0.20 (-0.90 – 0.30)		
Not clinically hypertensive	-0.40 (-1.10 – 0.45)	0.46	
Current infection	-0.20 (-0.90 – 0.40)		

No current infection	-0.50 (-1.00 – 0.00)	0.12	x

7.4.7 Description of change in T cells surface immune phenotypes between baseline and week 44 visits

Overall, the absolute CD4 count increased from screening to the week 44 visit from 44 to 150 cells/mm^3 as shown in Figure 7-8 ($p<0.00001$). An improvement was also seen in the CD4/CD8 ratio in the HIV infected participants ($p<0.01$), but remained much lower than HIV uninfected participants (Figure 7-9). CD4 and CD8 activation at baseline (2 weeks after ART initiation) and at week 44 visit for HIV uninfected and HIV infected participants is shown in Figure 7-10. CD4 activation but not CD8 activation decreased significantly at week 44 compared to baseline ($p<0.00001$ and $p=0.21$ respectively). The percentage of exhausted cells decreased over the same period for both CD4 and CD8 T cells, with the bigger effect seen in the CD4 T cell population ($p<0.00001$ and <0.01 respectively – see Figure 7-11). However, there was no improvement in T cell senescence between the two time points and CD8 T cell senescence actually increased ($p=1.00$ and <0.001 respectively – see Figure 7-12). There were no differences between the baseline and week 44 immune parameters within the HIV uninfected group.

Figure 7-8 Absolute CD4 counts for HIV infected participants at ART initiation and at week 44 visit

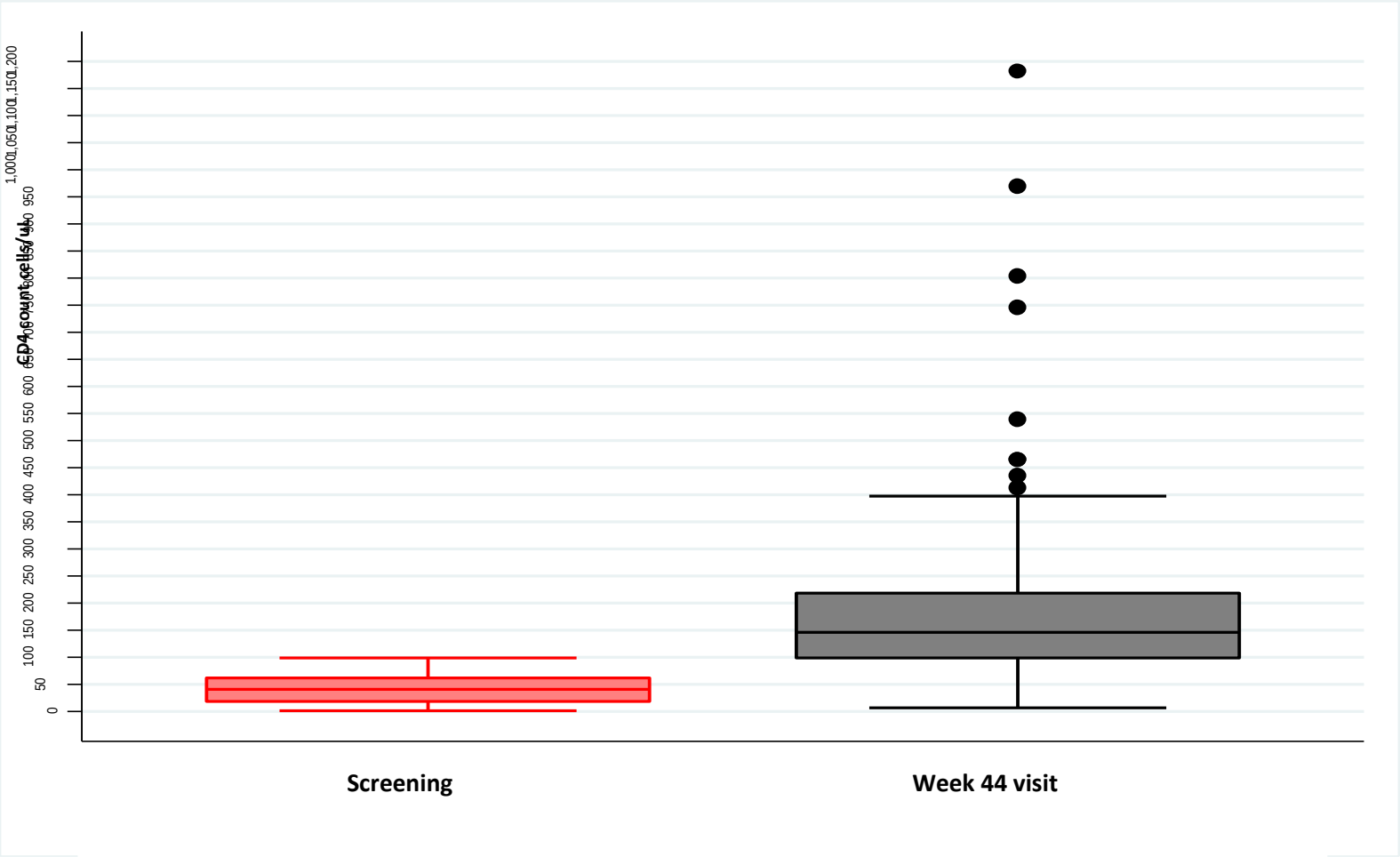


Figure 7-9 CD4/CD8 ratio in HIV uninfected and infected adults at baseline and week 44

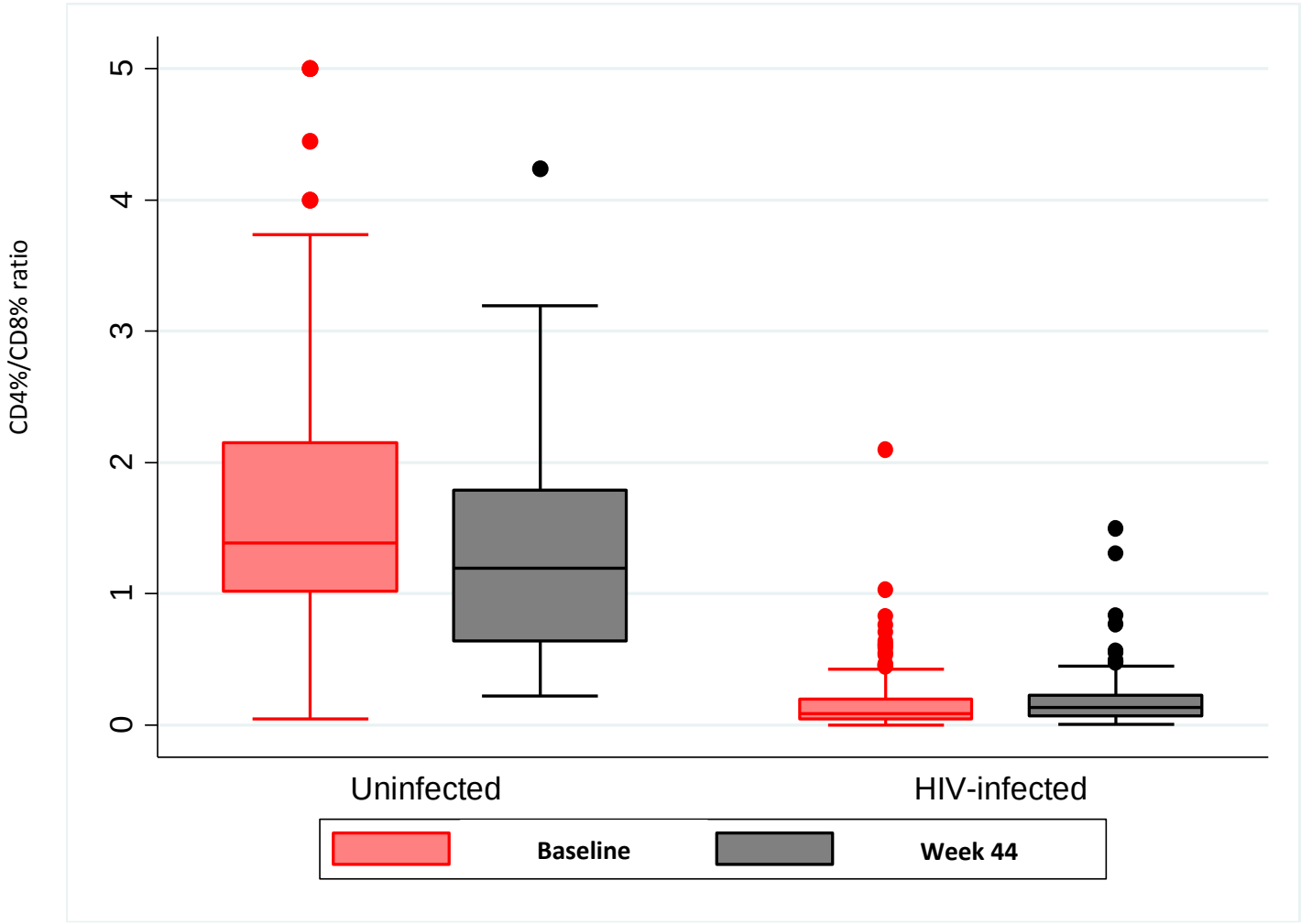


Figure 7-10 % Activated CD4 and CD8 T cells at baseline and week 44 in HIV uninfected and infected participants

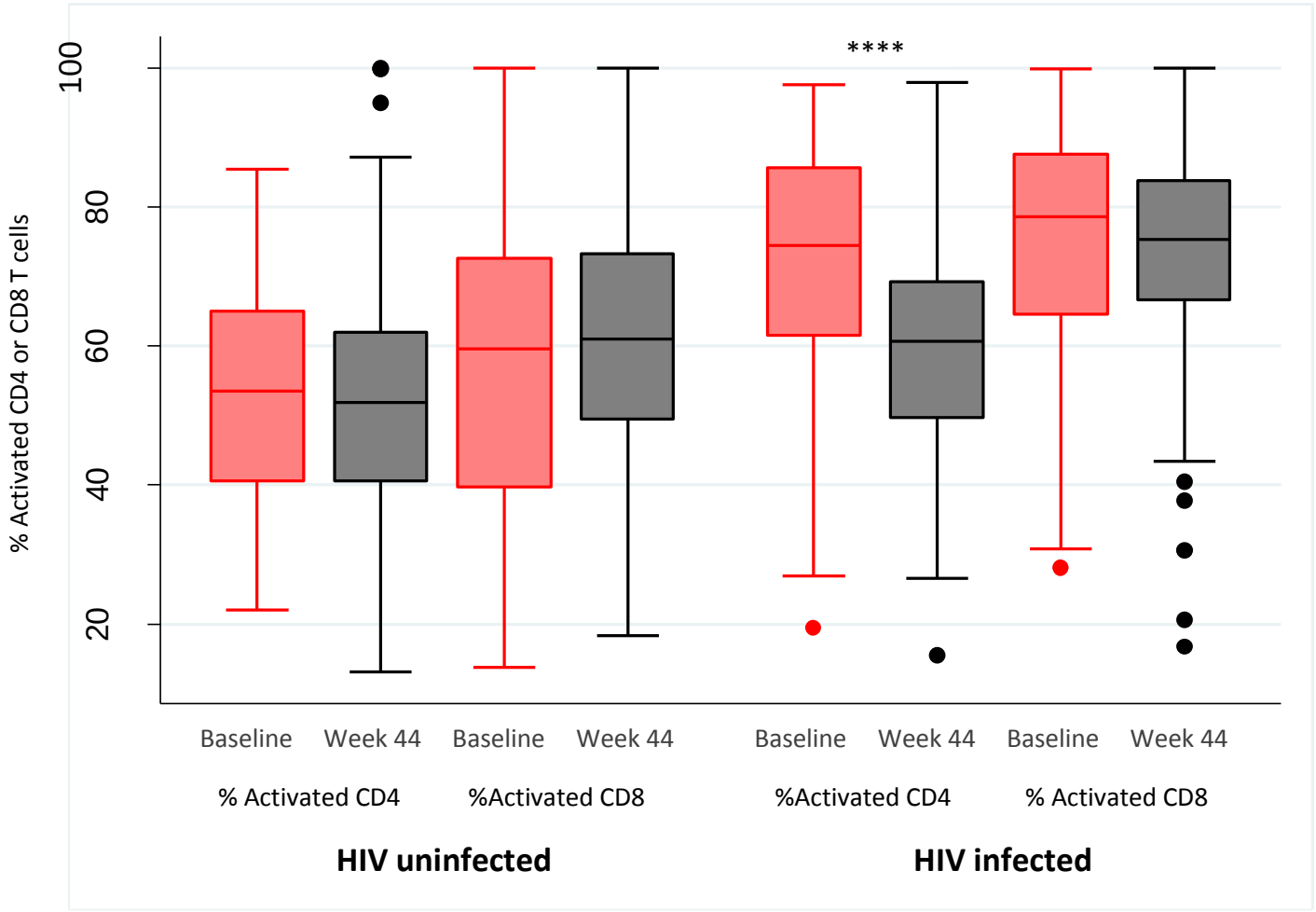


Figure 7-11 % Exhausted CD4 and CD8 T cells at baseline and week 44 in HIV uninfected and infected participants

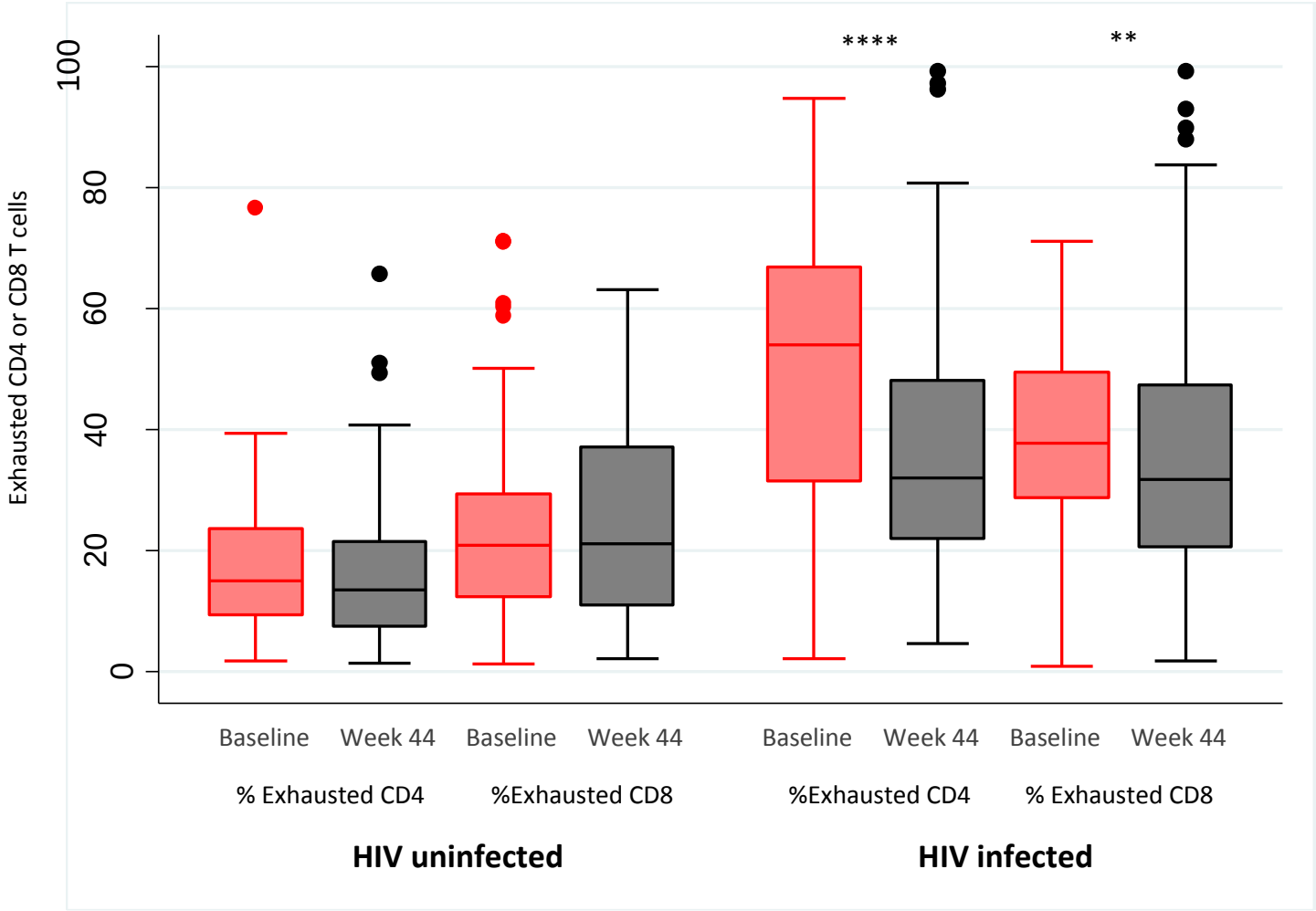
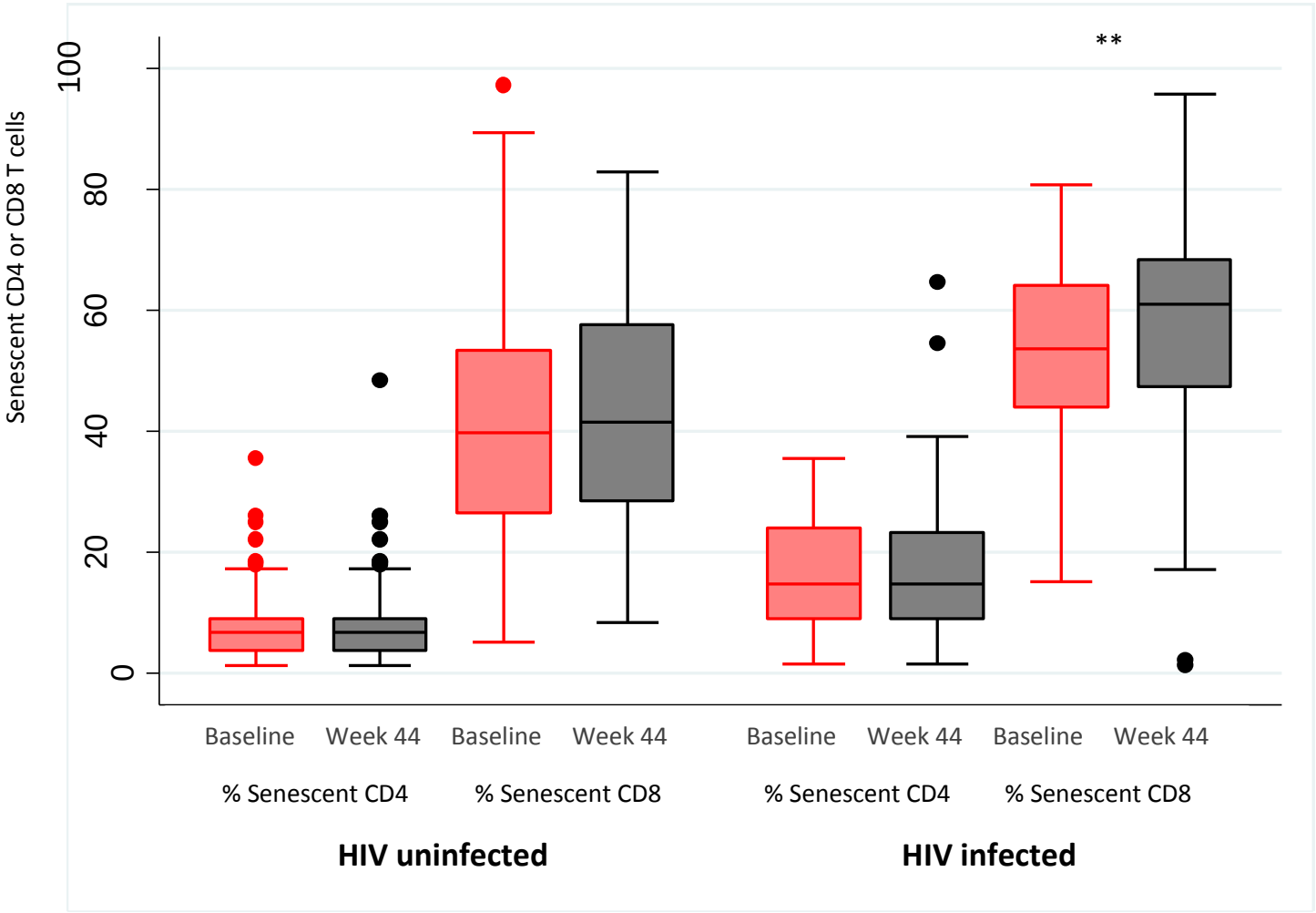


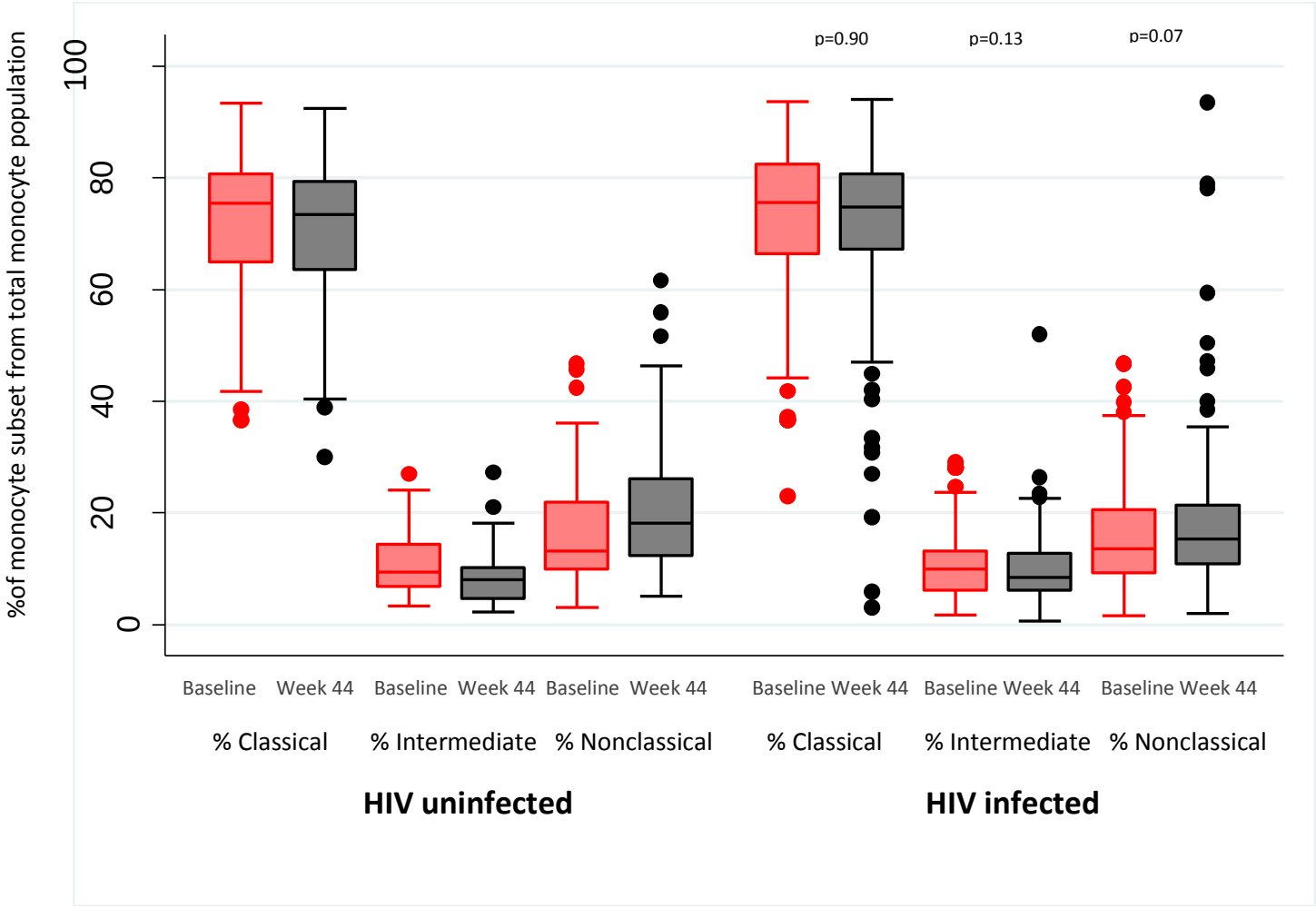
Figure 7-12 % Senescent CD4 and CD8 T cells at baseline and week 44 in HIV uninfected and infected participants



7.4.8 Description of change in monocyte cell surface subtypes between baseline and week 44 visit

There was no change in the percentage of classical or intermediate monocytes within the HIV infected population between baseline and week 44 visit. There was a trend towards an increase in nonclassical monocytes but this may have been influenced by outliers (see Figure 7-13). There were no significant differences in monocyte subsets within the HIV uninfected population over the same time period.

Figure 7-13 Proportion of monocyte subsets at baseline and week 44 in HIV uninfected and infected participants



7.4.9 The role of immune activation markers in adding to clinical predictors of absolute PWV at 44 weeks

7.4.9.1 At the week 44 visit, is immune activation associated with PWV?

CD4 and CD8 T cell exhaustion were associated with PWV at ART initiation (see section 5.4.4.2). We are now interested in whether this association remains after 44 weeks of ART therapy. Table 7-10 gives univariate associations between immune markers and PWV both measured at week 44. A detectable HIV viral load was not associated with PWV at 44 weeks. The proportion of CD4 T cells that express only CD38 (and are negative for HLA-DR) is negatively associated with PWV. This subset is likely to explain the negative association found between CD4 activated cells (in other words all CD4 T cells expressing CD38 or HLA-DR) and PWV at Week 44. It is possible that the CD4+CD38+HLA-DR- phenotype is an immune marker associated with a favourable immune activation profile (as indicated in the principal component analysis at baseline – see 5.4.5). CD4CD38+HLA-DR- T cells

were retained in the multivariate model when replaced for HIV, but at a p value which was not statistically significant with this number of participants (see Table 7-11).

Table 7-10 Univariate associations between immune markers at Week 44 and absolute PWV at Week 44

		Spearman rho n=273	p value
Absolute CD4 cell count (cells/L)		-0.09	0.23
CD4 T cells	**CD4/CD8 ratio**	0.02	0.69
	CD4 %	0.03	0.67
	CD4 CD38+HLA-DR-	-0.13	0.03
	CD4 CD38+HLA-DR+	-0.06	0.32
	CD4 CD38-HLA-DR+	0.03	0.58
	CD4 Activated	-0.17	<0.01
	CD4 CD57+PD1-	0.07	0.23
	CD4 CD57+PD1+	-0.005	0.94
	CD4 CD57-PD1+	-0.05	0.41
	CD4 Exhausted	-0.04	0.56
	CD4 Senescent	-0.01	0.84
CD8 T cells	**CD8%**	-0.004	0.95
	CD8 CD38+HLA-DR-	0.02	0.77
	CD8 CD38+HLA-DR+	0.004	0.94
	CD8 CD38-HLA-DR+	0.06	0.33
	CD8 Activated	0.01	0.93
	CD8 CD57+PD1-	0.06	0.33
	CD8 CD57+PD1+	0.03	0.66
	CD8 CD57-PD1+	-0.03	0.58
	CD8 Exhausted	0.02	0.78
	CD8 Senescent	0.07	0.25
Monocytes	**Classical**	-0.08	0.21

	Intermediate	-0.02	0.69
	Nonclassical	0.10	0.12

Table 7-11 Multivariate analysis for association between CD438+HLA-DR- CD4 T cells and absolute PWV at Week 44 after adjusting for confounders

Variable	Fold change in PWV	P value	95% CI (min)	95% CI (max)
Age (per 10-year increase)	1.26	<0.0001	1.21	1.32
Female sex	0.91	0.03	0.84	0.99
Diastolic BP (per 10 mmHg increase)	1.12	<0.0001	1.08	1.16
%CD4 CD38+HLA-DR-	0.98	0.15	0.95	1.01

7.4.9.2 Can baseline immune markers predict change in PWV over 44 weeks?

Next, we examined whether the effects of immune activation on PWV identified at baseline impacted PWV at week 44. Table 7-12 shows the univariate associations between immune markers measured at baseline and week 44 PWV. Higher proportions of CD4 activation (and, in particular, the CD38+HLA-DR+ phenotype), CD4 exhaustion, CD4 senescence and intermediate monocytes at baseline are associated with a lower PWV at Week 44. There is also a trend towards a less favourable CD4/CD8 T cell ratio also being associated with lower PWV at week 44. Taken together, this would suggest that those with the least favourable CD4 T cell percentages and immune activation profiles at baseline are those that are more likely to have a lower PWV after 44 weeks of ART. On multivariate analysis of the association between HIV and week 44 PWV, adjusted for confounders, there was a trend towards HIV being associated with a lower PWV at week 44 (see Table 7-13). When substituted with HIV in this model, CD4/CD8 ratio, percentage of CD4 CD38+HLA-DR+ and percentage of intermediate monocytes at baseline were all retained as predictors for PWV at week 44, albeit with p values above statistical significance (see Table 7-14, Table 7-15 and Table 7-16).

Table 7-12 Univariate associations between immune markers at baseline and PWV at Week 44

		Spearman rho n= 248	p value
Absolute CD4 count (cells/uL)		0.07	0.26
HIV viral load (copies/ml)		0.03	0.71
CD4 T cells	**CD4/CD8 ratio**	0.11	0.08
	CD4 %	0.11	0.08
	CD4 CD38+HLA-DR-	0.09	0.23
	CD4 CD38+HLA-DR+	-0.18	0.02
	CD4 CD38-HLA-DR+	-0.10	0.18
	CD4 Activated	-0.17	0.03
	CD4 CD57+PD1-	-0.09	0.23
	CD4 CD57+PD1+	-0.22	<0.01
	CD4 CD57-PD1+	-0.14	0.07
	CD4 Exhausted	-0.16	0.04
	CD4 Senescent	-0.20	<0.01
CD8 T cells	**CD8%**	-0.11	0.08
	CD8 CD38+HLA-DR-	-0.002	0.97
	CD8 CD38+HLA-DR+	-0.03	0.66
	CD8 CD38-HLA-DR+	-0.03	0.60
	CD8 Activated	0.03	0.66
	CD8 CD57+PD1-	-0.01	0.85
	CD8 CD57+PD1+	-0.10	0.12
	CD8 CD57-PD1+	-0.03	0.59
	CD8 Exhausted	-0.06	0.34
	CD8 Senescent	-0.07	0.29
Monocytes	**Classical**	0.04	0.51
	Intermediate	-0.19	<0.01

	Nonclassical	0.08	0.21

Table 7-13 Multivariate analysis for association between HIV status and PWV at week 44 after adjusting for confounders

Variable	Fold change in PWV	P value	95% CI (min)	95% CI (max)
Age (per 10-year increase)	-0.01	0.90	-0.18	0.16
Female sex	0.07	0.67	-0.25	0.39
Diastolic BP (per 10 mmHg increase)	0.14	0.08	0.00	0.28
HIV	-0.31	0.08	-0.63	0.05

Table 7-14 Multivariate analysis for association between CD4/CD8 ratio at baseline and PWV at week 44 after adjusting for confounders

Variable	Fold change in PWV	P value	95% CI (min)	95% CI (max)
Age (per 10-year increase)	0.20	0.05	0.01	0.39
Female sex	0.29	0.09	-0.05	0.63
Diastolic BP (per 10 mmHg increase)	0.15	0.06	-0.01	0.30
CD4/CD8 ratio (per 0.1-unit increase)	0.01	0.17	0.00	0.03

Table 7-15 Multivariate analysis for association between HIV %CD38+HLA-DR+ CD4 T cells at baseline and PWV at week 44 after adjusting for confounders

Variable	Fold change in PWV	P value	95% CI (min)	95% CI (max)
Age (per 10-year increase)	0.28	0.02	0.04	0.51
Female sex	0.49	0.03	0.06	0.91
Diastolic BP (per 10 mmHg increase)	0.16	0.12	-0.04	0.36
%CD4 CD38^{+}HLA-DR^{+} (per 10% increase)	-0.12	0.10	-0.25	0.02

Table 7-16 Multivariate analysis for association between %intermediate monocytes at baseline and PWV at week 44 after adjusting for confounders

Variable	Fold change in PWV	P value	95% CI (min)	95% CI (max)
Age (per 10-year increase)	0.14	0.19	-0.07	0.36
Female sex	0.17	0.39	-0.21	0.54
Diastolic BP (per 10 mmHg increase)	0.19	0.04	0.01	0.37
%Intermediate monocytes	-0.28	0.10	-0.61	0.05

7.4.9.3 Is change in immune activation markers related to a change in PWV over 44 weeks?

Finally, we analysed whether the change in immune activation markers between baseline and week 44 was predictive of PWV at the three different time points it was measured over 44 weeks. Table 7-17 gives the univariate associations between the change in each immune marker from baseline to 44 weeks and PWV at the three time points. There is a trend towards an increase in the CD4 CD38+HLA-DR- phenotype being associated with PWV at all three time points, which again likely accounted for the association between an increase in CD4 activation and a lower PWV at week 44. It is possible that the CD4 CD38+HLA-DR- phenotype is an immune marker of protection against endothelial damage at week 44. In contrast, a decrease in the proportion of CD4 T cells expressing both CD57 and PD-1 markers was associated with a lower PWV at week 44. Interestingly, a decrease

in the percentage of CD8 T cells was associated a lower PWV at week 44, but this association wasn't seen for CD4 T cells or the overall CD4/CD8 T cell ratio, suggesting that a high proportion of CD8 T cells rather than a low proportion of CD4 cells over time might play a more important role in establishing endothelial damage. However, of note, changes in CD8 T cell activation, exhaustion or senescence were not associated with PWV at week 44 indicating that any resolution in endothelial damage comes from a reduction in CD4 T cell rather than CD8 T cell immune activation. Additionally, a reduction in intermediate monocytes is associated with PWV at both 24 and 42 weeks. Although no difference was found in the proportion of intermediate monocytes according to HIV status at baseline, improvement in these subsets is associated with improvement in PWV.

Table 7-17 Univariate analysis of associations between baseline immune markers and change in PWV from baseline at 10, 22 and 44 week visits

		Week 10		**Week 22**		**Week 44**	
		Spearman rho n=196	p value	Spearman rho n=199	p value	Spearman rho n= 210	p value
Absolute CD4 cell count (cells/uL)		0.02	0.76	0.09	0.20	0.06	0.39
HIV viral load (copies/ml)		-0.03	0.63	-0.02	0.77	0.08	0.27
CD4 T cells	**CD4/CD8 ratio**	0.09	0.19	-0.02	0.83	-0.07	0.29
	CD4 %	0.03	0.74	-0.16	0.04	-0.04	0.64
	CD4 CD38+HLA-DR-	-0.15	0.08	-0.13	0.11	-0.15	0.07
	CD4 CD38+HLA-DR+	-0.02	0.85	-0.04	0.62	0.06	0.49
	CD4 CD38-HLA-DR+	-0.04	0.63	-0.05	0.52	-0.06	0.51
	CD4 Activated	0.00	0.96	-0.06	0.44	-0.17	0.03
	CD4 CD57+PD1-	0.08	0.35	0.19	0.03	0.07	0.42
	CD4 CD57+PD1+	0.00	0.97	0.05	0.53	0.20	0.02

	CD4 CD57-PD1+	0.01	0.87	0.00	0.93	0.08	0.36
	CD4 Exhausted	-0.07	0.44	-0.10	0.26	0.04	0.67
	CD4 Senescent	0.03	0.69	0.06	0.45	0.10	0.22
CD8 T cells	**CD8%**	-0.06	0.43	0.08	0.24	0.29	<0.01
	CD8 CD38+HLA-DR-	-0.07	0.32	-0.04	0.62	-0.02	0.79
	CD8 CD38+HLA-DR+	-0.07	0.36	-0.04	0.62	-0.04	0.57
	CD8 CD38-HLA-DR+	0.07	0.31	0.00	0.97	0.04	0.61
	CD8 Activated	-0.09	0.19	-0.10	0.15	-0.06	0.36
	CD8 CD57+PD1-	0.04	0.56	-0.01	0.92	-0.01	0.90
	CD8 CD57+PD1+	0.06	0.42	-0.05	0.53	0.06	0.38
	CD8 CD57-PD1+	-0.06	0.43	-0.03	0.63	-0.01	0.87
	CD8 Exhausted	0.03	0.64	0.00	0.89	0.08	0.25
	CD8 Senescent	-0.02	0.77	-0.02	0.81	-0.01	0.87
Monocytes	**Classical**	-0.08	0.31	-0.10	0.19	-0.11	0.12
	Intermediate	0.07	0.36	0.18	0.02	0.14	0.06
	Nonclassical	0.00	0.95	0.03	0.65	0.00	0.98

7.4.10 To what extent does the diagnosis of another infection during the study period contribute to immune activation?

7.4.10.1 Immune activation related to infection diagnosis

Analysis so far has shown that higher immune activation at baseline is associated with a lower PWV at week 44. Although this is associated with HIV infection, it is possible that treatment of co-infections throughout the study period might contribute to improvement in immune activation, and therefore PWV, in some participants. We therefore examined whether those patients diagnosed

with an acute infection during the study period had higher immune activation at baseline showing an improvement at week 44. Table 7-18 shows the univariate associations between immune markers at baseline and week 44 and the diagnosis of an acute infection during the study period (see Table 7-2 for a breakdown of co-infection diagnoses throughout the study). Overall immune activation markers decrease in participants who had a clinically diagnosed infection during the study period. Although CD4 and CD8 T cell activation and exhaustion (and to some extent senescence) are associated with having a diagnosis of infection during the study period, the majority of participants diagnosed with an acute infection were HIV infected.

Table 7-19 and Table 7-20 present final models showing the association between an acute infection diagnosis and percentage of CD8 T cells expressing both CD38 and HLA-DR, adjusted for HIV status, age and sex. Acute infection is retained in both models, even after adjustment for HIV infection.

Table 7-18 Univariate association between immune markers at baseline and Week 44 and a diagnosis of an acute infection during the study period

		Baseline			**Week 44**		
		No infection n=173	Infection n=90	P value	No infection n=118	Infection n=56	P value
Absolute CD4 count (cells/uL)		39 (17 – 59)	43.0 (19.0 – 71.0)	0.25	146 (94 – 218)	147 (108 – 229)	0.40
HIV viral load (copies/ml)		110,977 (41,492 – 299,9965)	108,086 (39,451 – 261,238)	0.66	NA	NA	NA
CD4 T cells	**CD4/CD8 ratio**	0.23 (0.07 – 1.23)	0.15 (0.05 – 0.42)	0.02	0.24 (0.11 – 0.72)	0.16 (0.09 – 0.39)	0.02

	CD4 %	18.8 (6.7 – 55.2)	13.2 (5.0 – 29.6)	0.02	13.0 (7.1 – 30.2)	9.9 (4.5 – 19.1)	0.02
	CD4 CD38+HLA-DR-	32.3 (20.5 – 41.8)	31.6 (19.3 – 43.0)	0.97	31.8 (20.4 – 40.0)	26.1 (16.1 – 38.3)	0.09
	CD4 CD38+HLA-DR+	17.7 (6.2 – 31.9)	9.5 (4.2 – 22.3)	<0.01	7.8 (3.8 – 13.4)	9.9 (5.1 – 18.9)	0.03
	CD4 CD38-HLA-DR+	12.2 (6.0 – 20.5)	13.1 (6.0 – 18.9)	0.82	11.8 (6.6 – 20.5)	16.2 (8.1 – 24.4)	0.05
	CD4 Activated	60.9 (44.8 – 76.1)	72.7 (54.3 – 85.5)	0.02	56.6 (46.4 – 65.8)	61.0 (47.4 – 70.6)	0.07
	CD4 CD57+PD1-	3.4 (2.0 – 7.8)	5.2 (2.5 – 9.9)	0.12	4.2 (2.2 – 6.7)	4.7 (2.5 – 7.1)	0.48
	CD4 CD57+PD1+	4.0 (2.0 – 7.5)	7.7 (2.5 – 13.8)	<0.01	3.6 (1.9 – 5.8)	4.8 (2.0 – 8.4)	0.05
	CD4 CD57-PD1+	20.0 (10.2 – 38.4)	36.2 (13.5 – 51.8)	0.01	18.7 (11.5 – 31.7)	26.1 (16.9 – 39.4)	<0.01
	CD4 Exhausted	24.7 (13.6 – 50.3)	48.8 (17.1 – 63.2)	<0.01	23.4 (13.8 – 39.0)	30.9 (20.5 – 47.7)	<0.01
	CD4 Senescent	9.0 (4.7 – 14.8)	16.2 (7.3 – 25.1)	<0.01	9.0 (4.7 – 14.8)	16.2 (7.3 – 25.1)	<0.01
CD8 T cells	**CD8%**	81.2 (44.8 – 93.2)	86.7 (70.4 – 95.0)	0.02	54.0 (39.6 – 63.6)	58.2 (47.4 – 64.7)	0.09
	CD8 CD38+HLA-DR-	25.4 (16.4 – 37.3)	23.1 (16.7 – 38.9)	0.99	32.9 (22.1 – 44.4)	33.2 (20.8)	0.42

	CD8 CD38+HLA-DR+	22.4 (12.9 – 37.6)	35.2 (18.0 – 48.8)	<0.001	21.4 (12.1 – 31.9)	25.8 (15.7 – 39.1)	<0.01
	CD8 CD38-HLA-DR+	11.1 (6.0 – 18.4)	9.3 (5.4 – 17.4)	0.25	9.8 (4.4 – 15.7)	10.2 (5.8 – 16.4)	0.19
	CD8 Activated	69.1 (55.0 – 81.4)	79.0 (64.6 – 87.7)	<0.01	70.5 (57.8 – 80.5)	74.6 (66.0 – 84.1)	0.01
	CD8 CD57+PD1-	30.1 (20.7 – 40.7)	28.6 (22.7 – 38.7)	0.98	35.7 (22.8 – 49.9)	38.8 (26.1 – 49.2)	0.58
	CD8 CD57+PD1+	15.6 (8.2 – 22.4)	20.5 (10.9 – 27.7)	<0.01	14.5 (8.3 – 23.0)	17.4 (10.0 – 27.0)	0.11
	CD8 CD57-PD1+	13.8 (8.6 – 20.3)	17.5 (10.9 – 27.1)	0.01	10.7 (5.7 – 18.2)	13.7 (7.8 – 19.7)	0.05
	CD8 Exhausted	31.5 (20.1 – 43.4)	39.1 (25.6 – 50.3)	<0.01	26.7 (15.5 – 43.6)	31.3 (20.8 – 46.6)	0.07
	CD8 Senescent	48.2 (35.5 – 62.5)	51.2 (41.3 – 61.9)	0.17	55.4 (41.0 – 67.0)	56.8 (44.9 – 67.0)	0.43
Monocytes	**Classical**	75.6 (66.0 – 82.1)	74.5 (65.3 – 80.4)	0.79	74.0 (66.7 – 80.6)	74.7 (65.5 – 80.7)	0.79
	Intermediate	9.8 (6.7 – 13.6)	10.2 (6.3 – 13.7)	0.81	8.1 (5.7 – 11.8)	8.7 (6.3 – 13.1)	0.39
	Nonclassical	14.0 (8.8 – 21.0)	13.0 (9.3 – 21.6)	0.68	16.0 (10.9 – 22.0)	11.4 (22.0 – 2.0)	0.90

Table 7-19 Final linear regression model for predictors of baseline %CD8CD38+HLA-DR+ T cells

Variable	Fold change in square root of baseline %CD8CD38+HLA-DR+	P value	95% CI (min)	95% CI (max)
Acute co-infection	0.39	0.06	-0.01	0.80
HIV	2.00	<0.0001	1.55	2.41

Table 7-20 Final linear regression model for predictors of Week 44 %CD8CD38+HLA-DR+ T cells

Variable	Fold change in square root of %CD8CD38+HLA-DR+	P value	95% CI (min)	95% CI (max)
Female sex	-0.44	0.04	-0.85	-0.03
Acute co-infection	0.51	0.02	0.07	0.96
HIV	0.78	<0.01	0.33	1.24

7.4.10.2 Association between diagnosis of an acute infection and PWV

PWV at week 10, 22 and 44 did not differ in patients with and without the diagnosis of an acute infection overall.

When the HIV uninfected group was analysed separately, the diagnosis of an acute infection was associated with an increase in PWV during the study period [median change (IQR) 0.80 (0.10 – 1.5) in participants with an acute infection and decrease of -0.10 (-0.80 – 0.75) in those without (p=0.01]. All HIV uninfected participants were retested for HIV infection at the Week 44 visit and no patients had acquired a new infection during the study period.

7.4.11 Change in immune activation according to early enhanced HIV intervention groups

There were no differences in CD4 counts according to REALITY intervention groups either at baseline or week 44. In addition, there were no statistically significant differences in HIV viral load at baseline or the number of patient reaching viral suppression at week 44.

7.4.11.1 Raltegravir

The median (IQR) proportion of classical monocytes was significantly lower at baseline in HIV infected participants receiving Raltegravir compared to HIV infected participants not receiving Raltegravir [70.5 (63.2 – 79.20) versus 77.5 (67.4 – 83.4) respectively, p=0.01], with higher proportions of nonclassical monocytes [18.0 (12.7 – 23.9) versus 12.3 (8.2 – 16.9) respectively, p<0.001]. At week 44, the median (IQR) proportion of both CD4 and CD8 HLA-DR+CD38- cells was reduced in the Raltegravir group [14.8 (8.1 – 21.8) versus 17.7 (12.0 – 26.9), p=0.02 and 8.1 (5.0 – 15.6) versus 10.6 (6.2 – 17.1), p=0.07 respectively].

7.4.11.2 Opportunistic infection prophylaxis

The proportion of classical monocytes was also lower in HIV patients receiving enhanced opportunistic prophylaxis compared to those not receiving enhanced opportunistic prophylaxis [median (IQR) 69.7 (64.2 – 78.8) versus 78.4 (68.6 – 83.6), p<0.01] and nonclassical monocytes were reciprocally higher [median (IQR) 16.7 (12.6 – 24.1) versus 12.2 (7.9 – 18.0), p<0.0001]. This expansion of nonclassical monocytes in the enhanced opportunistic infection (OI) arm had resolved at week 44. The proportion of CD38-HLA-DR+ CD4 T cells and activated CD8 T cells were lower in the enhanced OI prophylaxis group at baseline [median (IQR) 13.1 (7.4 – 17.8) versus 18.8 (12.4 – 25.6), p<0.01 and 72.7 (62.2 – 82.9) versus 80.4 (68.3 – 89.4), p<0.01 respectively] and the proportion of CD4CD38+HLA-DR- CD4 T cells was higher [median (IQR) 37.5 (26.3 – 47.7) versus 20.5 (16.2 – 32.7), p<0.001]. Again, these differences did not remain at week 44. All of the markers that were significantly altered at baseline in the enhanced OI prophylaxis group showed a statistically significant change between baseline and Week 44.

7.4.11.3 RUSF

In HIV infected participants receiving RUSF, no significant differences in proportions of immune markers were found at baseline. However, the percentage of CD8 T cell exhaustion decreased more rapidly in this group [median change -29.4 (-43.4 - -10.0) versus -18.1 (-33.0 – 7.0), p=0.02] and remained significantly lower at week 44 compared to HIV infected participants not receiving RUSF [median 24.5 (16.6 – 42.6) versus 31.2 (21.9 – 49.5) p=0.03].

7.4.12 Change in pulse wave velocity according to early enhanced HIV intervention groups

To address the final component of objective 7, we examined whether any of the REALITY interventions affected PWV.

7.4.12.1 Raltegravir

Absolute PWV was significantly higher at baseline in HIV infected participants receiving Raltegravir compared to those who were not receiving it (see Table 7-21). In addition, there was a significant decrease in PWV in the Raltegravir group between baseline and week 10 and between baseline and week 22 (see Table 7-22). The change in PWV for the Raltegravir intervention group compared to the non-intervention group is shown in Figure 7-14.

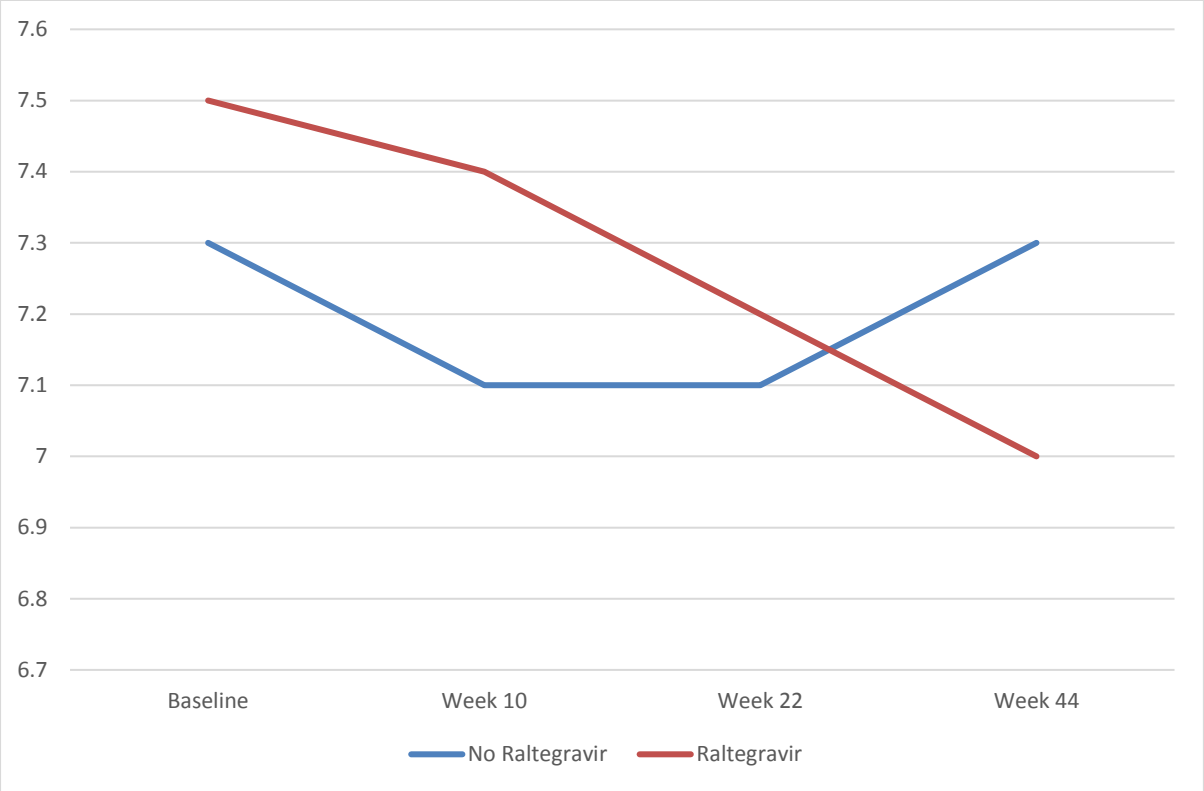
Table 7-21 Absolute PWV values according to intervention with Raltegravir for each study visit

Variable	Raltegravir	No Raltegravir	P value
Baseline	7.5 (7.0 – 8.3)	7.3 (6.3 – 8.2)	0.02
Week 10	7.4 (6.5 – 8.2)	7.1 (6.4 – 8.1)	0.28
Week 22	7.2 (6.3 – 7.7)	7.1 (6.3 – 8.0)	0.99
Week 44	7.3 (6.4 – 7.8)	7.0 (6.3 – 7.8)	0.58

Table 7-22 Change in PWV from baseline according to intervention with Raltegravir for each study visit

Variable	Raltegravir	No Raltegravir	P value
Week 10	-0.30 (-1 – 0.30)	-0.10 (-0.70 – 0.50)	0.06
Week 22	-0.40 (-1.20 – 0.10)	-0.20 (-0.85 -0.50)	0.03
Week 44	-0.60 (-1.20 – 0.10)	-0.25 (-0.90 – 0.20)	0.13

Figure 7-14 PWV in HIV infected patients receiving Raltegravir for the initial 12 weeks of ART therapy compared to those not receiving Raltegravir



Changes in individual PWV over time for patients who received Raltegravir and patients who did not receive Raltegravir are shown in Figure 7-15 and Figure 7-16 respectively. When Raltegravir was introduced into the mixed effects model looking at the effect of time on PWV, it was retained with a coefficient of 0.30 (-0.06 – 0.67, p=0.10). But when it was introduced as an interaction term, it did not significantly modify the slope of change in PWV (change in slope -0.003 (-0.01 – 0.006, p=0.54).

Figure 7-15 Spaghetti plot for changes in PWV in participants who received Raltegravir for 12 weeks

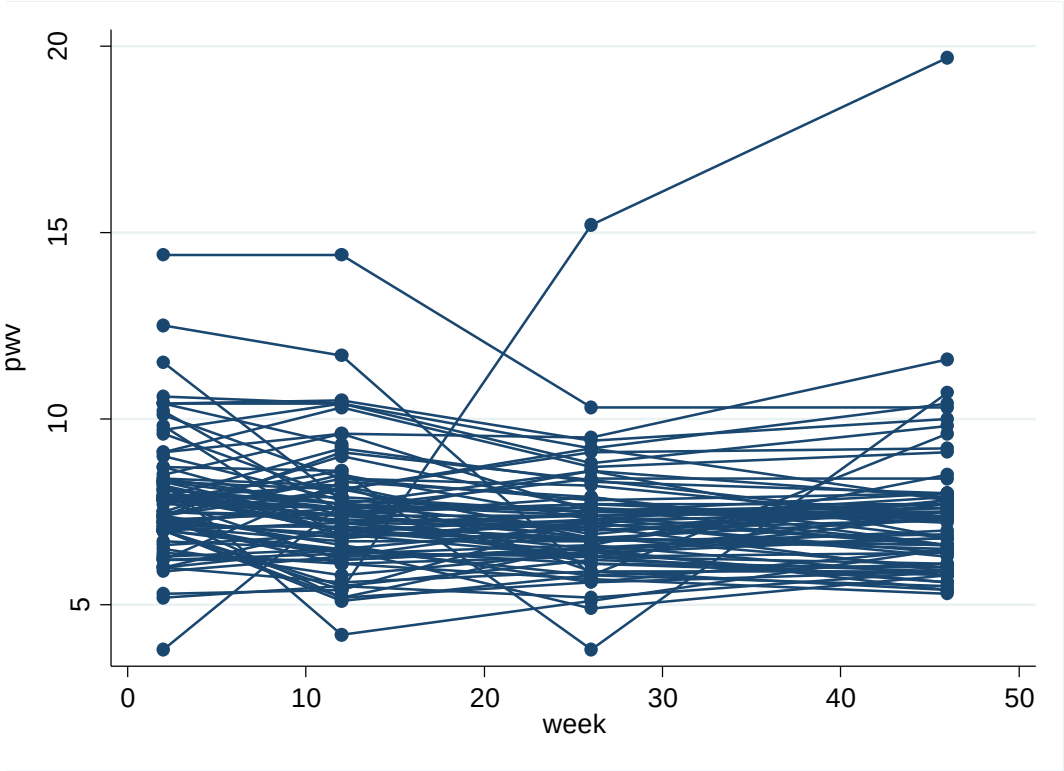
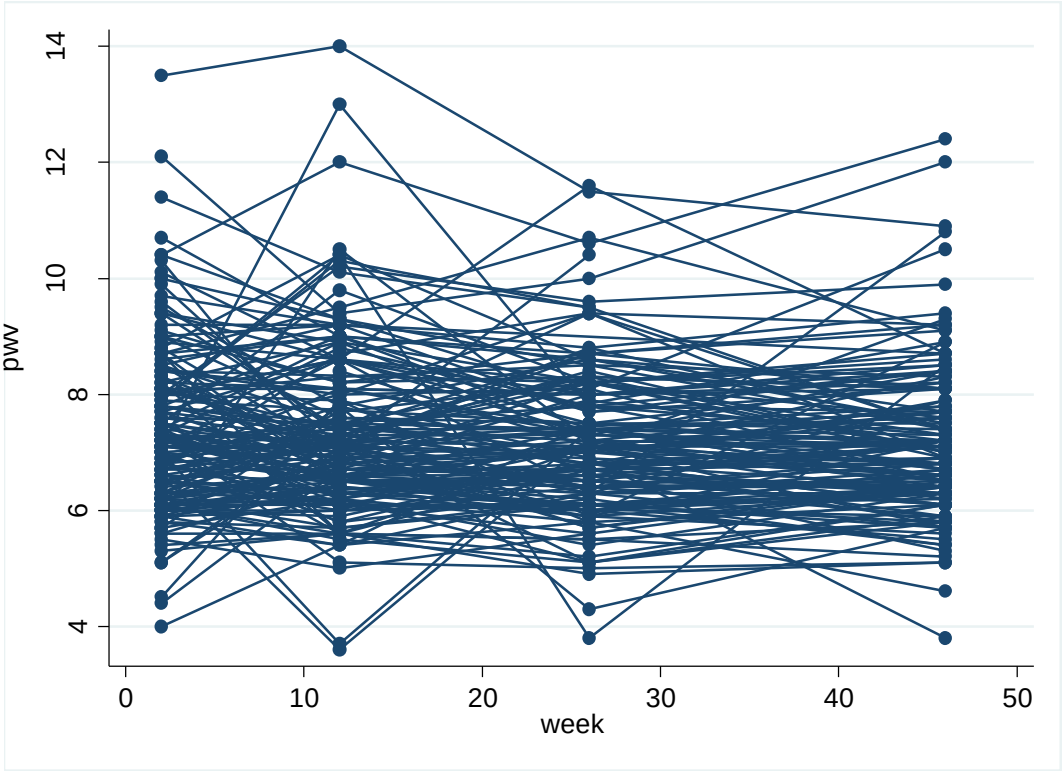


Figure 7-16 Spaghetti plot for changes in PWV in HIV infected participants who did not receive Raltegravir



7.4.12.2 Enhanced OI prophylaxis

There were no differences in PWV in patients who received enhanced OI prophylaxis compared to those who did not (see Table 7-23 and Table 7-24).

Table 7-23 Absolute PWV values according to intervention with enhanced OI prophylaxis for each study visit

Variable	OI prophylaxis	No OI prophylaxis	P value
Baseline	7.4 (6.4 – 8.3)	7.3 (6.5 – 8.2)	0.78
Week 10	7.2 (6.5 – 8.3)	7.2 (6.4 – 8.1)	0.88
Week 22	7.2 (6.4 – 7.8)	7.0 (6.3 – 8.0)	0.57
Week 44	7.1 (6.4 – 7.7)	7.0 (6.3 – 7.9)	0.64

Table 7-24 Change in PWV from baseline according to intervention with enhanced OI prophylaxis for each study visit

Variable	OI prophylaxis	No OI prophylaxis	P value
Week 10	-0.10 (-0.80 – 0.50)	-0.20 (-0.85 – 0.40)	0.80
Week 22	-0.10 (-1.20 – 0.40)	-0.40 (-0.90 – 0.40)	0.63
Week 44	-0.40 (-1.05 – 0.20)	-0.30 (-0.90 – 0.20)	0.62

7.4.12.3 RUSF

Equally, there were no differences in PWV in patients who received RUSF and those who did not (see Table 7-25 and Table 7-26).

Table 7-25 Absolute PWV values according to intervention with RUSF for each study visit

Variable	OI prophylaxis	No OI prophylaxis	P value
Baseline	7.5 (6.6 – 8.3)	7.4 (6.6 – 8.2)	0.79
Week 10	7.3 (6.3 – 8.2)	7.2 (6.5- 8.3)	0.84
Week 22	7.0 (6.1 – 7.8)	7.2 (6.4 – 7.9)	0.27
Week 44	7.0 (6.3 – 7.7)	7.1 (6.4 – 7.8)	0.69

Table 7-26 Change in PWV from baseline according to intervention with RUSF for each study visit

Variable	OI prophylaxis	No OI prophylaxis	P value
Week 10	-0.2 (-0.8 – 0.4)	-0.3 (-0.9 – 0.6)	0.91
Week 22	-0.4 (-1.2 – 0.2)	-0.4 (-0.9 – 0.3)	0.53
Week 44	-0.4 (-1.1 – 0.1)1	-0.4 (-1.1 – 0.2)	0.87

7.5 Discussion

The elevation in PWV found in HIV infected participants compared to uninfected at baseline persisted at 10 weeks but then resolved by 22 weeks. Patients with HIV infection showed an overall decrease in PWV over the study period and this was most pronounced in patients who had higher immune activation levels at baseline. Although T cell activation has previously been shown to decrease over 6 months without any change in subclinical atherosclerosis as assessed

by cIMT [495], T cell activation over 6.5 years of ART correlates with carotid artery stiffness in the WIHS [164]. Interestingly this study did not find any correlation between T cell activation and artery stiffness in HIV uninfected controls. Peripheral augmentation index was measured using an EndoPAT device in a cohort of 33 undernourished Zambians during the first 12 weeks of HIV treatment and showed that persistent rise in CRP, TNFα and soluble CD163 was associated with endothelial damage over that time period [496].

Although only 6% of the overall cohort was lost to follow-up, there was a high overall mortality rate of 6% which was almost exclusively from the HIV infected cohort. The highest mortality rate was seen in the first two weeks following initiation of ART. Half of patient who died had been diagnosed with an acute infection (TB, pneumonia and gastroenteritis were more common in HIV infected participants but not malaria), and over half met blood pressure criteria for new hypertension (140/90mmHg) during their enrolment visit. PWV was also significantly higher in patients who died but this sample size was too small to evaluate independent predictors of mortality. PWV has been consistently associated with higher all-cause mortality in the literature - the risk of death is approximately twice as high for those with the highest tertile of arterial stiffness values compared to the lowest tertile [497]. Patients who had a raised BP during enrolment were managed according to clinical guidelines - therapeutic intervention and / or repeat measurements were taken as appropriate. However, BP measurements for study purposes were only captured on record at enrolment and exit visits. Despite the limited data to support the diagnosis of hypertension, it would be expected that overall those patients with HIV infection who were sick with opportunistic infection might have a lower blood pressure and this was indeed reflected when comparing BP in the HIV uninfected and infected cohorts. However, when specifically looking at those who died, there was a high proportion with elevated BP. Higher diastolic BP and creatinine at enrolment both independently predicted PWV at week 44.

A greater resolution in CD4 activation than in CD8 activation was seen on ART. Exhausted CD4 and CD8 T cells both decreased over time but, again, a more important decrease was seen in exhausted CD4 T cells. This might either represent a continued stimulus for CD8 activation over and above HIV infection such as CMV, EBV, TB or helminth infection. Or, because activation and exhaustion here are reported as percentages of the whole T cell compartment (CD3 positive lymphocytes), it is possible that the decrease in CD4 activation and exhaustion actually represents a relative increase in the proportion of naïve CD4 T cells through release and new production following ART initiation. In other words, rather than a reduction in activation of

existing CD4 T cells, an increase in the production of naïve non-activated CD4 T cells is making up a higher proportion of the T cell pool.

There was no resolution seen in T cell senescence and it actually increased in CD8 T cells over the study period. This is in keeping with immunopathology of T cell senescence which results in an irreversible state of cell hyporesponsiveness meaning that these particular T cells are unable to return to the non-senescent pool. However, the maintenance of senescent T cells even on ART would suggest that the rate of generation of senescent T cells is more or less balanced with their rate of destruction implying that new senescent T cells continue to be produced. However, thymic migration markers were not measured in this study to support this hypothesis. It is likely that patients with such severe immunosuppression and likely resultant lymph node fibrosis would require longer periods on ART to restore immune function closer to that of HIV uninfected participants [8]. Consistent with literature from South Africa, markers of immune activation persisted even with ART treatment over and above those in HIV uninfected participants [411]. Persistent perturbations in CD4/CD8 ratio are associated with ongoing T cell activation and senescence and may be a useful biomarker for immune activation on ART [323]. Other studies have supported the observation that T cell population normalisation is slow on ART [498]. CD8% and /or CD4:CD8% ratio were important in predicting a change in PWV at 10 weeks. Although CD4 count increased by week 44, the vast majority of participants remained with a CD4 less than 200 cells/uL. Inflammatory biomarkers normalised in HIV infected participants starting ART almost to HIV uninfected levels, but with some exceptions (including CRP, sGP130, sCD14 and TNF). Most inflammation that was going to resolve did so by one year [499].

The trend towards an increase in nonclassical monocytes in the HIV infected population during the study period was probably influenced by a few outliers and was otherwise similar to the changes seen in the HIV uninfected group. However, the trend towards an association between a decrease in intermediate monocytes and PWV was seen at week 22 and, to a lesser degree, at week 44. The decrease in inflammatory monocytes on ART is consistent with previous results published from a South African cohort, however the proportion of intermediate monocytes in this untreated HIV population was approximately twice as high as our data from Malawi [117]. Greater declines in CD14 levels and IL-6 predicted lower cIMT in older Ugandans established on ART for 7 years [179].

CD4 and CD8 activation was lower in the Raltegravir group at week 44. Addition of Raltegravir to a standard ART regime has been shown to reduce HIV DNA reservoirs without translation into a reduction in immune activation [500]. Here we show that both CD4 and CD8 T cell activation

parameters were significantly lower at week 44 in those who had 12 weeks or Raltegravir at ART initiation [442]. This finding is supported by a higher CD4 count in patients who received Raltegravir during the overall REALITY trial analysis [501]. This reduction did not translate into a reduction in PWV at week 44, but PWV was initially higher in those receiving the Raltegravir arm. PWV began higher in the Raltegravir arm but reduced more quickly in models of summary estimates but was not significant in the linear effects model. Only one previous study has assessed the effects of Raltegravir on endothelial damage, using Flow Mediated Dilatation as a measure of endothelial dysfunction. They showed that there was no overall reduction in cardiovascular risk when Raltegravir was added to a standard regime for 6 months in patients already virologically suppressed on ART [502]. However, it is likely that the greatest effect of Raltegravir would be during ART initiation when there is still the potential to reduce the viral set point which may, in itself, be a risk factor for chronic immune activation on ART. The use of Raltegravir in a group of severely immune compromised participants, with a more rapid drop in viral load, may have led to a more vigorous inflammatory response causing the acute and temporary increase in PWV seen in the patients who received Raltegravir. It is possible that this initial increase in PWV may have offset any benefit incurred by the reduction in immune activation at week 44. This assessment is limited by the lack of serial data on HIV viral loads and immune activation markers during the early stages of ART as the dynamics associated with Raltegravir addition have been shown to evolve rapidly during ART initiation [503].

For the OI prophylaxis group the proportion of activated CD4 and CD8 T cells was lower at baseline compared to those who did not receive OI prophylaxis, but this benefit did not persist at week 44. This is an acute effect and may reflect treatment of bacterial infections with azithromycin or intestinal helminth infections with albendazole. Even in the first two weeks of therapy, OI prophylaxis may reduce T cell activation by preventing TB and cryptococcal meningitis. Although there were too few of these outcomes to assess this relationship within the SHIELD study, results from the REALITY trial showed that patients receiving OI prophylaxis had fewer TB and cryptococcal meningitis events [501]. Macrolides, including azithromycin demonstrate direct anti-inflammatory effects both in vitro and in vivo and are being used in trials of chronic lung disease [504, 505]. Although there was no difference in PWV between those who did and did not receive OI intervention, having an acute infection episode diagnosed at enrolment was associated with a trend towards a decrease in PWV at week 22 and at week 44. Immune activation markers, activated CD8 T cells in particular, also decreased in those patients who had been diagnosed with an acute infection during the study period. Taken together these results could suggest that treatment of acute infections during early ART leads to

an acute reduction in immune activation but, possibly, a more sustained reduction in PWV. The likely importance of treating acute infections to modify endothelial damage has been considered previously [506]. CD8 T cell activation was higher in patients with TB than with HIV [507]. Schistosomiasis may increase [508] activation of T cell subsets as well as intestinal parasites [509]. In vitro malaria infection of PBMCs leads to increased productive HIV infection [510]. Non classical monocytes were higher at baseline in patients who were receiving Raltegravir and OI prophylaxis compared to those not receiving these interventions. This resolved by week 44. Switching from Efavirenz to Raltegravir in a previous study didn't improve endothelial function, but did lead to a decrease in markers of monocyte activation [511]. It is also important to consider the role of cotrimoxazole in potentially treating and preventing acute infections. The routine use of cotrimoxazole in all HIV infected participants may account for the decrease in PWV in HIV infected participants even to below that of HIV uninfected participants. Cotrimoxazole has been shown to reduce infections caused by malaria, TB and helminths in SSA and also reduces all-cause mortality [512-515].

CD8 T cell exhaustion decreased more rapidly in the RUSF group and was lower at week 44 compared to HIV uninfected participants not receiving RUSF. Improving nutritional intake may lead to a decrease in inflammation in the gut mucosa, thereby reducing microbial translocation and activation of gut CD4 T cells [516]. The addition of RUSF may also have led to the generation of a more immunologically favourable gut microbiome [517]. The contribution of weight, undernutrition and body composition in low-income sub-Saharan Africa is challenging to decipher [518]. Although the REALITY study showed that the addition of RUSF led to increased BMI, this mostly consisted of increases in adipose tissue rather than lean muscle bulk (personal communication, Jane Mallewa, College of Medicine University of Malawi). This may not necessarily confer a long term benefit in terms of immune activation because recent evidence shows that adipocytes may be reservoirs for the accumulation of activated T cells [519]. But on the other hand, high levels of inflammation and increased cardiovascular risk have been identified in a small group of South African patients with low BMI during the first 12 weeks of ART [496].

HIV infection treated with ART and cotrimoxazole and resolution of immune activation are predictors of improvement in PWV over 44 weeks in adult Malawians. Treatment of an acute infection during this period is also a predictor of lower PWV, but the association is less clear and

further studies specifically designed to look at the effect of acute infection and its treatment on endothelial damage are warranted.

8 CHAPTER 8: General Discussion

Overall this study reports data from a cohort which is relatively young in terms of cardiovascular risk and has few traditional cardiovascular risk factors, apart from hypertension which was highly prevalent. Despite this seemingly low traditional cardiovascular risk, the age adjusted proportion of adults reaching criteria for arterial stiffness or subclinical atherosclerosis in this cohort is higher than reported in previous cohorts from high income settings. PWV values were 1 m/s faster in our cohort and subclinical atherosclerosis occurred 5 years earlier compared to healthy European controls [408, 416].

8.1 Aetiology of endothelial damage in adult Malawians

8.1.1 Background immune activation is high in HIV uninfected adult Malawians

The finding that background immune activation is high, even in apparently healthy HIV uninfected adults, suggests that immune activation has a different epidemiology in low-resource sub-Saharan Africa. HIV may only be one of several important infectious agents. This work challenges the paradigm of traditional versus HIV related risk factors from high income countries. Both subclinical infections (such as CMV, EBV and latent TB) and recurrent acute infections (such as malaria and gastroenteritis) may contribute to chronic immune activation in HIV uninfected and HIV infected participants and the distinction between traditional risk factors and infection related risk factors is less clear. Figure 8-1 shows an overview of the proposed working hypothesis on determinants of endothelial damage in an adult Malawian population, highlighting that overlap exists between background and HIV related risk factors.

8.1.2 HIV and immune activation impact vascular health during the first year of ART in adult Malawians with advanced HIV infection

At ART initiation in severely immunocompromised patients, HIV and immune activation play an important role in arterial stiffness. Patients with HIV infection and high levels of immune activation see the greatest improvements in PWV on ART and cotrimoxazole and treatment of acute infections likely contributes to this picture. Immune activation and hypertension were the most important reversible determinants of arterial stiffness but treatment of acute infections may also have contributed to improvements in immune activation. Subclinical atherosclerosis, as measured by cIMT, although raised compared to cohorts in this age group from high income

settings, was less well defined in terms of risk factors. It is likely that arterial stiffness is more important in this cohort because they are younger and have hypertension as their main traditional risk factor. With the evolution of cardiovascular disease, arterial stiffness usually precedes atherosclerosis and this study may have captured a cohort earlier in this evolution where arterial stiffness is the main feature of arterial damage.

The important limitations of this study were that dyslipidaemia and hypertension were not assessed in depth. It is possible that there may have been a derangement in cholesterol subtypes without seeing an increase in total cholesterol. This limited our ability to assess for metabolic syndrome which is an increasingly prevalent risk factor for CVD in SSA. Further research into cardiovascular risk in HIV should aim to include metabolic factors, especially in those patients established on ART [520]. Acute illness may have had an important effect on both blood pressure and, therefore PWV at enrolment. However, PWV was adjusted for blood pressure and haemoglobin (as a marker for plasma viscosity) and so these results should remain accurate. Blood pressure remained a consistent predictor of PWV both during the enrolment visit and the exit visit. Arterial stiffness, the main outcome measurement, is a proxy measure for cardiovascular risk. It has not been validated in sub-Saharan Africa and therefore it is difficult to quantify the increased risk associated with HIV and immune activation in this study. However, PWV had a high intra-operator reliability, was associated with the expected variables as previously reported in the literature (blood pressure, haemoglobin and age) and had absolute values comparable to research published elsewhere. Additionally, viral loads were taken at different time points around enrolment for SHIELD and REALITY participants which may have limited interpretation of the viral load data and, in particular, the contribution of viral load to arterial stiffness at the baseline study visit. However, there was no correlation between HIV viral load and immune activation or PWV even within the SHIELD participants alone. A large number of immune markers was assessed and so it is possible that some of the associations identified may have been significant by chance. This was taken into account during the reporting of the results, which focussed on those results which presented biologically plausible patterns involving two or more markers or a pattern in change of related markers over time. The number of patients with acute infections was low and this study was not specifically designed to assess their contribution. Therefore, firm conclusions cannot be made about the contribution of acute infection to either immune activation or endothelial damage. It is possible that some acute infections went undiagnosed, especially in the HIV uninfected cohort and there may have been differences in management of those infections given that new symptoms in the HIV uninfected cohort would not have been managed in the ART clinic.

Overall, this is the first study to characterise changes in PWV during the first year of ART. We have shown that immune activation is an important component of endothelial damage during this time and is relatively more important than any contribution from HIV viral load or effects of ART itself. Figure 8-2 presents a basic overview of the proposed pathogenesis of endothelial damage at a cellular level in adults with HIV infection in a low resource SSA setting.

Figure 8-1 Overview of working hypothesis of the risk factors for endothelial damage in adult Malawians

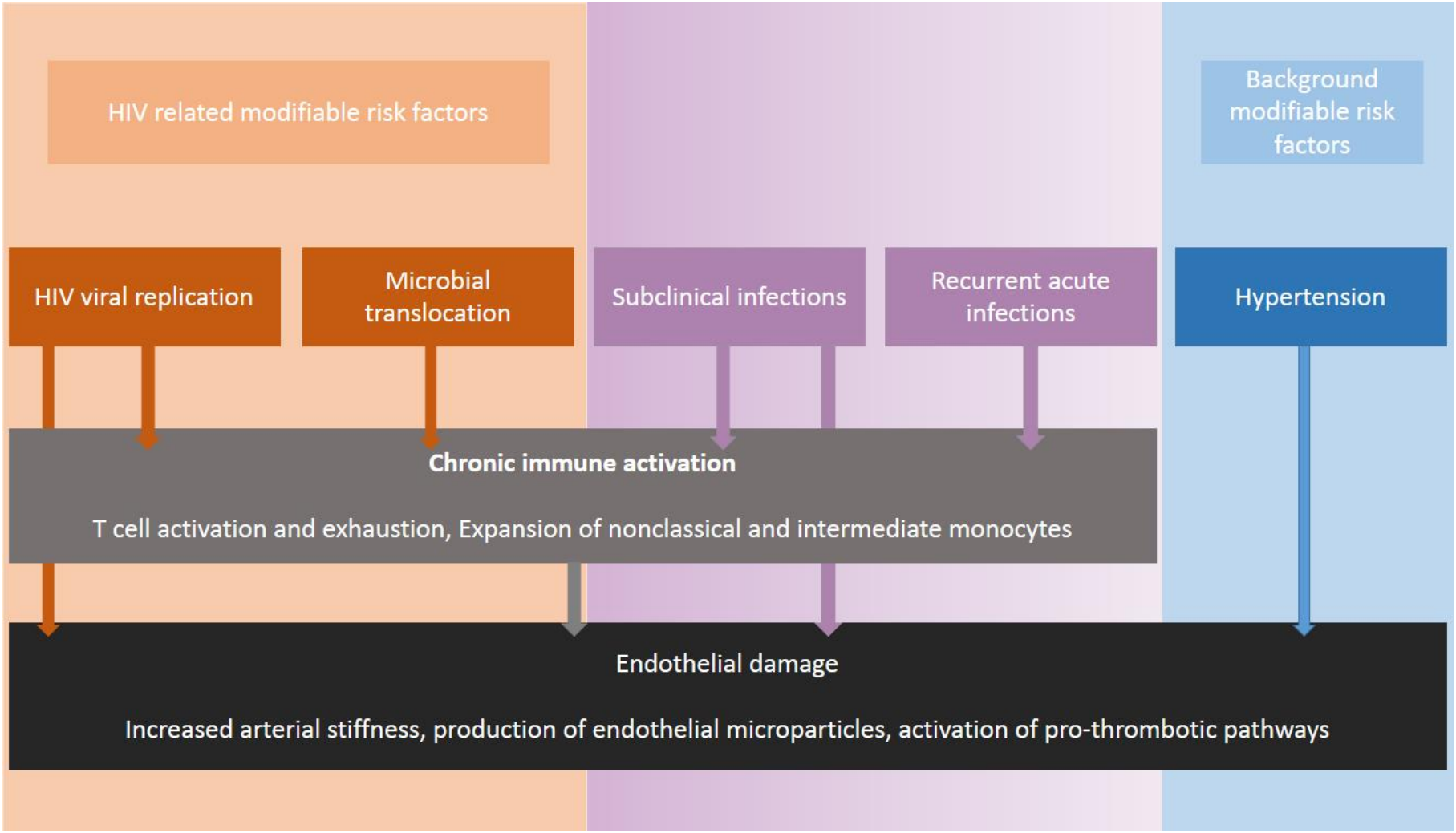
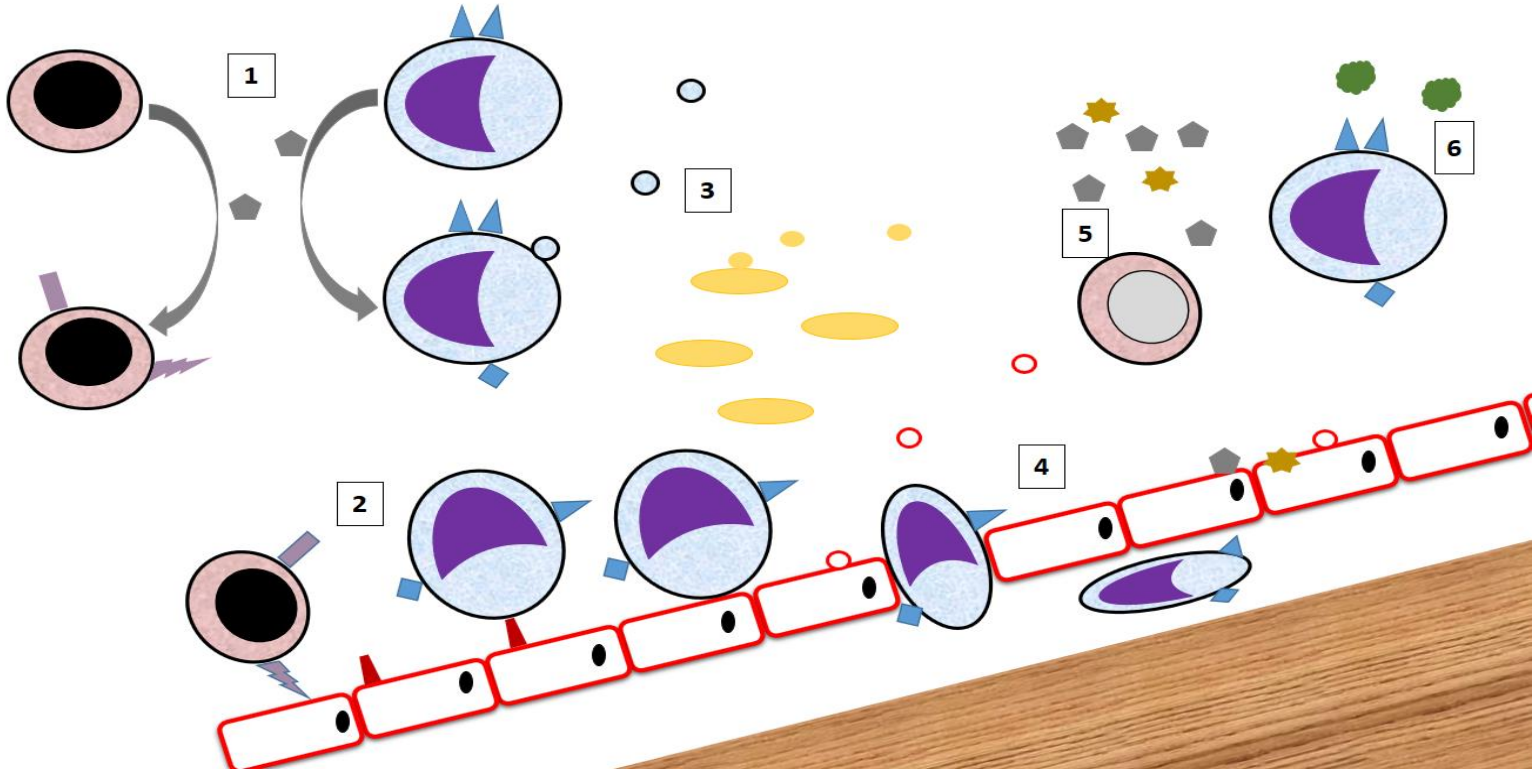


Figure 8-2 Overview of the working hypothesis of the pathogenesis of endothelial damage in Malawian adults



1.HIV infection activated T lymphocytes leading to the expression of HLA-DR, CD38 and PD1. CD16 expressing monocyte subsets are also expanded. **2.** Activated T cells interact with the endothelium via ICAM and VCAM initiating endothelial activation. This produces a cascade of pro-inflammatory cytokines and chemokines which stimulate the recruitment of other leucocytes and platelets. **3**. Leucocyte, platelet and endothelial cell activation leads to the production of microparticles which can also act as messengers to propagate the inflammation cascade. **4**. Nonclassical monocytes expressing CD16 roll along the endothelium where they transcytose into the intima layer producing a subendothelial inflammatory reaction. The pro-inflammatory milieu favours elastin degradation and enables artery wall stiffening**. 5**. T lymphocytes, and possibly monocytes, eventually become senescent and depleted and are no longer able to respond to antigenic stimuli. Both HIV infection and CMV infection may directly bind to and infect endothelial cells. **6**. Bacterial products either as a result of systemic infection or through microbial translocation further stimulate the innate immune system, continuing the cycle of immune activation and endothelial damage.

8.1.3 Aetiology of endothelial damage in the context of long term ART

This work has focussed on the early stages of HIV treatment. As the immune system reconstitutes and viral load decreases, the relative toxic effect of ART to the endothelium, which is accumulated over time, becomes more important. Efavirenz, the first line NNRTI in Malawi national guidelines, has been shown to lead to increase in arterial stiffness but not cIMT in ApoE (-/-) mice [521]. It also increases rolling and adhesion of leucocytes inducing emigration of neutrophils and monocytes in rat venules [522]. In a human coronary artery endothelial cell model, Efavirenz increased endothelial permeability and decreased tight junction proteins [523]. Clinical studies have also demonstrated a higher risk of endothelial damage with Efavirenz [524]. Strong evidence exists implicating Tenofovir in the development of renal disease as well as osteoporosis in patients with HIV infection [525, 526]. Abacavir also increases the risk of cardiovascular disease as demonstrated in the DAD study and has been shown to induce leucocyte-endothelial interactions when compared with Tenofovir [151, 152, 527]. Although several of the components of the current ART regime in Malawi may pose an increased risk for the development of cardiovascular disease, several studies have shown that long term ART, in general, can increase arterial stiffness [137, 528]. Given the increasing numbers of patients living for several decades on long term ART in sub -Saharan Africa, further research into optimum ART regimes to minimise risk of cardiovascular and other non-communicable complications is needed.

Over and above the direct metabolic effects of ART itself, come cardiovascular risks associated with treatment default and treatment failure. A recent report from patients established on ART in QECH clinic for at least 18 months showed that ART default and detectable viral loads were associated with a higher PWV [442]. Further, poor virological response to ART is associated with higher levels of CD8 T cell activation in an African cohort [529]. Therefore, in the longer term, better identification and management of viral break through and ART default may also help minimise cardiovascular risk in patients with HIV in Malawi [442].

8.2 Improving vascular health in adult Malawians

8.2.1 HIV, immune activation and recurrent acute infection are modifiable risk factors for endothelial damage in adult Malawians

The three main points of potential therapeutic intervention to modify cardiovascular risk in adults living with HIV in Malawi is shown in Figure 8-3. Firstly, drivers of chronic immune activation could

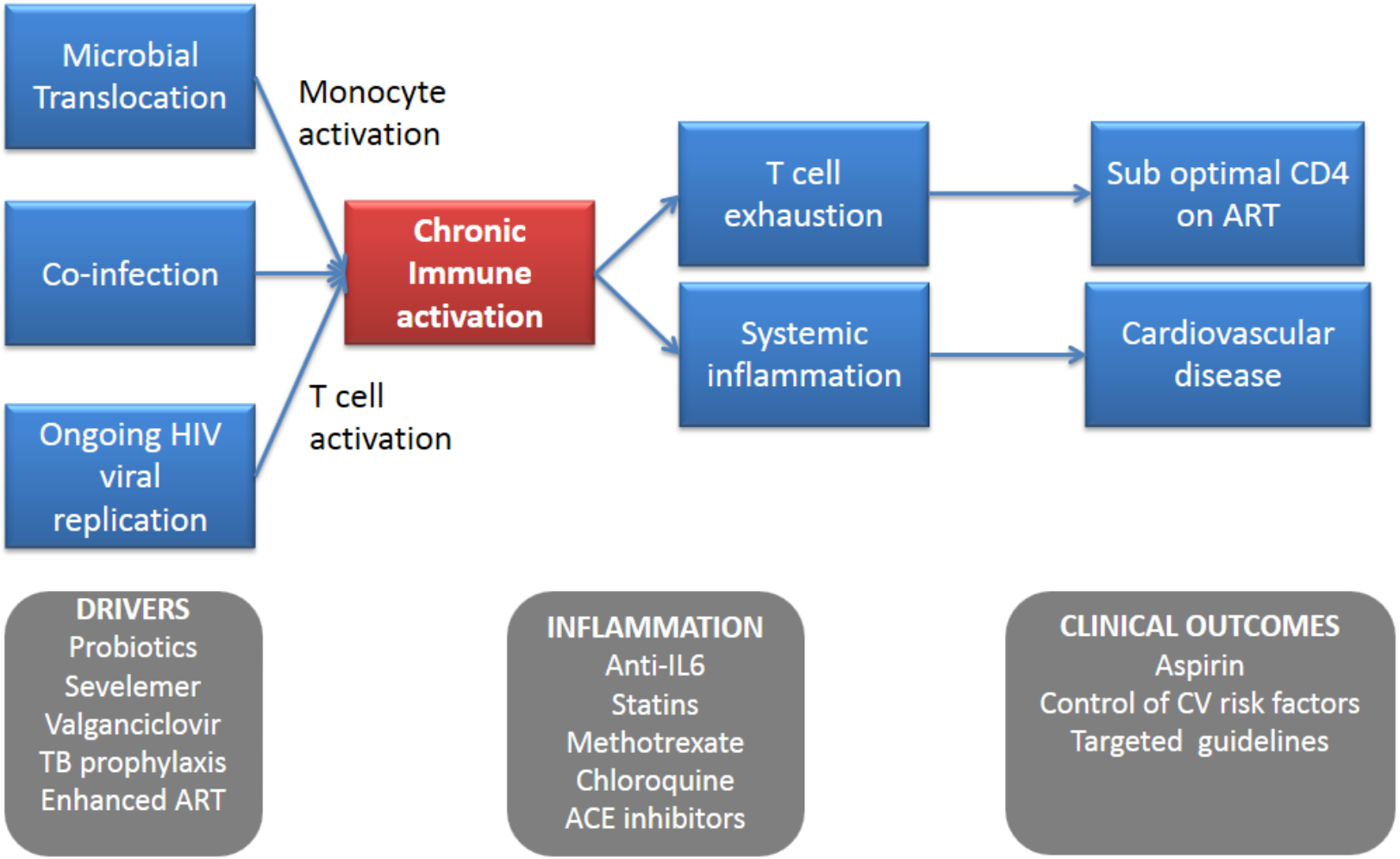
be targeted. Interventions directed towards improved health of the gut mucosa could reduce microbial translocation. So far these have included agents such as probiotics and sevelemer [530]. It is possible in this setting that anti-infective, (such as albendazole for intestinal helminths and azithromycin for bacterial gastrointestinal infections) and nutritional supplements (such as RUSF) may reduce mucosal inflammation and therefore microbial translocation. Anti-CMV agents such as valganciclovir have shown limited results in previous studies aiming to reduce chronic immune activation but, in this setting, where the majority of adults have latent CMV infection and around a third of people presenting with HIV have a CD4<100 cells/mm^3, reducing CMV viraemia could potentially have a more important impact [103]. Further investigation would be warranted into the value of screening for and treating latent TB infection and TB disease. The fact that this study has shown a decrease in PWV in those who had been diagnosed with an acute infection may mean that other participants had higher PWV values due to unrecognised infection. Given that TB is one of the most common co-infections in this setting, its role in chronic immune activation deserves further attention [531]. Reducing ongoing HIV replication may also be of relatively increased benefit in this setting. In patients with a high background immune activation, including expansion of the more HIV permissive CD16+ monocyte subset, it is possible that the viral set point could be higher. Use of drugs such as Raltegravir during early ART may help drive down that viral set point thereby reducing chronic immune activation and allowing better CD4 reconstitution in the longer term. We have shown here that the addition of Raltegravir acutely does reduce T cell activation in the longer term and the REALITY trial results have shown in the overall cohort that use of Raltegravir leads to higher CD4 counts at an earlier stage in this cohort. This is in contrast to studies from high income countries that have shown no immunological benefit to Raltegravir [532].

Secondly, modulation of the immune response can be considered. The main stay of current trials around modification of inflammation in adults with HIV in high income countries revolves around statin therapy. Rosuvastatin, in particular, has been shown to reduce both monocyte and T cell activation after 24 weeks of therapy [533]. ACE inhibitors may confer benefits over and above reduction in blood pressure and are the only agents that have been shown to reduce arterial stiffness independently of effects on BP [534]. Chloroquine based agents have been trialled with some success in high income settings but may be more interesting in countries like Malawi where they are cheap and available [535]. Like co-trimoxazole, the potential mechanisms for reduction in cardiovascular risk could include anti-inflammatory actions as well as prevention of common infections. A separate analysis of the role of cotrimoxazole in reducing cardiovascular risk in adult Malawians could shed light on the importance of prevention of infectious diseases in HIV uninfected adults for reduction of chronic immune activation. More specialised agents such as anti-IL6 and anti-

TNF monoclonal antibodies which are in use clinically for the treatment of rheumatoid arthritis, and PD-1 inhibitors which are being trialled as cancer therapy, are unlikely to be applicable in this setting due to the likelihood they would leave patients further immunosuppressed and at risk of increased infections [536, 537].

Lastly, it is important to consider more general down-stream targets include management of traditional risk factors, modification of pro-thrombotic pathways and protection of the endothelium. Because hypertension was the other important risk factor for endothelial damage in patients with HIV, guidelines for screening and targeted management of hypertension should be introduced in ART clinics to reduce the overall risk of cardiovascular disease. A simple blood pressure measurement could be added on to standard metrics recorded at ART clinic booking in addition to height and weight. An agreed guideline could then inform the need for further measurements and treatment recommendations. The use of 375mg of aspirin in all patients with HIV for prevention of cardiovascular events is currently being trialled in a high resource setting (NCT00783614, www.clinicaltrials.gov), but more data on region specific cardiovascular risk is needed before such trials are carried out in low income sub-Saharan Africa.

Figure 8-3 Potential points of therapeutic intervention to modify cardiovascular risk in adult Malawians



8.2.2 Areas for future research

Based on the results of this study, three main prongs of future research are warranted:

8.2.2.1 Prong 1. Characterisation of the determinants and burden of chronic immune activation in low income SSA

Firstly, it is necessary to better define the nature of chronic immune activation in this setting. This would include an assessment of relative types of T cells within the T cell pool using proliferation studies and cell surface phenotyping to identify naïve, memory and effector T cell subsets. Characterisation of cell function is required and, in particular, examination of the relative functions of monocyte subsets in this setting. Investigation of the extent of lymph node fibrosis would be an important addition to characterising immune exhaustion and senescence and PCR based methods could be used to examine T cell receptor repertoire. These studies should include both HIV uninfected adults as well as patients starting ART at higher CD4 counts. With the advent of "Test and Treat" it will be important to quantify the risk of chronic immune activation in patients starting ART across the spectrum of immune suppression. In addition, studies including a wide range of age groups, including younger children may be helpful in pinpointing the development of chronic immune activation. It is possible that a genetic component exists to chronic immune activation which has been developed in the face of recurrent infections over many generations in SSA.

Next, the pathophysiological consequences of chronic immune activation in this setting should be investigated. It is possible that if a high inflammatory state has been developed since childhood a tolerance of inflammation may have been developed and the threshold required to predispose to disease may be different. Or, it is possible that the relatively low levels of hyperlipidaemia, diabetes and obesity mean that the effects of inflammation are not yet manifest. To answer this question, future studies should assess the impact of chronic immune activation in patients with higher cardiovascular risk profiles. Ultimately, studies using interventions which reduce chronic immune activation would help to decipher whether reduction of chronic immune activation also leads to a reduction in clinical outcomes. Pilot studies into agents such as cotrimoxazole and chloroquine would be interesting in the first instance.

Lastly, the determinants of chronic immune activation in this setting should be investigated. In terms of subclinical or recurrent acute infections CMV, TB, helminths, malaria and

gastrointestinal infections are all of interest. A cohort study that closely characterises acute and subclinical infections and their association with immune activation would also allow for the examination of anti-infective treatment on chronic immune activation in the short term as well as in the longer term. This study did not focus on the role of the gut inflammation and microbial translocation in chronic immune activation in low income SSA and given the increased prevalence of important environmental and socio-economic risk factors and the likely role of recurrent gut infections and malnutrition in facilitating microbial translocation, this warrants particular attention. Urban versus rural populations and participants from different socio-economic backgrounds would provide interesting comparisons to help tease out the aetiologies.

8.2.2.2 Prong 2. Characterisation of clinical cardiovascular disease and validating physiological markers in low income SSA

In order to better understand the incidence and clinical phenotypes of cardiovascular diseases in this setting, a large observational cohort study would need to run for 5 to 10 years. We have shown that cIMT and PWV are feasible and potentially useful tools in a resource limited setting. However, accurate validation of these tools in this setting is essential if results are to be used to inform policy decisions. A clinical cohort study in an older population, with a higher prevalence of traditional cardiovascular risk factors could help validate these tools. The relative contribution of HIV related risk factors for cardiovascular disease are likely to change over time on ART, with the toxicity of the drugs themselves likely to become more important as the virus is controlled. Future cohort studies should include patients on ART and perform an in-depth analysis of metabolic factors including obesity, dyslipidaemia and glucose intolerance.

8.2.2.3 Prong 3. Formulation of a cardiovascular risk assessment strategy and guidelines for screening and prevention

Further to having a practical outcome measure, further data are required in order to generate tools for cardiovascular risk assessment in low income countries. If confirmed to be important, normative values for chronic immune activation would need to be established. Risk assessment tools should be generated from data produced in low income SSA and should be pragmatic. A simple guideline introducing the assessment of hypertension in people with HIV infection could

be assessed for feasibility and clinical benefit. Lastly, agents such as aspirin, ACE inhibitors or statins could be trialled in those deemed to be at highest risk.

In conclusion, cardiovascular risk in low income sub-Saharan Africa is a different playing field. Many of the potential risk factors identified here may be modifiable with simple interventions. A research platform is needed that can better characterise these risks and respond to changing risk factors and a dynamic clinical phenotype. The HIV epidemic has led to the rapid scale up of chronic disease infrastructure in SSA, which in turn could support the assessment and management of cardiovascular risk during the different stages of HIV treatment [203] and perhaps in the general population as well.

References

1. IUMS. *Virus taxonomy*. 2014 [cited 2016 29th March]; Available from: http://www.ictvonline.org/virustaxonomy.asp.
2. Perrin, L., L. Kaiser, and S. Yerly, *Travel and the spread of HIV-1 genetic variants.* The Lancet Infectious Diseases. **3**(1): p. 22-27.
3. Royce, R.A., et al., *Sexual transmission of HIV.* N Engl J Med, 1997. **336**(15): p. 1072-8.
4. Shen, R., H.E. Richter, and P.D. Smith, *Interactions between HIV-1 and mucosal cells in the female reproductive tract.* Am J Reprod Immunol, 2014. **71**(6): p. 608-17.
5. Kaul, R., et al., *Inflammation and HIV Transmission in Sub-Saharan Africa.* Curr HIV/AIDS Rep, 2015. **12**(2): p. 216-22.
6. Zhang, Z., et al., *Sexual transmission and propagation of SIV and HIV in resting and activated CD4+ T cells.* Science, 1999. **286**(5443): p. 1353-7.
7. Murooka, T.T., et al., *HIV-infected T cells are migratory vehicles for viral dissemination.* Nature, 2012. **490**(7419): p. 283-287.
8. Jambo, K.C., et al., *Asymptomatic HIV-infected individuals on antiretroviral therapy exhibit impaired lung CD4(+) T-cell responses to mycobacteria.* Am J Respir Crit Care Med, 2014. **190**(8): p. 938-47.
9. Resnick, L., et al., *Early penetration of the blood-brain-barrier by HIV.* Neurology, 1988. **38**(1): p. 9-14.
10. Grossman, Z., et al., *CD4+ T-cell depletion in HIV infection: are we closer to understanding the cause?* Nat Med, 2002. **8**(4): p. 319-23.
11. Wilson, J.D., et al., *Direct visualization of HIV-1-specific cytotoxic T lymphocytes during primary infection.* AIDS, 2000. **14**(3): p. 225-33.
12. Hazenberg, M.D., et al., *T-cell division in human immunodeficiency virus (HIV)-1 infection is mainly due to immune activation: a longitudinal analysis in patients before and during highly active antiretroviral therapy (HAART).* Blood, 2000. **95**(1): p. 249-55.
13. Sousa, A.E., et al., *CD4 T cell depletion is linked directly to immune activation in the pathogenesis of HIV-1 and HIV-2 but only indirectly to the viral load.* J Immunol, 2002. **169**(6): p. 3400-6.
14. Douek, D.C., et al., *Changes in thymic function with age and during the treatment of HIV infection.* Nature, 1998. **396**(6712): p. 690-5.
15. Freguja, R., et al., *Regulatory T cells and chronic immune activation in human immunodeficiency virus 1 (HIV-1)-infected children.* Clin Exp Immunol, 2011. **164**(3): p. 373-80.
16. Elahi, S., et al., *Protective HIV-specific CD8+ T cells evade Treg cell suppression.* Nat Med, 2011. **17**(8): p. 989-95.
17. Chun, T.W., et al., *Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection.* Nature, 1997. **387**(6629): p. 183-8.
18. Perelson, A.S., et al., *Decay characteristics of HIV-1-infected compartments during combination therapy.* Nature, 1997. **387**(6629): p. 188-91.
19. Rodger, A.J., M.A. Johnson, and T.W. Mahungu, *HIV/AIDS : An Atlas of Investigation and Management*. Atlas: HIV2011, Oxford: Clinical Publishing.
20. *Pneumocystis pneumonia--Los Angeles.* MMWR Morb Mortal Wkly Rep, 1981. **30**(21): p. 250-2.
21. Barre-Sinoussi, F., et al., *Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS).* Science, 1983. **220**(4599): p. 868-71.
22. Gelmann, E.P., et al., *Proviral DNA of a retrovirus, human T-cell leukemia virus, in two patients with AIDS.* Science, 1983. **220**(4599): p. 862-5.
23. Serwadda, D., et al., *Slim disease: a new disease in Uganda and its association with HTLV-III infection.* Lancet, 1985. **2**(8460): p. 849-52.

24. Ward, J.W., et al., *Laboratory and epidemiologic evaluation of an enzyme immunoassay for antibodies to HTLV-III.* JAMA, 1986. **256**(3): p. 357-61.
25. Yerly, S., et al., *A critical assessment of the prognostic value of HIV-1 RNA levels and CD4+ cell counts in HIV-infected patients. The Swiss HIV Cohort Study.* Arch Intern Med, 1998. **158**(3): p. 247-52.
26. Mellors, J.W., et al., *Prognosis in HIV-1 infection predicted by the quantity of virus in plasma.* Science, 1996. **272**(5265): p. 1167-70.
27. Fischl, M.A., et al., *The efficacy of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex. A double-blind, placebo-controlled trial.* N Engl J Med, 1987. **317**(4): p. 185-91.
28. Hirsch, M.S., et al., *Antiretroviral drug resistance testing in adults with HIV infection: implications for clinical management. International AIDS Society--USA Panel.* JAMA, 1998. **279**(24): p. 1984-91.
29. Hogg, R.S., et al., *Decline in deaths from AIDS due to new antiretrovirals.* Lancet, 1997. **349**(9061): p. 1294.
30. Hammer, S.M., et al., *A trial comparing nucleoside monotherapy with combination therapy in HIV-infected adults with CD4 cell counts from 200 to 500 per cubic millimeter. AIDS Clinical Trials Group Study 175 Study Team.* N Engl J Med, 1996. **335**(15): p. 1081-90.
31. Montaner, J.S., et al., *A randomized, double-blind trial comparing combinations of nevirapine, didanosine, and zidovudine for HIV-infected patients: the INCAS Trial. Italy, The Netherlands, Canada and Australia Study.* JAMA, 1998. **279**(12): p. 930-7.
32. Hammer, S.M., et al., *A controlled trial of two nucleoside analogues plus indinavir in persons with human immunodeficiency virus infection and CD4 cell counts of 200 per cubic millimeter or less. AIDS Clinical Trials Group 320 Study Team.* N Engl J Med, 1997. **337**(11): p. 725-33.
33. Feinmann, J., *Antiretrovirals planned for developing world.* Lancet, 1997. **350**(9093): p. 1759.
34. WHO. *Scaling up antiretroviral therapy in resource limited settings*. 2002 [cited 2016 01 April]; Available from: http://www.who.int/hiv/pub/prev_care/en/ScalingUp_E.pdf.
35. Egger, M., et al., *Impact of new antiretroviral combination therapies in HIV infected patients in Switzerland: prospective multicentre study. Swiss HIV Cohort Study.* BMJ, 1997. **315**(7117): p. 1194-9.
36. Palella, F.J., Jr., et al., *Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV Outpatient Study Investigators.* N Engl J Med, 1998. **338**(13): p. 853-60.
37. Palmisano, L. and S. Vella, *A brief history of antiretroviral therapy of HIV infection: success and challenges.* Ann Ist Super Sanita, 2011. **47**(1): p. 44-8.
38. Ledergerber, B., et al., *Predictors of trend in CD4-positive T-cell count and mortality among HIV-1-infected individuals with virological failure to all three antiretroviral-drug classes.* Lancet, 2004. **364**(9428): p. 51-62.
39. Steigbigel, R.T., et al., *Raltegravir with optimized background therapy for resistant HIV-1 infection.* N Engl J Med, 2008. **359**(4): p. 339-54.
40. El-Sadr, W.M., et al., *CD4+ count-guided interruption of antiretroviral treatment.* N Engl J Med, 2006. **355**(22): p. 2283-96.
41. WHO. *Global Health Observatory Data*. 2013 [cited 2016 31st March]; Available from: www.who.int/gho/hiv/en/.
42. *The Millenium Development Goals Report*. 2012; Available from: http://www.un.org/millenniumgoals/pdf/MDG%20Report%202012.pdf.
43. WHO, *GUIDELINE ON WHEN TO START ANTIRETROVIRAL THERAPY AND ON PRE-EXPOSURE PROPHYLAXIS FOR HIV*, 2015.
44. UNAIDS. *Fact Sheet*. 2015 [cited 2016 31st March]; Available from: http://www.unaids.org/en/resources/campaigns/HowAIDSchangedeverything/factsheet.

45. IPU, *Fast tracking HIV treatment: Parliamentary action and policy options*, UNAIDS, Editor 2015.
46. UNAIDS, *Core Epidemiology Slides*, 2015.
47. Siedner, M.J., et al., *Trends in CD4 count at presentation to care and treatment initiation in sub-Saharan Africa, 2002-2013: a meta-analysis.* Clin Infect Dis, 2015. **60**(7): p. 1120-7.
48. Mutimura, E., et al., *Trends in and correlates of CD4+ cell count at antiretroviral therapy initiation after changes in national ART guidelines in Rwanda.* AIDS, 2015. **29**(1): p. 67-76.
49. UNAIDS, *HIV TREATMENT IN AFRICA: A looming crisis*, in *ISSUES BRIEF*2015.
50. UNAIDS, *Onthe Fast-Track to end AIDS*, 2016.
51. UNAIDS, *MALAWI DEVELOPING SUBNATIONAL ESTIMATES OF HIV PREVALENCE AND THE NUMBER OF PEOPLE LIVING WITH HIV*, 2014.
52. AVERT. *HIV AND AIDS IN MALAWI*. Gap report 2014 2014 [cited 2016 31st March]; Available from: http://www.avert.org/professionals/hiv-around-world/sub-saharan-africa/malawi.
53. MoH. *Malawi AIDS response progress 2015*. 2015 [cited 2016 4th April]; Available from: http://www.unaids.org/sites/default/files/country/documents/MWI_narrative_report_2015.pdf.
54. Grinspoon, S. and A. Carr, *Cardiovascular risk and body-fat abnormalities in HIV-infected adults.* N Engl J Med, 2005. **352**(1): p. 48-62.
55. Lederman, M.M., et al., *'Modeling' relationships among HIV-1 replication, immune activation and CD4+ T-cell losses using adjusted correlative analyses.* AIDS, 2000. **14**(8): p. 951-8.
56. Deeks, S.G., *HIV infection, inflammation, immunosenescence, and aging.* Annu Rev Med, 2011. **62**: p. 141-55.
57. Nakanjako, D., et al., *Sub-optimal CD4 reconstitution despite viral suppression in an urban cohort on antiretroviral therapy (ART) in sub-Saharan Africa: frequency and clinical significance.* AIDS Res Ther, 2008. **5**: p. 23.
58. Nakanjako, D., et al., *High T-cell immune activation and immune exhaustion among individuals with suboptimal CD4 recovery after 4 years of antiretroviral therapy in an African cohort.* BMC Infect Dis, 2011. **11**: p. 43.
59. Eller, M.A., et al., *Innate and adaptive immune responses both contribute to pathological CD4 T cell activation in HIV-1 infected Ugandans.* PLoS One, 2011. **6**(4): p. e18779.
60. Glennie, S.J., et al., *Defective pneumococcal-specific Th1 responses in HIV-infected adults precedes a loss of control of pneumococcal colonization.* Clin Infect Dis, 2013. **56**(2): p. 291-9.
61. Glennie, S.J., N.A. Williams, and R.S. Heyderman, *Mucosal immunity in resource-limited setting: is the battle ground different?* Trends Microbiol, 2010. **18**(11): p. 487-93.
62. Iwajomo, O.H., et al., *Deteriorating pneumococcal-specific B-cell memory in minimally symptomatic African children with HIV infection.* J Infect Dis, 2011. **204**(4): p. 534-43.
63. Glennie, S.J., et al., *Impaired CD4 T cell memory response to Streptococcus pneumoniae precedes CD4 T cell depletion in HIV-infected Malawian adults.* PLoS One, 2011. **6**(9): p. e25610.
64. Hellerstein, M., et al., *Directly measured kinetics of circulating T lymphocytes in normal and HIV-1-infected humans.* Nat Med, 1999. **5**(1): p. 83-9.
65. Papagno, L., et al., *Immune activation and CD8+ T-cell differentiation towards senescence in HIV-1 infection.* PLoS Biol, 2004. **2**(2): p. E20.
66. Campillo-Gimenez, L., et al., *Neutrophils in antiretroviral therapy-controlled HIV demonstrate hyperactivation associated with a specific IL-17/IL-22 environment.* J Allergy Clin Immunol, 2014. **134**(5): p. 1142-52 e5.
67. Fauci, A.S., D. Mavilio, and S. Kottilil, *NK cells in HIV infection: paradigm for protection or targets for ambush.* Nat Rev Immunol, 2005. **5**(11): p. 835-43.
68. Moir, S. and A.S. Fauci, *B-cell exhaustion in HIV infection: the role of immune activation.* Curr Opin HIV AIDS, 2014. **9**(5): p. 472-7.

69. O'Brien, M., et al., *Spatiotemporal trafficking of HIV in human plasmacytoid dendritic cells defines a persistently IFN-alpha-producing and partially matured phenotype.* J Clin Invest, 2011. **121**(3): p. 1088-101.
70. Giorgi, J.V., et al., *Elevated levels of CD38+ CD8+ T cells in HIV infection add to the prognostic value of low CD4+ T cell levels: results of 6 years of follow-up. The Los Angeles Center, Multicenter AIDS Cohort Study.* J Acquir Immune Defic Syndr, 1993. **6**(8): p. 904-12.
71. Liu, Z., et al., *CD8+ T-lymphocyte activation in HIV-1 disease reflects an aspect of pathogenesis distinct from viral burden and immunodeficiency.* J Acquir Immune Defic Syndr Hum Retrovirol, 1998. **18**(4): p. 332-40.
72. Deeks, S.G., et al., *Immune activation set point during early HIV infection predicts subsequent CD4+ T-cell changes independent of viral load.* Blood, 2004. **104**(4): p. 942-7.
73. Imamichi, H., et al., *The CD8(+)HLA-DR(+) T cells expanded in HIV-1 infection are qualitatively identical to those from healthy controls.* European Journal of Immunology, 2012. **42**(10): p. 10.1002/eji.201142046.
74. Effros, R.B., et al., *Shortened telomeres in the expanded CD28-CD8+ cell subset in HIV disease implicate replicative senescence in HIV pathogenesis.* AIDS, 1996. **10**(8): p. F17-22.
75. Brenchley, J.M., et al., *Expression of CD57 defines replicative senescence and antigen-induced apoptotic death of CD8+ T cells.* Blood, 2003. **101**(7): p. 2711-20.
76. Lee, S.A., et al., *Low proportions of CD28- CD8+ T cells expressing CD57 can be reversed by early ART initiation and predict mortality in treated HIV infection.* J Infect Dis, 2014. **210**(3): p. 374-82.
77. Appay, V., et al., *Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections.* Nat Med, 2002. **8**(4): p. 379-85.
78. Webster, A., et al., *Cytomegalovirus (CMV) infection, CD4+ lymphocyte counts and the development of AIDS in HIV-1-infected haemophiliac patients.* Clin Exp Immunol, 1992. **88**(1): p. 6-9.
79. Lichtner, M., et al., *Cytomegalovirus coinfection is associated with an increased risk of severe non-AIDS-defining events in a large cohort of HIV-infected patients.* J Infect Dis, 2015. **211**(2): p. 178-86.
80. Appay, V., et al., *Old age and anti-cytomegalovirus immunity are associated with altered T-cell reconstitution in HIV-1-infected patients.* AIDS, 2011. **25**(15): p. 1813-22.
81. Akbar, A.N. and S.M. Henson, *Are senescence and exhaustion intertwined or unrelated processes that compromise immunity?* Nat Rev Immunol, 2011. **11**(4): p. 289-95.
82. Cockerham, L.R., et al., *Programmed death-1 expression on CD4(+) and CD8(+) T cells in treated and untreated HIV disease.* AIDS, 2014. **28**(12): p. 1749-58.
83. Passlick, B., D. Flieger, and H.W. Ziegler-Heitbrock, *Identification and characterization of a novel monocyte subpopulation in human peripheral blood.* Blood, 1989. **74**(7): p. 2527-34.
84. Ziegler-Heitbrock, L., *The CD14+ CD16+ blood monocytes: their role in infection and inflammation.* J Leukoc Biol, 2007. **81**(3): p. 584-92.
85. Skinner, N.A., et al., *Regulation of Toll-like receptor (TLR)2 and TLR4 on CD14dimCD16+ monocytes in response to sepsis-related antigens.* Clin Exp Immunol, 2005. **141**(2): p. 270-8.
86. Han, J., et al., *CD14(high)CD16(+) rather than CD14(low)CD16(+) monocytes correlate with disease progression in chronic HIV-infected patients.* J Acquir Immune Defic Syndr, 2009. **52**(5): p. 553-9.
87. Ellery, P.J., et al., *The CD16+ monocyte subset is more permissive to infection and preferentially harbors HIV-1 in vivo.* J Immunol, 2007. **178**(10): p. 6581-9.
88. Andrade, B.B., et al., *Mycobacterial antigen driven activation of CD14++CD16- monocytes is a predictor of tuberculosis-associated immune reconstitution inflammatory syndrome.* PLoS Pathog, 2014. **10**(10): p. e1004433.
89. Tacke, F., et al., *Monocyte subsets differentially employ CCR2, CCR5, and CX3CR1 to accumulate within atherosclerotic plaques.* J Clin Invest, 2007. **117**(1): p. 185-94.

90. Younas, M., et al., *Immune activation in the course of HIV-1 infection: Causes, phenotypes and persistence under therapy.* HIV Med, 2016. **17**(2): p. 89-105.
91. Fernandez, S., et al., *Low CD4+ T-cell counts in HIV patients receiving effective antiretroviral therapy are associated with CD4+ T-cell activation and senescence but not with lower effector memory T-cell function.* Clin Immunol, 2006. **120**(2): p. 163-70.
92. Hunt, P.W., et al., *Relationship between T cell activation and CD4+ T cell count in HIV-seropositive individuals with undetectable plasma HIV RNA levels in the absence of therapy.* J Infect Dis, 2008. **197**(1): p. 126-33.
93. Hunt, P.W., et al., *T cell activation is associated with lower CD4+ T cell gains in human immunodeficiency virus-infected patients with sustained viral suppression during antiretroviral therapy.* J Infect Dis, 2003. **187**(10): p. 1534-43.
94. Hatano, H., et al., *Cell-based measures of viral persistence are associated with immune activation and programmed cell death protein 1 (PD-1)-expressing CD4+ T cells.* J Infect Dis, 2013. **208**(1): p. 50-6.
95. Beignon, A.S., et al., *Endocytosis of HIV-1 activates plasmacytoid dendritic cells via Toll-like receptor-viral RNA interactions.* J Clin Invest, 2005. **115**(11): p. 3265-75.
96. Doitsh, G., et al., *Abortive HIV infection mediates CD4 T cell depletion and inflammation in human lymphoid tissue.* Cell, 2010. **143**(5): p. 789-801.
97. Burdo, T.H., et al., *Soluble CD163 made by monocyte/macrophages is a novel marker of HIV activity in early and chronic infection prior to and after anti-retroviral therapy.* J Infect Dis, 2011. **204**(1): p. 154-63.
98. Finkel, T.H., et al., *Apoptosis occurs predominantly in bystander cells and not in productively infected cells of HIV- and SIV-infected lymph nodes.* Nat Med, 1995. **1**(2): p. 129-34.
99. Chahroudi, A., et al., *Natural SIV hosts: showing AIDS the door.* Science, 2012. **335**(6073): p. 1188-93.
100. Choudhary, S.K., et al., *Low immune activation despite high levels of pathogenic human immunodeficiency virus type 1 results in long-term asymptomatic disease.* J Virol, 2007. **81**(16): p. 8838-42.
101. Gonzalez, V.D., et al., *High levels of chronic immune activation in the T-cell compartments of patients coinfected with hepatitis C virus and human immunodeficiency virus type 1 and on highly active antiretroviral therapy are reverted by alpha interferon and ribavirin treatment.* J Virol, 2009. **83**(21): p. 11407-11.
102. Crane, M., et al., *Lipopolysaccharide, immune activation, and liver abnormalities in HIV/hepatitis B virus (HBV)-coinfected individuals receiving HBV-active combination antiretroviral therapy.* J Infect Dis, 2014. **210**(5): p. 745-51.
103. Hunt, P.W., et al., *Valganciclovir reduces T cell activation in HIV-infected individuals with incomplete CD4+ T cell recovery on antiretroviral therapy.* J Infect Dis, 2011. **203**(10): p. 1474-83.
104. Sheth, P.M., et al., *Coinfection with herpes simplex virus type 2 is associated with reduced HIV-specific T cell responses and systemic immune activation.* J Infect Dis, 2008. **197**(10): p. 1394-401.
105. Petrara, M.R., et al., *Epstein-Barr virus load and immune activation in human immunodeficiency virus type 1-infected patients.* J Clin Virol, 2012. **53**(3): p. 195-200.
106. Siddiqui, S., et al., *Tuberculosis specific responses following therapy for TB: Impact of HIV co-infection.* Clin Immunol, 2015. **159**(1): p. 1-12.
107. Sullivan, Z.A., et al., *Latent and Active Tuberculosis Infection Increase Immune Activation in Individuals Co-Infected with HIV.* EBioMedicine, 2015. **2**(4): p. 334-340.
108. Tenforde, M.W., et al., *C-reactive protein (CRP), interferon gamma-inducible protein 10 (IP-10), and lipopolysaccharide (LPS) are associated with risk of tuberculosis after initiation of antiretroviral therapy in resource-limited settings.* PLoS One, 2015. **10**(2): p. e0117424.

109. Mulu, A., et al., *Effect of deworming on Th2 immune response during HIV-helminths co-infection.* J Transl Med, 2015. **13**.
110. Mattapallil, J.J., et al., *Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection.* Nature, 2005. **434**(7037): p. 1093-7.
111. Guadalupe, M., et al., *Severe CD4+ T-cell depletion in gut lymphoid tissue during primary human immunodeficiency virus type 1 infection and substantial delay in restoration following highly active antiretroviral therapy.* J Virol, 2003. **77**(21): p. 11708-17.
112. Veazey, R.S., et al., *Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection.* Science, 1998. **280**(5362): p. 427-31.
113. Brenchley, J.M., et al., *CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract.* J Exp Med, 2004. **200**(6): p. 749-59.
114. Brenchley, J.M., et al., *Microbial translocation is a cause of systemic immune activation in chronic HIV infection.* Nat Med, 2006. **12**(12): p. 1365-71.
115. Hunt, P.W., et al., *Gut epithelial barrier dysfunction and innate immune activation predict mortality in treated HIV infection.* J Infect Dis, 2014. **210**(8): p. 1228-38.
116. Caron, G., et al., *Direct stimulation of human T cells via TLR5 and TLR7/8: flagellin and R-848 up-regulate proliferation and IFN-gamma production by memory CD4+ T cells.* J Immunol, 2005. **175**(3): p. 1551-7.
117. Cassol, E., et al., *Persistent microbial translocation and immune activation in HIV-1-infected South Africans receiving combination antiretroviral therapy.* J Infect Dis, 2010. **202**(5): p. 723-33.
118. Ross, R., *Atherosclerosis is an inflammatory disease.* Am Heart J, 1999. **138**(5 Pt 2): p. S419-20.
119. Murphy, K., *Janeway's immunobiology*. 8th ed2011: Garland Sciences.
120. Sima, A.V., C.S. Stancu, and M. Simionescu, *Vascular endothelium in atherosclerosis.* Cell Tissue Res, 2009. **335**(1): p. 191-203.
121. Cybulsky, M.I., et al., *A major role for VCAM-1, but not ICAM-1, in early atherosclerosis.* J Clin Invest, 2001. **107**(10): p. 1255-62.
122. An, G., et al., *P-selectin glycoprotein ligand-1 is highly expressed on Ly-6Chi monocytes and a major determinant for Ly-6Chi monocyte recruitment to sites of atherosclerosis in mice.* Circulation, 2008. **117**(25): p. 3227-37.
123. Hioe, C.E., et al., *HIV envelope gp120 activates LFA-1 on CD4 T-lymphocytes and increases cell susceptibility to LFA-1-targeting leukotoxin (LtxA).* PLoS One, 2011. **6**(8): p. e23202.
124. Lahoute, C., et al., *Adaptive immunity in atherosclerosis: mechanisms and future therapeutic targets.* Nat Rev Cardiol, 2011. **8**(6): p. 348-58.
125. Doran, A.C., N. Meller, and C.A. McNamara, *Role of smooth muscle cells in the initiation and early progression of atherosclerosis.* Arterioscler Thromb Vasc Biol, 2008. **28**(5): p. 812-9.
126. Lo, J. and J. Plutzky, *The biology of atherosclerosis: general paradigms and distinct pathogenic mechanisms among HIV-infected patients.* J Infect Dis, 2012. **205 Suppl 3**: p. S368-74.
127. Martin-Iguacel, R., J.M. Llibre, and N. Friis-Moller, *Risk of Cardiovascular Disease in an Aging HIV Population: Where Are We Now?* Curr HIV/AIDS Rep, 2015. **12**(4): p. 375-87.
128. Triant, V.A., J.B. Meigs, and S.K. Grinspoon, *Association of C-reactive protein and HIV infection with acute myocardial infarction.* J Acquir Immune Defic Syndr, 2009. **51**(3): p. 268-73.
129. Armah, K.A., et al., *Prehypertension, hypertension, and the risk of acute myocardial infarction in HIV-infected and -uninfected veterans.* Clin Infect Dis, 2014. **58**(1): p. 121-9.
130. Lorgis, L., et al., *Outcomes after acute myocardial infarction in HIV-infected patients: analysis of data from a French nationwide hospital medical information database.* Circulation, 2013. **127**(17): p. 1767-74.

131. Hsue, P.Y., et al., *Impact of HIV infection on diastolic function and left ventricular mass.* Circ Heart Fail, 2010. **3**(1): p. 132-9.
132. Ovbiagele, B. and A. Nath, *Increasing incidence of ischemic stroke in patients with HIV infection.* Neurology, 2011. **76**(5): p. 444-50.
133. Papita, A.M., et al., *Markers of preclinical vascular disease and left ventricular diastolic dysfunction in patients with HIV infection.* Med Ultrason, 2012. **14**(1): p. 10-8.
134. Papita, A., et al., *Arterial stiffness and carotid intima-media thickness in HIV infected patients.* Med Ultrason, 2011. **13**(2): p. 127-34.
135. Lekakis, J., et al., *Association of highly active antiretroviral therapy with increased arterial stiffness in patients infected with human immunodeficiency virus.* Am J Hypertens, 2009. **22**(8): p. 828-34.
136. Siedner, M.J., et al., *HIV infection and arterial stiffness among older-adults taking antiretroviral therapy in rural Uganda.* AIDS, 2015.
137. van Vonderen, M.G., et al., *Carotid intima-media thickness and arterial stiffness in HIV-infected patients: the role of HIV, antiretroviral therapy, and lipodystrophy.* J Acquir Immune Defic Syndr, 2009. **50**(2): p. 153-61.
138. Phillips, A.N., et al., *Interruption of antiretroviral therapy and risk of cardiovascular disease in persons with HIV-1 infection: exploratory analyses from the SMART trial.* Antivir Ther, 2008. **13**(2): p. 177-87.
139. Scheglovitova, O., et al., *CD4-positive lymphoid cells rescue HIV-1 replication from abortively infected human primary endothelial cells.* Arch Virol, 1993. **132**(3-4): p. 267-80.
140. Steffan, A.M., et al., *Primary cultures of endothelial cells from the human liver sinusoid are permissive for human immunodeficiency virus type 1.* Proc Natl Acad Sci U S A, 1992. **89**(5): p. 1582-6.
141. Edinger, A.L., et al., *CD4-independent, CCR5-dependent infection of brain capillary endothelial cells by a neurovirulent simian immunodeficiency virus strain.* Proc Natl Acad Sci U S A, 1997. **94**(26): p. 14742-7.
142. Andras, I.E., et al., *HIV-1 Tat protein alters tight junction protein expression and distribution in cultured brain endothelial cells.* J Neurosci Res, 2003. **74**(2): p. 255-65.
143. Maisa, A., et al., *Monocytes from HIV-infected individuals show impaired cholesterol efflux and increased foam cell formation after transendothelial migration.* AIDS, 2015. **29**(12): p. 1445-57.
144. Nordell, A.D., et al., *Severity of cardiovascular disease outcomes among patients with HIV is related to markers of inflammation and coagulation.* J Am Heart Assoc, 2014. **3**(3): p. e000844.
145. Butt, A.A., et al., *Risk of heart failure with human immunodeficiency virus in the absence of prior diagnosis of coronary heart disease.* Arch Intern Med, 2011. **171**(8): p. 737-43.
146. Lundgren, J.D., et al., *Initiation of Antiretroviral Therapy in Early Asymptomatic HIV Infection.* N Engl J Med, 2015. **373**(9): p. 795-807.
147. Zuber, J.P., et al., *Pulmonary arterial hypertension related to HIV infection: improved hemodynamics and survival associated with antiretroviral therapy.* Clin Infect Dis, 2004. **38**(8): p. 1178-85.
148. Kristoffersen, U.S., et al., *Reduction in circulating markers of endothelial dysfunction in HIV-infected patients during antiretroviral therapy.* HIV Med, 2009. **10**(2): p. 79-87.
149. Freiberg, M.S., et al., *HIV infection and the risk of acute myocardial infarction.* JAMA Intern Med, 2013. **173**(8): p. 614-22.
150. Rodger, A.J., et al., *Mortality in well controlled HIV in the continuous antiretroviral therapy arms of the SMART and ESPRIT trials compared with the general population.* AIDS, 2013. **27**(6): p. 973-9.
151. Friis-Moller, N., et al., *Combination antiretroviral therapy and the risk of myocardial infarction.* N Engl J Med, 2003. **349**(21): p. 1993-2003.

152. Friis-Moller, N., et al., *Class of antiretroviral drugs and the risk of myocardial infarction.* N Engl J Med, 2007. **356**(17): p. 1723-35.
153. Lopez-Sublet, M., et al., *Protease inhibitor treatment effect on aortic stiffness in normotensive patients with human immunodeficiency virus infection.* J Mal Vasc, 2012. **37**(4): p. 179-85.
154. Gupta, S.K., et al., *Worsening endothelial function with efavirenz compared to protease inhibitors: a 12-month prospective study.* PLoS One, 2012. **7**(9): p. e45716.
155. Hsue, P.Y., et al., *Association of abacavir and impaired endothelial function in treated and suppressed HIV-infected patients.* AIDS, 2009. **23**(15): p. 2021-7.
156. Dillon, D.G., et al., *Association of HIV and ART with cardiometabolic traits in sub-Saharan Africa: a systematic review and meta-analysis.* Int J Epidemiol, 2013. **42**(6): p. 1754-71.
157. Sinn, K., R. Richardson, and A. Carr, *Lower arterial stiffness and Framingham score after switching abacavir to tenofovir in men at high cardiovascular risk.* AIDS, 2010. **24**(15): p. 2403-5.
158. Baker, J.V., et al., *HIV replication, inflammation, and the effect of starting antiretroviral therapy on plasma asymmetric dimethylarginine, a novel marker of endothelial dysfunction.* J Acquir Immune Defic Syndr, 2012. **60**(2): p. 128-34.
159. Francisci, D., et al., *HIV type 1 infection, and not short-term HAART, induces endothelial dysfunction.* AIDS, 2009. **23**(5): p. 589-96.
160. Ngatchou, W., et al., *Effects of antiretroviral therapy on arterial stiffness in Cameroonian HIV-infected patients.* Blood Press Monit, 2013. **18**(5): p. 247-51.
161. Lazar, J.M., et al., *Arterial wave reflection in HIV-infected and HIV-uninfected Rwandan women.* AIDS Res Hum Retroviruses, 2009. **25**(9): p. 877-82.
162. Currier, J.S., et al., *Coronary heart disease in HIV-infected individuals.* J Acquir Immune Defic Syndr, 2003. **33**(4): p. 506-12.
163. Kaplan, R.C., et al., *T cell activation predicts carotid artery stiffness among HIV-infected women.* Atherosclerosis, 2011. **217**(1): p. 207-13.
164. Karim, R., et al., *T-cell activation, both pre- and post-HAART levels, correlates with carotid artery stiffness over 6.5 years among HIV-infected women in the WIHS.* J Acquir Immune Defic Syndr, 2014. **67**(3): p. 349-56.
165. Tincati, C., et al., *CD8+ hyperactivation and senescence correlate with early carotid intima-media thickness in HIV+ patients with no cardiovascular disease.* J Acquir Immune Defic Syndr, 2009. **51**(5): p. 642-4.
166. Goulenok, T., et al., *Increased carotid intima-media thickness is not associated with T-cell activation nor with cytomegalovirus in HIV-infected never-smoker patients.* AIDS, 2015. **29**(3): p. 287-93.
167. Hsue, P.Y., et al., *Increased carotid intima-media thickness in HIV patients is associated with increased cytomegalovirus-specific T-cell responses.* AIDS, 2006. **20**(18): p. 2275-83.
168. Crowe, S.M., et al., *The macrophage: the intersection between HIV infection and atherosclerosis.* J Leukoc Biol, 2010. **87**(4): p. 589-98.
169. Funderburg, N.T., et al., *Increased tissue factor expression on circulating monocytes in chronic HIV infection: relationship to in vivo coagulation and immune activation.* Blood, 2010. **115**(2): p. 161-7.
170. Sandler, N.G., et al. *Plasma levels of soluble CD14 independently predict mortality in HIV infection*. The Journal of infectious diseases, 2011. 780-90.
171. Longenecker, C.T., et al., *Soluble CD14 is independently associated with coronary calcification and extent of subclinical vascular disease in treated HIV infection.* AIDS, 2014. **28**(7): p. 969-77.
172. Funderburg, N.T., et al., *Shared monocyte subset phenotypes in HIV-1 infection and in uninfected subjects with acute coronary syndrome.* Blood, 2012. **120**(23): p. 4599-608.

173. Tsujioka, H., et al., *Impact of heterogeneity of human peripheral blood monocyte subsets on myocardial salvage in patients with primary acute myocardial infarction.* J Am Coll Cardiol, 2009. **54**(2): p. 130-8.
174. Pedersen, K.K., et al., *Monocyte activation, but not microbial translocation, is independently associated with markers of endovascular dysfunction in HIV-infected patients receiving cART.* J Acquir Immune Defic Syndr, 2014. **67**(4): p. 370-4.
175. Pedersen, K.K., et al., *Microbial translocation in HIV infection is associated with dyslipidemia, insulin resistance, and risk of myocardial infarction.* J Acquir Immune Defic Syndr, 2013. **64**(5): p. 425-33.
176. Kuller, L.H., et al., *Inflammatory and coagulation biomarkers and mortality in patients with HIV infection.* PLoS Med, 2008. **5**(10): p. e203.
177. Tien, P.C., et al., *Inflammation and mortality in HIV-infected adults: analysis of the FRAM study cohort.* J Acquir Immune Defic Syndr, 2010. **55**(3): p. 316-22.
178. Ledwaba, L., et al., *Pre-ART levels of inflammation and coagulation markers are strong predictors of death in a South African cohort with advanced HIV disease.* PLoS One, 2012. **7**(3): p. e24243.
179. Siedner, M.J., et al., *Persistent Immune Activation and Carotid Atherosclerosis in HIV-Infected Ugandans Receiving Antiretroviral Therapy.* J Infect Dis, 2016. **213**(3): p. 370-8.
180. Armah, K.A., et al., *HIV status, burden of comorbid disease, and biomarkers of inflammation, altered coagulation, and monocyte activation.* Clin Infect Dis, 2012. **55**(1): p. 126-36.
181. Guaraldi, G., et al., *Premature age-related comorbidities among HIV-infected persons compared with the general population.* Clin Infect Dis, 2011. **53**(11): p. 1120-6.
182. Ho, J.E., et al., *The association of CD4+ T-cell counts and cardiovascular risk in treated HIV disease.* AIDS, 2012. **26**(9): p. 1115-20.
183. Kaplan, R.C., et al., *Low CD4+ T-cell count as a major atherosclerosis risk factor in HIV-infected women and men.* AIDS, 2008. **22**(13): p. 1615-24.
184. Monteiro, P., et al., *Is arterial stiffness in HIV-infected individuals associated with HIV-related factors?* Braz J Med Biol Res, 2012. **45**(9): p. 818-26.
185. Schillaci, G., et al., *Symmetric ambulatory arterial stiffness index and 24-h pulse pressure in HIV infection: results of a nationwide cross-sectional study.* J Hypertens, 2013. **31**(3): p. 560-7; discussion 567.
186. Silverberg, M.J., et al., *Immunodeficiency and risk of myocardial infarction among HIV-positive individuals with access to care.* J Acquir Immune Defic Syndr, 2014. **65**(2): p. 160-6.
187. Lichtenstein, K.A., et al., *Low CD4+ T cell count is a risk factor for cardiovascular disease events in the HIV outpatient study.* Clin Infect Dis, 2010. **51**(4): p. 435-47.
188. Sabin, C.A., et al., *Associations between immune depression and cardiovascular events in HIV infection.* AIDS, 2013.
189. Baker, J.V., et al., *CD4+ count and risk of non-AIDS diseases following initial treatment for HIV infection.* AIDS, 2008. **22**(7): p. 841-8.
190. Menozzi, M., et al., *CD4/CD8 ratio is not predictive of multi-morbidity prevalence in HIV-infected patients but identify patients with higher CVD risk.* J Int AIDS Soc, 2014. **17**(4 Suppl 3): p. 19709.
191. Lang, S., et al., *HIV replication and immune status are independent predictors of the risk of myocardial infarction in HIV-infected individuals.* Clin Infect Dis, 2012. **55**(4): p. 600-7.
192. Lozano, R., et al., *Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010.* Lancet, 2012. **380**(9859): p. 2095-128.
193. Cappuccio, F.P., *Commentary: epidemiological transition, migration, and cardiovascular disease.* Int J Epidemiol, 2004. **33**(2): p. 387-8.

194. Narayan, K.M., et al., *HIV and noncommunicable disease comorbidities in the era of antiretroviral therapy: a vital agenda for research in low- and middle-income country settings.* J Acquir Immune Defic Syndr, 2014. **67 Suppl 1**: p. S2-7.
195. Walker, R., et al., *Stroke incidence in rural and urban Tanzania: a prospective, community-based study.* Lancet Neurol, 2010. **9**(8): p. 786-92.
196. Walker, R.W., et al., *Electrocardiographic Assessment of Coronary Artery Disease and Stroke Risk Factors in Rural and Urban Tanzania: A Case-Control Study.* J Stroke Cerebrovasc Dis, 2013.
197. Wester, C.W., et al., *Non-AIDS-defining events among HIV-1-infected adults receiving combination antiretroviral therapy in resource-replete versus resource-limited urban setting.* AIDS, 2011. **25**(12): p. 1471-9.
198. Balti, E.V., et al., *Metabolic syndrome and fatal outcomes in the post-stroke event: a 5-year cohort study in cameroon.* PLoS One, 2013. **8**(4): p. e60117.
199. Bugnicourt, J.M., et al., *[A comparison of stroke patients' characteristics between a sub-Saharan Africa population (Abidjan) and a French stroke unit (Amiens): An observational study].* Rev Neurol (Paris), 2013. **169**(3): p. 258-65.
200. Schwartz, T., et al., *HIV as a risk factor for cardiac disease in Botswana: a cross-sectional study.* Int Health, 2012. **4**(1): p. 30-7.
201. Twagirumukiza, M., et al., *Prevalence of dilated cardiomyopathy in HIV-infected African patients not receiving HAART: a multicenter, observational, prospective, cohort study in Rwanda.* Curr HIV Res, 2007. **5**(1): p. 129-37.
202. Meghji, J., et al., *Non-communicable Lung Disease in Sub Saharan Africa: a Community-based Cross-sectional Study of Adults in Urban Malawi.* Am J Respir Crit Care Med, 2016.
203. Atun, R., et al., *Improving responsiveness of health systems to non-communicable diseases.* Lancet, 2013. **381**(9867): p. 690-7.
204. Thorogood, M., et al., *Secondary prevention of stroke--results from the Southern Africa Stroke Prevention Initiative (SASPI) study.* Bull World Health Organ, 2004. **82**(7): p. 503-8.
205. Heikinheimo, T., et al., *Stroke outcomes in Malawi, a country with high prevalence of HIV: a prospective follow-up study.* PLoS One, 2012. **7**(3): p. e33765.
206. Abboud, H., et al., *Demographics, socio-economic characteristics, and risk factor prevalence in patients with non-cardioembolic ischaemic stroke in low- and middle-income countries: the OPTIC registry.* Int J Stroke, 2012.
207. Muronya, W., et al., *Cardiovascular risk factors in adult Malawians on long-term antiretroviral therapy.* Trans R Soc Trop Med Hyg, 2011. **105**(11): p. 644-9.
208. D'Agostino, R.B., Sr., et al., *General cardiovascular risk profile for use in primary care: the Framingham Heart Study.* Circulation, 2008. **117**(6): p. 743-53.
209. [cited 2016 4th October]; Available from: http://www.escardio.org/Education/Practice-Tools/CVD-prevention-toolbox/SCORE-Risk-Charts.
210. Thompson-Paul, A.M., et al., *Cardiovascular disease risk prediction in the HIV Outpatient Study.* Clin Infect Dis, 2016.
211. Monroe, A.K., et al., *Cardiovascular disease risk scores' relationship to subclinical cardiovascular disease among HIV-infected and HIV-uninfected men.* AIDS, 2016. **30**(13): p. 2075-84.
212. Kingery, J.R., et al., *Short-term and long-term cardiovascular risk, metabolic syndrome and HIV in Tanzania.* Heart, 2016. **102**(15): p. 1200-5.
213. Palombo, C. and M. Kozakova, *Arterial stiffness, atherosclerosis and cardiovascular risk: Pathophysiologic mechanisms and emerging clinical indications.* Vascul Pharmacol, 2015.
214. Sehgel, N.L., et al., *Increased vascular smooth muscle cell stiffness: a novel mechanism for aortic stiffness in hypertension.* Am J Physiol Heart Circ Physiol, 2013. **305**(9): p. H1281-7.
215. Lorentzen, K.A., et al., *Mechanisms involved in extracellular matrix remodeling and arterial stiffness induced by hyaluronan accumulation.* Atherosclerosis, 2016. **244**: p. 195-203.

216. Pikilidou, M., et al., *The Contribution of Osteoprogenitor Cells to Arterial Stiffness and Hypertension.* J Vasc Res, 2015. **52**(1): p. 32-40.
217. van Popele, N.M., et al., *Aortic stiffness is associated with atherosclerosis of the coronary arteries in older adults: the Rotterdam Study.* J Hypertens, 2006. **24**(12): p. 2371-6.
218. Laurent, S., et al., *Aortic stiffness as a tissue biomarker for predicting future cardiovascular events in asymptomatic hypertensive subjects.* Ann Med, 2012. **44 Suppl 1**: p. S93-7.
219. Palombo, C., et al., *Circulating endothelial progenitor cells and large artery structure and function in young subjects with uncomplicated type 1 diabetes.* Cardiovasc Diabetol, 2011. **10**: p. 88.
220. Wagenseil, J.E. and R.P. Mecham, *Elastin in large artery stiffness and hypertension.* J Cardiovasc Transl Res, 2012. **5**(3): p. 264-73.
221. Gotschy, A., et al., *Local arterial stiffening assessed by MRI precedes atherosclerotic plaque formation.* Circ Cardiovasc Imaging, 2013. **6**(6): p. 916-23.
222. Haynes, W.G., *ATVB in focus: noninvasive assessment of atherosclerosis--from structure to function.* Arterioscler Thromb Vasc Biol, 2002. **22**(7): p. 1064.
223. Van Bortel, L.M., et al., *Expert consensus document on the measurement of aortic stiffness in daily practice using carotid-femoral pulse wave velocity.* J Hypertens, 2012. **30**(3): p. 445-8.
224. Laurent, S., et al., *Expert consensus document on arterial stiffness: methodological issues and clinical applications.* Eur Heart J, 2006. **27**(21): p. 2588-605.
225. Mitchell, G.F., et al., *Arterial stiffness and cardiovascular events: the Framingham Heart Study.* Circulation, 2010. **121**(4): p. 505-11.
226. Vlachopoulos, C., et al., *The role of vascular biomarkers for primary and secondary prevention. A position paper from the European Society of Cardiology Working Group on peripheral circulation: Endorsed by the Association for Research into Arterial Structure and Physiology (ARTERY) Society.* Atherosclerosis, 2015. **241**(2): p. 507-32.
227. Mancia, G., et al., *2013 ESH/ESC Practice Guidelines for the Management of Arterial Hypertension.* Blood Press, 2014. **23**(1): p. 3-16.
228. Vlachopoulos, C., K. Aznaouridis, and C. Stefanadis, *Aortic stiffness for cardiovascular risk prediction: just measure it, just do it!* J Am Coll Cardiol, 2014. **63**(7): p. 647-9.
229. Rossignol, P., et al., *Target organ damage assessment in French hypertensive patients without established cardiovascular or renal disease: results of the PREVENT-A study.* J Hypertens, 2013. **31**(1): p. 177-85.
230. Laurent, S., M. Briet, and P. Boutouyrie, *Arterial stiffness as surrogate end point: needed clinical trials.* Hypertension, 2012. **60**(2): p. 518-22.
231. Hlatky, M.A., et al., *Criteria for evaluation of novel markers of cardiovascular risk: a scientific statement from the American Heart Association.* Circulation, 2009. **119**(17): p. 2408-16.
232. Vlachopoulos, C., K. Aznaouridis, and C. Stefanadis, *Prediction of cardiovascular events and all-cause mortality with arterial stiffness: a systematic review and meta-analysis.* J Am Coll Cardiol, 2010. **55**(13): p. 1318-27.
233. Ben-Shlomo, Y., et al., *Aortic pulse wave velocity improves cardiovascular event prediction: an individual participant meta-analysis of prospective observational data from 17,635 subjects.* J Am Coll Cardiol, 2014. **63**(7): p. 636-46.
234. Mattace-Raso, F.U., et al., *Arterial stiffness and risk of coronary heart disease and stroke: the Rotterdam Study.* Circulation, 2006. **113**(5): p. 657-63.
235. Pereira, T., et al., *Aortic pulse wave velocity and HeartSCORE: Improving cardiovascular risk stratification. A sub-analysis of the EDIVA (Estudo de DIstensibilidade VAscular) project.* Blood Press, 2014. **23**(2): p. 109-15.
236. van Sloten, T.T., et al., *Carotid Stiffness Is Associated With Incident Stroke: A Systematic Review and Individual Participant Data Meta-Analysis.* J Am Coll Cardiol, 2015. **66**(19): p. 2116-25.

237. Berard, E., et al., *Pulse wave velocity, pulse pressure and number of carotid or femoral plaques improve prediction of cardiovascular death in a population at low risk.* J Hum Hypertens, 2013. **27**(9): p. 529-34.
238. Kavousi, M., et al., *Evaluation of newer risk markers for coronary heart disease risk classification: a cohort study.* Ann Intern Med, 2012. **156**(6): p. 438-44.
239. Shahin, Y., J.A. Khan, and I. Chetter, *Angiotensin converting enzyme inhibitors effect on arterial stiffness and wave reflections: a meta-analysis and meta-regression of randomised controlled trials.* Atherosclerosis, 2012. **221**(1): p. 18-33.
240. Osuch, E., et al., *Effect of perindopril on pulse-wave velocity and endothelin-1 in black hypertensive patients.* Cardiovasc J Afr, 2012. **23**(7): p. 396-9.
241. Frimodt-Moller, M., et al., *Beneficial effects on arterial stiffness and pulse-wave reflection of combined enalapril and candesartan in chronic kidney disease--a randomized trial.* PLoS One, 2012. **7**(7): p. e41757.
242. Koumaras, C., et al., *Effects of renin-angiotensin-aldosterone system inhibitors and beta-blockers on markers of arterial stiffness.* J Am Soc Hypertens, 2014. **8**(2): p. 74-82.
243. Rizos, E.C., M.S. Agouridis Ap Fau - Elisaf, and M.S. Elisaf, *The effect of statin therapy on arterial stiffness by measuring pulse wave velocity: a systematic review.* (1875-6212 (Electronic)).
244. Kanaki, A.I., et al., *Effects of low-dose atorvastatin on arterial stiffness and central aortic pressure augmentation in patients with hypertension and hypercholesterolemia.* Am J Hypertens, 2013. **26**(5): p. 608-16.
245. Wang, J., et al., *Improvement of arterial stiffness by reducing oxidative stress damage in elderly hypertensive patients after 6 months of atorvastatin therapy.* J Clin Hypertens (Greenwich), 2012. **14**(4): p. 245-9.
246. Tam, L.S., G.D. Kitas, and M.A. Gonzalez-Gay, *Can suppression of inflammation by anti-TNF prevent progression of subclinical atherosclerosis in inflammatory arthritis?* Rheumatology (Oxford), 2014.
247. McInnes, I.B., et al., *Effect of interleukin-6 receptor blockade on surrogates of vascular risk in rheumatoid arthritis: MEASURE, a randomised, placebo-controlled study.* Ann Rheum Dis, 2013.
248. Tam, L.S., et al., *The effects of golimumab on subclinical atherosclerosis and arterial stiffness in ankylosing spondylitis--a randomized, placebo-controlled pilot trial.* Rheumatology (Oxford), 2014.
249. Wolffenbuttel, B.H., et al., *Breakers of advanced glycation end products restore large artery properties in experimental diabetes.* (0027-8424 (Print)).
250. Kozakova, M., et al., *The impact of age and risk factors on carotid and carotid-femoral pulse wave velocity.* J Hypertens, 2015. **33**(7): p. 1446-51.
251. AlGhatrif, M., et al., *Longitudinal trajectories of arterial stiffness and the role of blood pressure: the Baltimore Longitudinal Study of Aging.* Hypertension, 2013. **62**(5): p. 934-41.
252. Pettersen, K.H., et al., *Arterial stiffening provides sufficient explanation for primary hypertension.* PLoS Comput Biol, 2014. **10**(5): p. e1003634.
253. Cecelja, M. and P. Chowienczyk, *Dissociation of aortic pulse wave velocity with risk factors for cardiovascular disease other than hypertension: a systematic review.* Hypertension, 2009. **54**(6): p. 1328-36.
254. Mitchell, G.F., *Arterial stiffness and hypertension: chicken or egg?* Hypertension, 2014. **64**(2): p. 210-4.
255. Zheng, X., et al., *Arterial Stiffness as a Predictor of Clinical Hypertension.* J Clin Hypertens (Greenwich), 2015. **17**(8): p. 582-91.
256. *2013 Practice guidelines for the management of arterial hypertension of the European Society of Hypertension (ESH) and the European Society of Cardiology (ESC): ESH/ESC Task Force for the Management of Arterial Hypertension.* J Hypertens, 2013. **31**(10): p. 1925-38.

257. Thompson, J.E., et al., *Masked hypertension and its associated cardiovascular risk in young individuals: the African-PREDICT study.* Hypertens Res, 2015.
258. Ware, L.J., et al., *Masked Hypertension in Low-Income South African Adults.* J Clin Hypertens (Greenwich), 2016.
259. Schutte, A.E., et al., *6A.08: HIGH FREQUENCY OF MASKED HYPERTENSION AND ASSOCIATED ARTERIAL STIFFNESS IN AFRICANS.* J Hypertens, 2015. **33 Suppl 1**: p. e75.
260. Gottsater, M., et al., *Non-hemodynamic predictors of arterial stiffness after 17 years of follow-up: the Malmo Diet and Cancer study.* J Hypertens, 2015. **33**(5): p. 957-65.
261. Ho, C.T., et al., *Arterial stiffness is strongly associated with insulin resistance in Chinese--a population-based study (Taichung Community Health Study, TCHS).* J Atheroscler Thromb, 2011. **18**(2): p. 122-30.
262. Stehouwer, C.D., R.M. Henry, and I. Ferreira, *Arterial stiffness in diabetes and the metabolic syndrome: a pathway to cardiovascular disease.* Diabetologia, 2008. **51**(4): p. 527-39.
263. Lemogoum, D., et al., *Effects of hunter-gatherer subsistence mode on arterial distensibility in Cameroonian pygmies.* Hypertension, 2012. **60**(1): p. 123-8.
264. Yoo, H.J., et al., *Association of metabolically abnormal but normal weight (MANW) and metabolically healthy but obese (MHO) individuals with arterial stiffness and carotid atherosclerosis.* Atherosclerosis, 2014. **234**(1): p. 218-223.
265. Ngatchou, W., et al., *Increased burden and severity of metabolic syndrome and arterial stiffness in treatment-naive HIV+ patients from Cameroon.* Vasc Health Risk Manag, 2013. **9**: p. 509-16.
266. Huisman, H.W., et al., *Low BMI is inversely associated with arterial stiffness in Africans.* Br J Nutr, 2015. **113**(10): p. 1621-7.
267. Wang, F., et al., *Association of serum lipids with arterial stiffness in a population-based study in Beijing.* Eur J Clin Invest, 2011. **41**(9): p. 929-36.
268. Wang, J., et al., *Increased fasting glucose and the prevalence of arterial stiffness: a cross-sectional study in Chinese adults.* Neurol Res, 2014. **36**(5): p. 427-33.
269. Wang, X., et al., *Relationships between HDL-C, hs-CRP, with central arterial stiffness in apparently healthy people undergoing a general health examination.* PLoS One, 2013. **8**(12): p. e81778.
270. Medda, E., et al., *Heritability of arterial stiffness and carotid intima-media thickness: An Italian twin study.* Nutr Metab Cardiovasc Dis, 2013.
271. Mitchell, G.F., et al., *Heritability and a genome-wide linkage scan for arterial stiffness, wave reflection, and mean arterial pressure: the Framingham Heart Study.* Circulation, 2005. **112**(2): p. 194-9.
272. Mitchell, G.F., et al., *Common genetic variation in the 3'-BCL11B gene desert is associated with carotid-femoral pulse wave velocity and excess cardiovascular disease risk: the AortaGen Consortium.* Circ Cardiovasc Genet, 2012. **5**(1): p. 81-90.
273. Cecelja, M., et al., *Arterial stiffening relates to arterial calcification but not to noncalcified atheroma in women. A twin study.* J Am Coll Cardiol, 2011. **57**(13): p. 1480-6.
274. Cecelja, M., et al., *Association of Cross-Sectional and Longitudinal Change in Arterial Stiffness With Gene Expression in the Twins UK Cohort.* Hypertension, 2016. **67**(1): p. 70-6.
275. Morris, A.A., et al., *Racial differences in arterial stiffness and microcirculatory function between Black and White Americans.* J Am Heart Assoc, 2013. **2**(2): p. e002154.
276. Schutte, A.E., et al., *Arterial stiffness profiles: investigating various sections of the arterial tree of African and Caucasian people.* Clin Exp Hypertens, 2011. **33**(8): p. 511-7.
277. Schutte, A.E., et al., *Exploring soluble urokinase plasminogen activator receptor and its relationship with arterial stiffness in a bi-ethnic population: the SAfrEIC-study.* Thromb Res, 2012. **130**(2): p. 273-7.

278. Yan, H., et al., *Hemodynamic and arterial stiffness differences between African-Americans and Caucasians after maximal exercise.* Am J Physiol Heart Circ Physiol, 2014. **306**(1): p. H60-8.
279. Kruger, R., et al., *N-terminal prohormone B-type natriuretic peptide and cardiovascular function in Africans and Caucasians: the SAfrEIC study.* Heart Lung Circ, 2012. **21**(2): p. 88-95.
280. Pignoli, P., et al., *Intimal plus medial thickness of the arterial wall: a direct measurement with ultrasound imaging.* Circulation, 1986. **74**(6): p. 1399-406.
281. Singh, R.B., et al., *Pathogenesis of atherosclerosis: A multifactorial process.* Experimental & Clinical Cardiology, 2002. **7**(1): p. 40-53.
282. Johri, A.M., et al., *ICEBERG: Intimal Carotid Evaluation Before Echocardiography Reveals Global Vascular Risk.* Can J Cardiol, 2014. **30**(10): p. 1183-9.
283. Glagov, S., et al., *Hemodynamics and atherosclerosis. Insights and perspectives gained from studies of human arteries.* Arch Pathol Lab Med, 1988. **112**(10): p. 1018-31.
284. Masawa, N., S. Glagov, and C.K. Zarins, *Quantitative morphologic study of intimal thickening at the human carotid bifurcation: II. The compensatory enlargement response and the role of the intima in tensile support.* Atherosclerosis, 1994. **107**(2): p. 147-55.
285. Rosvall, M., et al., *Incident coronary events and case fatality in relation to common carotid intima-media thickness.* J Intern Med, 2005. **257**(5): p. 430-7.
286. Greenland, P., et al., *2010 ACCF/AHA guideline for assessment of cardiovascular risk in asymptomatic adults: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines.* J Am Coll Cardiol, 2010. **56**(25): p. e50-103.
287. Davis, P.H., et al., *Carotid intimal-medial thickness is related to cardiovascular risk factors measured from childhood through middle age: The Muscatine Study.* Circulation, 2001. **104**(23): p. 2815-9.
288. Urbina, E.M., et al., *Impact of multiple coronary risk factors on the intima-media thickness of different segments of carotid artery in healthy young adults (The Bogalusa Heart Study).* Am J Cardiol, 2002. **90**(9): p. 953-8.
289. Koskinen, J., et al., *Conventional cardiovascular risk factors and metabolic syndrome in predicting carotid intima-media thickness progression in young adults: the cardiovascular risk in young Finns study.* Circulation, 2009. **120**(3): p. 229-36.
290. Scuteri, A., et al., *Metabolic syndrome amplifies the age-associated increases in vascular thickness and stiffness.* J Am Coll Cardiol, 2004. **43**(8): p. 1388-95.
291. Touboul, P.J., et al., *Mannheim carotid intima-media thickness and plaque consensus (2004-2006-2011). An update on behalf of the advisory board of the 3rd, 4th and 5th watching the risk symposia, at the 13th, 15th and 20th European Stroke Conferences, Mannheim, Germany, 2004, Brussels, Belgium, 2006, and Hamburg, Germany, 2011.* Cerebrovasc Dis, 2012. **34**(4): p. 290-6.
292. Goff, D.C., Jr., et al., *2013 ACC/AHA guideline on the assessment of cardiovascular risk: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines.* Circulation, 2014. **129**(25 Suppl 2): p. S49-73.
293. Den Ruijter, H.M., et al., *Common carotid intima-media thickness measurements in cardiovascular risk prediction: a meta-analysis.* JAMA, 2012. **308**(8): p. 796-803.
294. Polak, J.F., et al., *Carotid-wall intima-media thickness and cardiovascular events.* N Engl J Med, 2011. **365**(3): p. 213-21.
295. Lorenz, M.W., et al., *Prediction of clinical cardiovascular events with carotid intima-media thickness: a systematic review and meta-analysis.* Circulation, 2007. **115**(4): p. 459-67.
296. Chambless, L.E., et al., *Carotid wall thickness is predictive of incident clinical stroke: the Atherosclerosis Risk in Communities (ARIC) study.* Am J Epidemiol, 2000. **151**(5): p. 478-87.
297. Rosvall, M., et al., *Incidence of stroke is related to carotid IMT even in the absence of plaque.* Atherosclerosis, 2005. **179**(2): p. 325-31.

298. Johnsen, S.H., et al., *Carotid atherosclerosis is a stronger predictor of myocardial infarction in women than in men: a 6-year follow-up study of 6226 persons: the Tromso Study.* Stroke, 2007. **38**(11): p. 2873-80.
299. Lorenz, M.W., et al., *Carotid intima-media thickening indicates a higher vascular risk across a wide age range: prospective data from the Carotid Atherosclerosis Progression Study (CAPS).* Stroke, 2006. **37**(1): p. 87-92.
300. Lorenz, M.W., et al., *Carotid intima-media thickness progression to predict cardiovascular events in the general population (the PROG-IMT collaborative project): a meta-analysis of individual participant data.* Lancet, 2012. **379**(9831): p. 2053-62.
301. Crouse, J.R., 3rd, et al., *Effect of rosuvastatin on progression of carotid intima-media thickness in low-risk individuals with subclinical atherosclerosis: the METEOR Trial.* JAMA, 2007. **297**(12): p. 1344-53.
302. Yu, C.M., et al., *Comparison of intensive and low-dose atorvastatin therapy in the reduction of carotid intimal-medial thickness in patients with coronary heart disease.* Heart, 2007. **93**(8): p. 933-9.
303. Kelly, C., et al., *Discordant Immune Response with Antiretroviral Therapy in HIV-1: A Systematic Review of Clinical Outcomes.* PLoS One, 2016. **11**(6): p. e0156099.
304. May, M., et al., *Prognosis of HIV-1-infected patients up to 5 years after initiation of HAART: collaborative analysis of prospective studies.* AIDS, 2007. **21**(9): p. 1185-97.
305. *Initiation of Antiretroviral Therapy in Early Asymptomatic HIV Infection.* N Engl J Med, 2015.
306. *A Trial of Early Antiretrovirals and Isoniazid Preventive Therapy in Africa.* N Engl J Med, 2015.
307. Hunt, P.W., et al., *Impact of CD8+ T Cell Activation on CD4+ T Cell Recovery and Mortality in HIV-infected Ugandans Initiating Antiretroviral Therapy.* AIDS, 2011. **25**(17): p. 2123-31.
308. Pakker, N.G., et al., *Immune restoration does not invariably occur following long-term HIV-1 suppression during antiretroviral therapy. INCAS Study Group.* AIDS, 1999. **13**(2): p. 203-12.
309. Gilson, R.J., et al., *Discordant responses on starting highly active antiretroviral therapy: suboptimal CD4 increases despite early viral suppression in the UK Collaborative HIV Cohort (UK CHIC) Study.* HIV Med, 2010. **11**(2): p. 152-60.
310. Moore, D.M., et al., *Discordant immunologic and virologic responses to highly active antiretroviral therapy are associated with increased mortality and poor adherence to therapy.* J Acquir Immune Defic Syndr, 2005. **40**(3): p. 288-93.
311. Tan, R., et al., *Clinical outcome of HIV-infected antiretroviral-naive patients with discordant immunologic and virologic responses to highly active antiretroviral therapy.* J Acquir Immune Defic Syndr, 2008. **47**(5): p. 553-8.
312. Tuboi, S.H., et al., *Mortality associated with discordant responses to antiretroviral therapy in resource-constrained settings.* J Acquir Immune Defic Syndr, 2010. **53**(1): p. 70-7.
313. Zoufaly, A., et al., *Clinical outcome of HIV-infected patients with discordant virological and immunological response to antiretroviral therapy.* J Infect Dis, 2011. **203**(3): p. 364-71.
314. http://www.unaids.org/sites/default/files/documents/20141118_FS_WADreport_en.pdf. *UNAIDS Global Statistics Fact Sheet 2014*. 2014 [cited 2015 23rd March].
315. http://apps.who.int/iris/bitstream/10665/85321/1/9789241505727_eng.pdf?ua=1. [cited 2014 15th December].
316. Mocroft, A., et al., *The incidence of AIDS-defining illnesses at a current CD4 count >/= 200 cells/muL in the post-combination antiretroviral therapy era.* Clin Infect Dis, 2013. **57**(7): p. 1038-47.
317. Lawn, S.D., et al., *Changing mortality risk associated with CD4 cell response to antiretroviral therapy in South Africa.* AIDS, 2009. **23**(3): p. 335-42.
318. Bandera, A., et al., *CD4+ T cell depletion, immune activation and increased production of regulatory T cells in the thymus of HIV-infected individuals.* PLoS One, 2010. **5**(5): p. e10788.

319. Vrisekoop, N., et al., *Restoration of the CD4 T cell compartment after long-term highly active antiretroviral therapy without phenotypical signs of accelerated immunological aging.* J Immunol, 2008. **181**(2): p. 1573-81.
320. Li, T., et al., *Reduced thymic output is a major mechanism of immune reconstitution failure in HIV-infected patients after long-term antiretroviral therapy.* Clin Infect Dis, 2011. **53**(9): p. 944-51.
321. Zeng, M., et al., *Lymphoid tissue damage in HIV-1 infection depletes naive T cells and limits T cell reconstitution after antiretroviral therapy.* PLoS Pathog, 2012. **8**(1): p. e1002437.
322. Diaz, A., et al., *Factors associated with collagen deposition in lymphoid tissue in long-term treated HIV-infected patients.* AIDS, 2010. **24 (13)**: p. 2029-2039.
323. Serrano-Villar, S., et al., *HIV-infected individuals with low CD4/CD8 ratio despite effective antiretroviral therapy exhibit altered T cell subsets, heightened CD8+ T cell activation, and increased risk of non-AIDS morbidity and mortality.* PLoS Pathog, 2014. **10**(5): p. e1004078.
324. Delobel, P., et al., *Naive T-cell depletion related to infection by X4 human immunodeficiency virus type 1 in poor immunological responders to highly active antiretroviral therapy.* J Virol, 2006. **80**(20): p. 10229-36.
325. Funderburg, N.T., et al., *Dynamics of immune reconstitution and activation markers in HIV+ treatment-naive patients treated with raltegravir, tenofovir disoproxil fumarate and emtricitabine.* PLoS One, 2013. **8**(12): p. e83514.
326. Shive, C.L., et al., *Inflammatory cytokines drive CD4+ T-cell cycling and impaired responsiveness to interleukin 7: implications for immune failure in HIV disease.* J Infect Dis, 2014. **210**(4): p. 619-29.
327. Gandhi, R.T., et al., *Effect of baseline- and treatment-related factors on immunologic recovery after initiation of antiretroviral therapy in HIV-1-positive subjects: results from ACTG 384.* J Acquir Immune Defic Syndr, 2006. **42**(4): p. 426-34.
328. Lapadula, G., et al., *Risk of Severe Non AIDS Events Is Increased among Patients Unable to Increase their CD4+ T-Cell Counts >200+/mul Despite Effective HAART.* PLoS One, 2015. **10**(5): p. e0124741.
329. Funderburg, N.T., *Markers of coagulation and inflammation often remain elevated in ART-treated HIV-infected patients.* Curr Opin HIV AIDS, 2014. **9**(1): p. 80-6.
330. Wallet, M.A., et al., *Microbial translocation induces persistent macrophage activation unrelated to HIV-1 levels or T-cell activation following therapy.* AIDS, 2010. **24**(9): p. 1281-90.
331. Mavigner, M., et al., *HIV-1 residual viremia correlates with persistent T-cell activation in poor immunological responders to combination antiretroviral therapy.* PLoS One, 2009. **4**(10): p. e7658.
332. Barrett, L., et al., *Immune resilience in HIV-infected individuals seronegative for cytomegalovirus.* AIDS, 2014. **28**(14): p. 2045-9.
333. Hermans, S.M., et al., *Incident tuberculosis during antiretroviral therapy contributes to suboptimal immune reconstitution in a large urban HIV clinic in sub-Saharan Africa.* PLoS One, 2010. **5**(5): p. e10527.
334. Hermans, S.M., et al., *Unrecognised tuberculosis at antiretroviral therapy initiation is associated with lower CD4+ T cell recovery.* Trop Med Int Health, 2012. **17**(12): p. 1527-33.
335. Somsouk, M., et al., *Gut epithelial barrier and systemic inflammation during chronic HIV infection.* AIDS, 2015. **29**(1): p. 43-51.
336. Sandler, N.G., et al., *Plasma levels of soluble CD14 independently predict mortality in HIV infection.* J Infect Dis, 2011. **203**(6): p. 780-90.
337. Lichtfuss, G.F., et al., *Virologically suppressed HIV patients show activation of NK cells and persistent innate immune activation.* J Immunol, 2012. **189**(3): p. 1491-9.
338. BenMarzouk-Hidalgo, O.J., et al., *Differential effects of viremia and microbial translocation on immune activation in HIV-infected patients throughout ritonavir-boosted darunavir monotherapy.* Medicine (Baltimore), 2015. **94**(17): p. e781.

339. Wilson, E.M. and I. Sereti, *Immune restoration after antiretroviral therapy: the pitfalls of hasty or incomplete repairs.* Immunol Rev, 2013. **254**(1): p. 343-54.
340. Gazzola, L., et al., *The absence of CD4+ T cell count recovery despite receipt of virologically suppressive highly active antiretroviral therapy: clinical risk, immunological gaps, and therapeutic options.* Clin Infect Dis, 2009. **48**(3): p. 328-37.
341. Gaardbo, J.C., et al., *Incomplete immune recovery in HIV infection: mechanisms, relevance for clinical care, and possible solutions.* Clin Dev Immunol, 2012. **2012**: p. 670957.
342. Corbeau, P. and J. Reynes, *Immune reconstitution under antiretroviral therapy: the new challenge in HIV-1 infection.* Blood, 2011. **117**(21): p. 5582-90.
343. Battegay, M., et al., *Immunological recovery and antiretroviral therapy in HIV-1 infection.* Lancet Infect Dis, 2006. **6**(5): p. 280-7.
344. Cenderello, G. and A. De Maria, *Discordant responses to cART in HIV-1 patients in the era of high potency antiretroviral drugs: clinical evaluation, classification, management prospects.* Expert Rev Anti Infect Ther, 2015: p. 1-12.
345. Schechter, M. and S.H. Tuboi, *Discordant immunological and virological responses to antiretroviral therapy.* J Antimicrob Chemother, 2006. **58**(3): p. 506-10.
346. Moher, D., et al., *Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement.* PLoS Med, 2009. **6**(7): p. e1000097.
347. http://www.who.int/hiv/pub/guidelines/HIVstaging150307.pdf. [cited 2015 31st July].
348. https://bmg.cochrane.org/sites/bmg.cochrane.org/files/uploads/Tool%20to%20Assess%20Risk%20of%20Bias%20in%20Cohort%20Studies.pdf. *Tool to Assess Risk of Bias in Cohort Studies*. [cited 2014 12th December].
349. Mugyenyi, P., et al., *Routine versus clinically driven laboratory monitoring of HIV antiretroviral therapy in Africa (DART): a randomised non-inferiority trial.* Lancet, 2010. **375**(9709): p. 123-31.
350. http://handbook.cochrane.org/chapter_8/8_8_2_2_studies_assessed_as_at_unclear_risk_of_bias.htm. Cochrane handbook for systematic reviews of interventions [cited 2015 5th August].
351. Baker, J.V., et al., *Poor initial CD4+ recovery with antiretroviral therapy prolongs immune depletion and increases risk for AIDS and non-AIDS diseases.* J Acquir Immune Defic Syndr, 2008. **48**(5): p. 541-6.
352. Dronda, F., et al., *Long-term outcomes among antiretroviral-naive human immunodeficiency virus-infected patients with small increases in CD4+ cell counts after successful virologic suppression.* Clin Infect Dis, 2002. **35**(8): p. 1005-9.
353. Engsig, F.N., et al., *Long-term mortality in HIV patients virally suppressed for more than three years with incomplete CD4 recovery: a cohort study.* BMC Infect Dis, 2010. **10**: p. 318.
354. Falster, K., et al., *Poor baseline immune function predicts an incomplete immune response to combination antiretroviral treatment despite sustained viral suppression.* J Acquir Immune Defic Syndr, 2009. **50**(3): p. 307-13.
355. Grabar, S., et al., *Clinical outcome of patients with HIV-1 infection according to immunologic and virologic response after 6 months of highly active antiretroviral therapy.* Ann Intern Med, 2000. **133**(6): p. 401-10.
356. Gutierrez, F., et al., *Patients' characteristics and clinical implications of suboptimal CD4 T-cell gains after 1 year of successful antiretroviral therapy.* Curr HIV Res, 2008. **6**(2): p. 100-7.
357. Kaufmann, G.R., et al., *Characteristics, determinants, and clinical relevance of CD4 T cell recovery to <500 cells/microL in HIV type 1-infected individuals receiving potent antiretroviral therapy.* Clin Infect Dis, 2005. **41**(3): p. 361-72.

358. Loutfy, M.R., et al., *A CD4+ cell count <200 cells per cubic millimeter at 2 years after initiation of combination antiretroviral therapy is associated with increased mortality in HIV-infected individuals with viral suppression.* J Acquir Immune Defic Syndr, 2010. **55**(4): p. 451-9.
359. Pacheco, Y.M., et al., *Risk factors, CD4 long-term evolution and mortality of HIV-infected patients who persistently maintain low CD4 counts, despite virological response to HAART.* Curr HIV Res, 2009. **7**(6): p. 612-9.
360. Zoufaly, A., et al., *Clinical Outcome of HIV-Infected Patients with Discordant Virological and Immunological Response to Antiretroviral Therapy.* J Infect Dis, 2011. **203**(3): p. 364-71.
361. Nicastri, E., et al., *Clinical outcome after 4 years follow-up of HIV-seropositive subjects with incomplete virologic or immunologic response to HAART.* J Med Virol, 2005. **76**(2): p. 153-60.
362. Taiwo, B.O., et al., *Higher risk of AIDS or death in patients with lower CD4 cell counts after virally suppressive HAART.* HIV Med, 2009. **10**(10): p. 657-60.
363. Batista, G., et al., *Initial suboptimal CD4 reconstitution with antiretroviral therapy despite full viral suppression in a cohort of HIV-infected patients in Senegal.* Med Mal Infect, 2015. **45**(6): p. 199-206.
364. Takuva, S., et al., *Poor CD4 recovery and risk of subsequent progression to AIDS or death despite viral suppression in a South African cohort.* J Int AIDS Soc, 2014. **17**: p. 18651.
365. Engsig, F.N., et al., *Long-term mortality in HIV positive individuals virally suppressed for more than three years with incomplete CD4 recovery.* Clinical Infectious Diseases, 2014.
366. Smith, C.J., et al., *The potential for CD4 cell increases in HIV-positive individuals who control viraemia with highly active antiretroviral therapy.* AIDS, 2003. **17**(7): p. 963-9.
367. Castro, P., et al., *Influence of episodes of intermittent viremia ("blips") on immune responses and viral load rebound in successfully treated HIV-infected patients.* AIDS Res Hum Retroviruses, 2013. **29**(1): p. 68-76.
368. Negredo, E., et al., *Early but limited effects of raltegravir intensification on CD4 T cell reconstitution in HIV-infected patients with an immunodiscordant response to antiretroviral therapy.* J Antimicrob Chemother, 2013. **68**(10): p. 2358-62.
369. Pallikkuth, S., M.A. Fischl, and S. Pahwa, *Combination antiretroviral therapy with raltegravir leads to rapid immunologic reconstitution in treatment-naive patients with chronic HIV infection.* J Infect Dis, 2013. **208**(10): p. 1613-23.
370. Yi, T.J., et al., *A randomized controlled pilot trial of valacyclovir for attenuating inflammation and immune activation in HIV/herpes simplex virus 2-coinfected adults on suppressive antiretroviral therapy.* Clin Infect Dis, 2013. **57**(9): p. 1331-8.
371. Stiksrud, B., et al., *Reduced Levels of D-dimer and Changes in Gut Microbiota Composition After Probiotic Intervention in HIV-Infected Individuals on Stable ART.* J Acquir Immune Defic Syndr, 2015. **70**(4): p. 329-37.
372. Ganesan, A., et al., *High dose atorvastatin decreases cellular markers of immune activation without affecting HIV-1 RNA levels: results of a double-blind randomized placebo controlled clinical trial.* J Infect Dis, 2011. **203**(6): p. 756-64.
373. Nakanjako, D., et al., *Atorvastatin reduces T-cell activation and exhaustion among HIV-infected cART-treated suboptimal immune responders in Uganda: a randomised crossover placebo-controlled trial.* Trop Med Int Health, 2015. **20**(3): p. 380-90.
374. Savarino, A. and I.L. Shytaj, *Chloroquine and beyond: exploring anti-rheumatic drugs to reduce immune hyperactivation in HIV/AIDS.* Retrovirology, 2015. **12**: p. 51.
375. Zanussi, S., et al., *Immunological changes in peripheral blood and in lymphoid tissue after treatment of HIV-infected subjects with highly active anti-retroviral therapy (HAART) or HAART + IL-2.* Clin Exp Immunol, 1999. **116**(3): p. 486-92.
376. Vogler, M.A., et al., *Daily low-dose subcutaneous interleukin-2 added to single- or dual-nucleoside therapy in HIV infection does not protect against CD4+ T-cell decline or improve other indices of immune function: results of a randomized controlled clinical trial (ACTG 248).* J Acquir Immune Defic Syndr, 2004. **36**(1): p. 576-87.

377. Imamichi, H., et al., *HIV-1 viruses detected during episodic blips following interleukin-7 administration are similar to the viruses present before and after interleukin-7 therapy.* AIDS, 2011. **25**(2): p. 159-64.
378. Beq, S., J.F. Delfraissy, and J. Theze, *Interleukin-7 (IL-7): immune function, involvement in the pathogenesis of HIV infection and therapeutic potential.* Eur Cytokine Netw, 2004. **15**(4): p. 279-89.
379. Plana, M., et al., *The reconstitution of the thymus in immunosuppressed individuals restores CD4-specific cellular and humoral immune responses.* Immunology, 2011. **133**(3): p. 318-28.
380. Jacobson, J.M., et al., *A randomized controlled trial of palifermin (recombinant human keratinocyte growth factor) for the treatment of inadequate CD4+ T-lymphocyte recovery in patients with HIV-1 infection on antiretroviral therapy.* J Acquir Immune Defic Syndr, 2014. **66**(4): p. 399-406.
381. Schuetz, A., et al., *Initiation of ART during early acute HIV infection preserves mucosal Th17 function and reverses HIV-related immune activation.* PLoS Pathog, 2014. **10**(12): p. e1004543.
382. Jain, V., et al., *Antiretroviral therapy initiated within 6 months of HIV infection is associated with lower T-cell activation and smaller HIV reservoir size.* J Infect Dis, 2013. **208**(8): p. 1202-11.
383. Hocqueloux, L., et al., *Long-term antiretroviral therapy initiated during primary HIV-1 infection is key to achieving both low HIV reservoirs and normal T cell counts.* J Antimicrob Chemother, 2013. **68**(5): p. 1169-78.
384. Okulicz, J.F., et al., *Influence of the timing of antiretroviral therapy on the potential for normalization of immune status in human immunodeficiency virus 1-infected individuals.* JAMA Intern Med, 2015. **175**(1): p. 88-99.
385. *Late presentation for HIV care across Europe: update from the Collaboration of Observational HIV Epidemiological Research Europe (COHERE) study, 2010 to 2013.* Euro Surveill, 2015. **20**(47).
386. Dickson, N., et al., *Late presentation of HIV infection among adults in New Zealand: 2005-2010.* HIV Med, 2012. **13**(3): p. 182-9.
387. https://www.google.co.uk/maps/place/Queen+Elizabeth+Central+Hospital, B., +Malawi/@-15.8030426,35.0168894,16z/data=!3m1!4b1!4m2!3m1!1s0x18d845947a5ca71d:0x143328d65dc6a84c. [cited 2016 22nd January]; Map of QECH].
388. SanJoaquin, M.A., et al., *Surveillance Programme of IN-patients and Epidemiology (SPINE): implementation of an electronic data collection tool within a large hospital in Malawi.* PLoS Med, 2013. **10**(3): p. e1001400.
389. Lewis, D.K., et al., *Prevalence and indicators of HIV and AIDS among adults admitted to medical and surgical wards in Blantyre, Malawi.* Trans R Soc Trop Med Hyg, 2003. **97**(1): p. 91-6.
390. *Malawi National Guidelines for the Management of HIV infection*. www.hiv.health.gov.mw/index.php/our-documents 2014 [cited 2015 22nd January].
391. Walker, A.S., et al., *Mortality in the Year Following Antiretroviral Therapy Initiation in HIV-Infected Adults and Children in Uganda and Zimbabwe.* Clin Infect Dis, 2012. **55**(12): p. 1707-18.
392. Mackenzie, I.S., I.B. Wilkinson, and J.R. Cockcroft, *Assessment of arterial stiffness in clinical practice.* QJM, 2002. **95**(2): p. 67-74.
393. Hu, F.S., et al., *A region-matching method for pulse transit time estimation: potential for improving the accuracy in determining carotid femoral pulse wave velocity.* J Hum Hypertens, 2015. **29**(11): p. 675-82.
394. Stojadinovic, B., et al., *Effect of viscosity on the wave propagation: Experimental determination of compression and expansion pulse wave velocity in fluid-fill elastic tube.* J Biomech, 2015. **48**(15): p. 3969-74.

395. Tanaka, H., et al., *Comparison between carotid-femoral and brachial-ankle pulse wave velocity as measures of arterial stiffness.* J Hypertens, 2009. **27**(10): p. 2022-7.
396. DeLoach, S.S. and R.R. Townsend, *Vascular stiffness: its measurement and significance for epidemiologic and outcome studies.* Clin J Am Soc Nephrol, 2008. **3**(1): p. 184-92.
397. Weber, T., et al., *Noninvasive methods to assess pulse wave velocity: comparison with the invasive gold standard and relationship with organ damage.* J Hypertens, 2015. **33**(5): p. 1023-31.
398. Bozec, E. and N. Girerd, *Carotid-Femoral Pulse Wave Velocity: An Urgent Need for a Harmonization of Denominations.* Am J Hypertens, 2015. **28**(7): p. 951.
399. Hickson, S.S., et al., *Validity and repeatability of the Vicorder apparatus: a comparison with the SphygmoCor device.* Hypertens Res, 2009. **32**(12): p. 1079-85.
400. van Leeuwen-Segarceanu, E.M., et al., *Comparison of two instruments measuring carotid-femoral pulse wave velocity: Vicorder versus SphygmoCor.* J Hypertens, 2010. **28**(8): p. 1687-91.
401. Crouse, J.R., et al., *Arterial enlargement in the atherosclerosis risk in communities (ARIC) cohort. In vivo quantification of carotid arterial enlargement. The ARIC Investigators.* Stroke, 1994. **25**(7): p. 1354-9.
402. Hulten, E., et al., *HIV positivity, protease inhibitor exposure and subclinical atherosclerosis: a systematic review and meta-analysis of observational studies.* Heart, 2009. **95**(22): p. 1826-35.
403. Benetos, A., et al., *Determinants of accelerated progression of arterial stiffness in normotensive subjects and in treated hypertensive subjects over a 6-year period.* Circulation, 2002. **105**(10): p. 1202-7.
404. Toyama, K., et al., *Combination treatment of rosuvastatin or atorvastatin, with regular exercise improves arterial wall stiffness in patients with coronary artery disease.* PLoS One, 2012. **7**(7): p. e41369.
405. Orlova, I.A., et al., *Prognostic value of changes in arterial stiffness in men with coronary artery disease.* Vasc Health Risk Manag, 2010. **6**: p. 1015-21.
406. Wilson, D.P., et al., *A critical epidemiological review of cardiovascular disease risk in HIV-infected adults: the importance of the HIV-uninfected comparison group, confounding, and competing risks--authors' reply.* HIV Med, 2013. **14**(3): p. 193-4.
407. Magalhães, P., et al., *Age- and gender-specific reference values of pulse wave velocity for African adults: preliminary results.* Age, 2013. **35**(6): p. 2345-2355.
408. *Determinants of pulse wave velocity in healthy people and in the presence of cardiovascular risk factors: 'establishing normal and reference values'.* Eur Heart J, 2010. **31**(19): p. 2338.
409. #xed, et al., *Reference Values of Pulse Wave Velocity in Healthy People from an Urban and Rural Argentinean Population.* Int J Hypertens, 2014. **2014**: p. 7.
410. Shiburi, C.P., et al., *Reference Values for SphygmoCor Measurements in South Africans of African Ancestry*.* Am J Hypertens, 2006. **19**(1): p. 40-46.
411. Fourie, C.M., et al., *Endothelial activation and cardiometabolic profiles of treated and never-treated HIV infected Africans.* Atherosclerosis, 2015. **240**(1): p. 154-60.
412. Kooij, K.W., et al., *Difference in Aortic Stiffness Between Treated Middle-Aged HIV Type 1-Infected and Uninfected Individuals Largely Explained by Traditional Cardiovascular Risk Factors, With an Additional Contribution of Prior Advanced Immunodeficiency.* J Acquir Immune Defic Syndr, 2016. **73**(1): p. 55-62.
413. Maia-Leite, L.H., et al., *Aortic stiffness aging is influenced by past profound immunodeficiency in HIV-infected individuals: results from the EVAS-HIV (EValuation of Aortic Stiffness in HIV-infected individuals).* J Hypertens, 2016. **34**(7): p. 1338-46.
414. Ho, J.E., et al., *Initiation of antiretroviral therapy at higher nadir CD4+ T-cell counts is associated with reduced arterial stiffness in HIV-infected individuals.* AIDS, 2010. **24**(12): p. 1897-905.

415. Gutierrez, J., et al., *Thinning of the arterial media layer as a possible preclinical stage in HIV vasculopathy: a pilot study.* Stroke, 2012. **43**(4): p. 1156-8.
416. Schoffelen, A.F., et al., *Carotid Intima Media Thickness in Mainly Female HIV-Infected Subjects in Rural South Africa: Association With Cardiovascular but Not HIV-Related Factors.* Clin Infect Dis, 2015. **61**(10): p. 1606-14.
417. Stein, J.H., et al., *Use of carotid ultrasound to identify subclinical vascular disease and evaluate cardiovascular disease risk: a consensus statement from the American Society of Echocardiography Carotid Intima-Media Thickness Task Force. Endorsed by the Society for Vascular Medicine.* J Am Soc Echocardiogr, 2008. **21**(2): p. 93-111; quiz 189-90.
418. O'Donnell, M.J., et al., *Global and regional effects of potentially modifiable risk factors associated with acute stroke in 32 countries (INTERSTROKE): a case-control study.* Lancet, 2016. **388**(10046): p. 761-75.
419. Imamichi, H., et al., *The CD8+ HLA-DR+ T cells expanded in HIV-1 infection are qualitatively identical to those from healthy controls.* Eur J Immunol, 2012. **42**(10): p. 2608-20.
420. Deeks, S.G., E. Verdin, and J.M. McCune, *Immunosenescence and HIV.* Curr Opin Immunol, 2012. **24**(4): p. 501-6.
421. Lee, S.A., et al., *Low Proportions of CD28- CD8+ T cells Expressing CD57 Can Be Reversed by Early ART Initiation and Predict Mortality in Treated HIV Infection.* J Infect Dis, 2014.
422. Agca, R., et al., *Atherosclerotic cardiovascular disease in patients with chronic inflammatory joint disorders.* Heart, 2016. **102**(10): p. 790-5.
423. Kitahata, M.M., et al., *Effect of early versus deferred antiretroviral therapy for HIV on survival.* N Engl J Med, 2009. **360**(18): p. 1815-26.
424. Hatano, H., et al., *Evidence of persistent low-level viremia in long-term HAART-suppressed, HIV-infected individuals.* AIDS, 2010. **24**(16): p. 2535-9.
425. Hanna, D.B., et al., *HIV Infection and Carotid Artery Intima-media Thickness: Pooled Analyses Across 5 Cohorts of the NHLBI HIV-CVD Collaborative.* Clin Infect Dis, 2016. **63**(2): p. 249-56.
426. Hua, S., et al., *Potential Role for HIV-Specific CD38(−)/HLA-DR(+) CD8(+) T Cells in Viral Suppression and Cytotoxicity in HIV Controllers.* PLoS One, 2014. **9**(7): p. e101920.
427. Eller, M.A., et al., *HIV Type 1 Disease Progression to AIDS and Death in a Rural Ugandan Cohort Is Primarily Dependent on Viral Load Despite Variable Subtype and T-Cell Immune Activation Levels.* J Infect Dis, 2015. **211**(10): p. 1574-84.
428. Lukwiya, M., et al., *Evaluation of immune activation in HIV-infected and uninfected African individuals by single-cell analysis of cytokine production.* J Acquir Immune Defic Syndr, 2001. **28**(5): p. 429-36.
429. Tsegaye, A., et al., *Immunophenotyping of blood lymphocytes at birth, during childhood, and during adulthood in HIV-1-uninfected Ethiopians.* Clin Immunol, 2003. **109**(3): p. 338-46.
430. Glennie, S.J., et al., *Do multiple concurrent infections in African children cause irreversible immunological damage?* Immunology, 2012. **135**(2): p. 125-32.
431. Kuebler, P.J., et al., *Persistent HIV Type 1 Seronegative Status Is Associated With Lower CD8+ T-Cell Activation.* J Infect Dis, 2016. **213**(4): p. 569-73.
432. Begaud, E., et al., *Reduced CD4 T cell activation and in vitro susceptibility to HIV-1 infection in exposed uninfected Central Africans.* Retrovirology, 2006. **3**: p. 35.
433. Jaworowski, A., et al., *CD16+ monocyte subset preferentially harbors HIV-1 and is expanded in pregnant Malawian women with Plasmodium falciparum malaria and HIV-1 infection.* J Infect Dis, 2007. **196**(1): p. 38-42.
434. Zawada, A.M., et al., *SuperSAGE evidence for CD14++CD16+ monocytes as a third monocyte subset.* Blood, 2011. **118**(12): p. e50-61.
435. Ancuta, P., L. Weiss, and N. Haeffner-Cavaillon, *CD14+CD16++ cells derived in vitro from peripheral blood monocytes exhibit phenotypic and functional dendritic cell-like characteristics.* Eur J Immunol, 2000. **30**(7): p. 1872-83.

436. Cros, J., et al., *Human CD14(dim) Monocytes Patrol and Sense Nucleic Acids and Viruses via TLR7 and TLR8 Receptors.* Immunity, 2010. **33**(3): p. 375-386.
437. Scriven, J., et al., *Monocyte deactivation is associated with mortality in HIV-associated cryptococcal meningitis.* Journal of Infection. **71**(6): p. 684-685.
438. Naranbhai, V., et al., *Neither microbial translocation nor TLR responsiveness are likely explanations for preexisting immune activation in women who subsequently acquired HIV in CAPRISA 004.* J Acquir Immune Defic Syndr, 2013. **63**(3): p. 294-8.
439. Krikke, M., et al., *The role of T cells in the development of cardiovascular disease in HIV-infected patients.* Atherosclerosis, 2014. **237**(1): p. 92-8.
440. Paris, R.M., et al., *Selective Loss of Early Differentiated, Highly Functional PD1high CD4 T Cells with HIV Progression.* PLoS One, 2015. **10**(12): p. e0144767.
441. Jensen, S.S., et al., *Initiation of Antiretroviral Therapy (ART) at Different Stages of HIV-1 Disease Is Not Associated with the Proportion of Exhausted CD8+ T Cells.* PLoS One, 2015. **10**(10): p. e0139573.
442. Peterson, I., et al., *Unstructured treatment interruption: an important risk factor for arterial stiffness in adult Malawian patients with antiretroviral treatment.* AIDS, 2016. **30**(15): p. 2373-8.
443. Kaplan, R.C., et al., *T cell activation and senescence predict subclinical carotid artery disease in HIV-infected women.* J Infect Dis, 2011. **203**(4): p. 452-63.
444. Chow, D.C., et al., *Non-classical monocytes predict progression of carotid artery bifurcation intima-media thickness in HIV-infected individuals on stable antiretroviral therapy.* HIV Clin Trials, 2016. **17**(3): p. 114-22.
445. Zungsontiporn, N., et al., *Non-Classical Monocytes and Monocyte Chemoattractant Protein-1 (MCP-1) Correlate with Coronary Artery Calcium Progression in Chronically HIV-1 Infected Adults on Stable Antiretroviral Therapy.* PLoS One, 2016. **11**(2): p. e0149143.
446. Longenecker, C.T., et al., *Markers of inflammation and CD8 T-cell activation, but not monocyte activation, are associated with subclinical carotid artery disease in HIV-infected individuals.* HIV Med, 2013. **14**(6): p. 385-90.
447. Yong, Y.K., et al., *Genetic polymorphisms in the CD14 gene are associated with monocyte activation and carotid intima-media thickness in HIV-infected patients on antiretroviral therapy.* Medicine (Baltimore), 2016. **95**(31): p. e4477.
448. Sauce, D., et al., *HIV disease progression despite suppression of viral replication is associated with exhaustion of lymphopoiesis.* Blood, 2011. **117**(19): p. 5142-51.
449. Xie, J., et al., *[The correlation between HLA-DR or CD38 expression in CD8(+)T cells and CD4(+)T cells count in treatment-naive HIV/AIDS patients].* Zhonghua Nei Ke Za Zhi, 2016. **55**(1): p. 36-9.
450. Lubaki, N.M., et al., *HIV-1-specific cytolytic T-lymphocyte activity correlates with lower viral load, higher CD4 count, and CD8+CD38-DR- phenotype: comparison of statistical methods for measurement.* J Acquir Immune Defic Syndr, 1999. **22**(1): p. 19-30.
451. Ramzaoui, S., et al., *During HIV infection, CD4+ CD38+ T-cells are the predominant circulating CD4+ subset whose HLA-DR positivity increases with disease progression and whose V beta repertoire is similar to that of CD4+ CD38- T-cells.* Clin Immunol Immunopathol, 1995. **77**(1): p. 33-41.
452. Liu, Z., et al., *Elevated CD38 antigen expression on CD8+ T cells is a stronger marker for the risk of chronic HIV disease progression to AIDS and death in the Multicenter AIDS Cohort Study than CD4+ cell count, soluble immune activation markers, or combinations of HLA-DR and CD38 expression.* J Acquir Immune Defic Syndr Hum Retrovirol, 1997. **16**(2): p. 83-92.
453. Vion, A.C., et al., *Shear stress regulates endothelial microparticle release.* Circ Res, 2013. **112**(10): p. 1323-33.
454. Zwaal, R.F.A., P. Comfurius, and E.M. Bevers, *Surface exposure of phosphatidylserine in pathological cells.* Cellular and Molecular Life Sciences CMLS, 2005. **62**(9): p. 971-988.

455. Loyer, X., et al., *Microvesicles as cell-cell messengers in cardiovascular diseases.* Circ Res, 2014. **114**(2): p. 345-53.
456. Eleftheriou, D., et al., *Endothelial injury in childhood stroke with cerebral arteriopathy: a cross-sectional study.* Neurology, 2012. **79**(21): p. 2089-96.
457. Schiro, A., et al., *Endothelial microparticles as conveyors of information in atherosclerotic disease.* Atherosclerosis, 2014. **234**(2): p. 295-302.
458. Amabile, N., et al., *Association of circulating endothelial microparticles with cardiometabolic risk factors in the Framingham Heart Study.* Eur Heart J, 2014. **35**(42): p. 2972-9.
459. Barry, O.P., et al., *Modulation of monocyte-endothelial cell interactions by platelet microparticles.* The Journal of Clinical Investigation. **102**(1): p. 136-144.
460. Stakos, D.A., et al., *Expression of functional tissue factor by neutrophil extracellular traps in culprit artery of acute myocardial infarction.* Eur Heart J, 2015. **36**(22): p. 1405-1414.
461. Martin, S., et al., *Shed membrane particles from T lymphocytes impair endothelial function and regulate endothelial protein expression.* Circulation, 2004. **109**(13): p. 1653-9.
462. Mayne, E., et al., *Increased platelet and microparticle activation in HIV infection: upregulation of Pselectin and tissue factor expression.* J Acquir Immune Defic Syndr, 2012. **59**(4): p. 340-346.
463. da Silva, E.F., et al., *Imbalance between endothelial progenitors cells and microparticles in HIV-infected patients naive for antiretroviral therapy.* AIDS, 2011. **25**(13): p. 1595-601.
464. Mayne, E., et al., *Increased platelet and microparticle activation in HIV infection: upregulation of P-selectin and tissue factor expression.* J Acquir Immune Defic Syndr, 2012. **59**(4): p. 340-6.
465. Arderiu, G., E. Pena, and L. Badimon, *Angiogenic microvascular endothelial cells release microparticles rich in tissue factor that promotes postischemic collateral vessel formation.* Arterioscler Thromb Vasc Biol, 2015. **35**(2): p. 348-57.
466. Switonska, M., et al., *Tissue-factor-bearing microparticles (MPs-TF) in patients with acute ischaemic stroke: the influence of stroke treatment on MPs-TF generation.* Eur J Neurol, 2015. **22**(2): p. 395-401, e28-9.
467. Baker, J.V., et al., *Circulating levels of tissue factor microparticle procoagulant activity are reduced with antiretroviral therapy and are associated with persistent inflammation and coagulation activation among HIV-positive patients.* J Acquir Immune Defic Syndr, 2013. **63**(3): p. 367-71.
468. Geddings, J.E., et al., *Tissue factor-positive tumor microvesicles activate platelets and enhance thrombosis in mice.* J Thromb Haemost, 2016. **14**(1): p. 153-66.
469. Varon, D. and E. Shai, *Platelets and their microparticles as key players in pathophysiological responses.* J Thromb Haemost, 2015. **13 Suppl 1**: p. S40-6.
470. Punyadee, N., et al., *Microparticles provide a novel biomarker to predict severe clinical outcomes of dengue virus infection.* J Virol, 2015. **89**(3): p. 1587-607.
471. Chen, Y., et al., *Plasma endothelial microparticles and their correlation with the presence of hypertension and arterial stiffness in patients with type 2 diabetes.* J Clin Hypertens (Greenwich), 2012. **14**(7): p. 455-60.
472. Feng, B., et al., *Circulating level of microparticles and their correlation with arterial elasticity and endothelium-dependent dilation in patients with type 2 diabetes mellitus.* Atherosclerosis, 2010. **208**(1): p. 264-9.
473. Chiva-Blanch, G., et al., *Microparticle Shedding by Erythrocytes, Monocytes and Vascular Smooth Muscular Cells Is Reduced by Aspirin in Diabetic Patients.* Rev Esp Cardiol (Engl Ed), 2016. **69**(7): p. 672-80.
474. Zu, L., et al., *Endothelial microparticles after antihypertensive and lipid-lowering therapy inhibit the adhesion of monocytes to endothelial cells.* Int J Cardiol, 2016. **202**: p. 756-9.

475. Brown, R.A., et al., *Epidemiology and pathogenesis of diffuse obstructive coronary artery disease: the role of arterial stiffness, shear stress, monocyte subsets and circulating microparticles.* Ann Med, 2016: p. 1-12.
476. Thomas, G.M., et al., *Tissue factor expressed by circulating cancer cell-derived microparticles drastically increases the incidence of deep vein thrombosis in mice.* J Thromb Haemost, 2015. **13**(7): p. 1310-9.
477. van Es, N., et al., *Clinical Significance of Tissue Factor-Exposing Microparticles in Arterial and Venous Thrombosis.* Semin Thromb Hemost, 2015. **41**(7): p. 718-27.
478. Berezin, A., et al., *Predictive role of circulating endothelial-derived microparticles in cardiovascular diseases.* Clin Biochem, 2015. **48**(9): p. 562-8.
479. Mack, M., et al., *Transfer of the chemokine receptor CCR5 between cells by membrane-derived microparticles: a mechanism for cellular human immunodeficiency virus 1 infection.* Nat Med, 2000. **6**(7): p. 769-75.
480. Xu, R., et al., *Extracellular vesicle isolation and characterization: toward clinical application.* J Clin Invest, 2016. **126**(4): p. 1152-62.
481. Eggena, M.P., et al., *T cell activation in HIV-seropositive Ugandans: differential associations with viral load, CD4+ T cell depletion, and coinfection.* J Infect Dis, 2005. **191**(5): p. 694-701.
482. Baker, C.A., et al., *Profile of immunologic recovery in HIV-infected Ugandan adults after antiretroviral therapy.* AIDS Res Hum Retroviruses, 2007. **23**(7): p. 900-5.
483. Miro, J.M., et al., *Immune Reconstitution in Severely Immunosuppressed Antiretroviral-Naive HIV-1-Infected Patients Starting Efavirenz, Lopinavir-Ritonavir, or Atazanavir-Ritonavir Plus Tenofovir/Emtricitabine: Final 48-Week Results (The Advanz-3 Trial).* J Acquir Immune Defic Syndr, 2015. **69**(2): p. 206-15.
484. Paiardini, M. and M. Muller-Trutwin, *HIV-associated chronic immune activation.* Immunol Rev, 2013. **254**(1): p. 78-101.
485. Ghislain, M., et al., *Late Antiretroviral Therapy (ART) Initiation Is Associated with Long-Term Persistence of Systemic Inflammation and Metabolic Abnormalities.* PLoS One, 2015. **10**(12): p. e0144317.
486. Mata-Marin, J.A., et al., *Effect of antiretroviral therapy on inflammatory markers of endothelial dysfunction in HIV treatment-naive infected patients.* J Med Virol, 2013. **85**(8): p. 1321-6.
487. Graham, S.M., et al., *A prospective study of endothelial activation biomarkers, including plasma angiopoietin-1 and angiopoietin-2, in Kenyan women initiating antiretroviral therapy.* BMC Infect Dis, 2013. **13**: p. 263.
488. Ronsholt, F.F., et al., *Persistent inflammation and endothelial activation in HIV-1 infected patients after 12 years of antiretroviral therapy.* PLoS One, 2013. **8**(6): p. e65182.
489. Jong, E., et al., *The effect of initiating combined antiretroviral therapy on endothelial cell activation and coagulation markers in South African HIV-infected individuals.* Thromb Haemost, 2010. **104**(6): p. 1228-34.
490. Arildsen, H., et al., *Endothelial dysfunction, increased inflammation, and activated coagulation in HIV-infected patients improve after initiation of highly active antiretroviral therapy.* HIV Med, 2013. **14**(1): p. 1-9.
491. Torriani, F.J., et al., *Endothelial function in human immunodeficiency virus-infected antiretroviral-naive subjects before and after starting potent antiretroviral therapy: The ACTG (AIDS Clinical Trials Group) Study 5152s.* J Am Coll Cardiol, 2008. **52**(7): p. 569-76.
492. Piconi, S., et al., *Atherosclerosis is associated with multiple pathogenic mechanisms in HIV-infected antiretroviral-naive or treated individuals.* AIDS, 2013. **27**(3): p. 381-9.
493. Mugyenyi, P., et al. *Routine versus clinically driven laboratory monitoring of HIV antiretroviral therapy in Africa (DART): a randomised non-inferiority trial*. Lancet, 2010. 123-31.

494. Benjamin, L.A., et al., *HIV, antiretroviral treatment, hypertension, and stroke in Malawian adults: A case-control study.* Neurology, 2016. **86**(4): p. 324-33.
495. Zanni, M.V., et al., *Effects of Antiretroviral Therapy on Immune Function and Arterial Inflammation in Treatment-Naive Patients With Human Immunodeficiency Virus Infection.* JAMA Cardiol, 2016. **1**(4): p. 474-80.
496. Bestawros, M., et al., *Increased systemic inflammation is associated with cardiac and vascular dysfunction over the first 12 weeks of antiretroviral therapy among undernourished, HIV-infected adults in Southern Africa.* J AIDS Clin Res, 2015. **6**(3).
497. Kim, J., et al., *Brachial-ankle pulse wave velocity is a strong predictor for mortality in patients with acute stroke.* Hypertension, 2014. **64**(2): p. 240-6.
498. Emu, B., et al., *Composition and function of T cell subpopulations are slow to change despite effective antiretroviral treatment of HIV disease.* PLoS One, 2014. **9**(1): p. e85613.
499. Wada, N.I., et al., *The effect of HAART-induced HIV suppression on circulating markers of inflammation and immune activation.* AIDS, 2015. **29**(4): p. 463-71.
500. Hey-Cunningham, W.J., et al., *Early antiretroviral therapy with raltegravir generates sustained reductions in HIV reservoirs but not lower T-cell activation levels.* AIDS, 2015. **29**(8): p. 911-9.
501. 2016 [cited 2016 2nd October]; Available from: http://www.ctu.mrc.ac.uk/news/103986/reality_trial_results.
502. Hatano, H., et al., *A randomized controlled trial assessing the effects of raltegravir intensification on endothelial function in treated HIV infection.* J Acquir Immune Defic Syndr, 2012. **61**(3): p. 317-25.
503. Andrade, A., et al., *Early HIV RNA decay during raltegravir-containing regimens exhibits two distinct subphases (1a and 1b).* AIDS, 2015. **29**(18): p. 2419-26.
504. Lin, S.J., et al., *Azithromycin modulates immune response of human monocyte-derived dendritic cells and CD4+ T cells.* Int Immunopharmacol, 2016. **40**: p. 318-326.
505. Porter, J.D., et al., *Identification of novel macrolides with antibacterial, anti-inflammatory and type I and III IFN-augmenting activity in airway epithelium.* J Antimicrob Chemother, 2016. **71**(10): p. 2767-81.
506. Mahan, C.S., et al. *Tuberculosis treatment in HIV infected Ugandans with CD4 counts>350 cells/mm reduces immune activation with no effect on HIV load or CD4 count*. PLoS One, 2010. e9138.
507. Wyndham-Thomas, C., et al., *Immune activation by Mycobacterium tuberculosis in HIV-infected and -uninfected subjects.* J Acquir Immune Defic Syndr, 2016.
508. Prodger, J.L., et al., *Schistosoma mansoni Infection in Ugandan Men Is Associated with Increased Abundance and Function of HIV Target Cells in Blood, but Not the Foreskin: A Cross-sectional Study.* PLoS Negl Trop Dis, 2015. **9**(9): p. e0004067.
509. Kassu, A., et al., *Role of incidental and/or cured intestinal parasitic infections on profile of CD4+ and CD8+ T cell subsets and activation status in HIV-1 infected and uninfected adult Ethiopians.* Clin Exp Immunol, 2003. **132**(1): p. 113-9.
510. Froebel, K., et al., *Activation by malaria antigens renders mononuclear cells susceptible to HIV infection and re-activates replication of endogenous HIV in cells from HIV-infected adults.* Parasite Immunol, 2004. **26**(5): p. 213-7.
511. Gupta, S.K., et al., *Effects of switching from efavirenz to raltegravir on endothelial function, bone mineral metabolism, inflammation, and renal function: a randomized, controlled trial.* J Acquir Immune Defic Syndr, 2013. **64**(3): p. 279-83.
512. Bwakura-Dangarembizi, M., et al., *A randomized trial of prolonged co-trimoxazole in HIV-infected children in Africa.* N Engl J Med, 2014. **370**(1): p. 41-53.
513. Crook, A.M., et al., *Tuberculosis incidence is high in HIV-infected African children but is reduced by co-trimoxazole and time on antiretroviral therapy.* BMC Med, 2016. **14**: p. 50.

514. Davis, N.L., et al., *Impact of daily cotrimoxazole on clinical malaria and asymptomatic parasitemias in HIV-exposed, uninfected infants.* Clin Infect Dis, 2015. **61**(3): p. 368-74.
515. Suthar, A.B., et al., *Co-trimoxazole prophylaxis in adults, including pregnant women, with HIV: a systematic review and meta-analysis.* Lancet HIV, 2015. **2**(4): p. e137-50.
516. Vazquez-Castellanos, J.F., et al., *Altered metabolism of gut microbiota contributes to chronic immune activation in HIV-infected individuals.* Mucosal Immunol, 2015. **8**(4): p. 760-72.
517. Mutlu, E.A., et al., *A compositional look at the human gastrointestinal microbiome and immune activation parameters in HIV infected subjects.* PLoS Pathog, 2014. **10**(2): p. e1003829.
518. Koethe, J.R., et al., *From Wasting to Obesity: The Contribution of Nutritional Status to Immune Activation in HIV Infection.* J Infect Dis, 2016. **214 Suppl 2**: p. S75-82.
519. Damouche, A., et al., *Adipose Tissue Is a Neglected Viral Reservoir and an Inflammatory Site during Chronic HIV and SIV Infection.* PLoS Pathog, 2015. **11**(9): p. e1005153.
520. Dave, J.A., et al., *Anti-Retroviral Therapy Increases the Prevalence of Dyslipidemia in South African HIV-Infected Patients.* PLoS One, 2016. **11**(3): p. e0151911.
521. Caulk, A.W., et al., *Efavirenz treatment causes arterial stiffening in apolipoprotein E-null mice.* J Biomech, 2015. **48**(10): p. 2176-80.
522. Orden, S., et al., *Efavirenz induces interactions between leucocytes and endothelium through the activation of Mac-1 and gp150,95.* J Antimicrob Chemother, 2014. **69**(4): p. 995-1004.
523. Jamaluddin, M.S., et al., *Non-nucleoside reverse transcriptase inhibitor efavirenz increases monolayer permeability of human coronary artery endothelial cells.* Atherosclerosis, 2010. **208**(1): p. 104-11.
524. Gleason, R.L., Jr., et al., *Current Efavirenz (EFV) or ritonavir-boosted lopinavir (LPV/r) use correlates with elevate markers of atherosclerosis in HIV-infected subjects in Addis Ababa, Ethiopia.* PLoS One, 2015. **10**(4): p. e0117125.
525. Mouton, J.P., K. Cohen, and G. Maartens, *Key toxicity issues with the WHO-recommended first-line antiretroviral therapy regimen.* Expert Rev Clin Pharmacol, 2016: p. 1-11.
526. Casado, J.L., *Renal and Bone Toxicity with the Use of Tenofovir: Understanding at the End.* AIDS Rev, 2016. **18**(2): p. 59-68.
527. De Pablo, C., et al., *Differential effects of tenofovir/emtricitabine and abacavir/lamivudine on human leukocyte recruitment.* Antivir Ther, 2012. **17**(8): p. 1615-9.
528. Charakida, M., et al., *Increased arterial stiffness in HIV-infected children: risk factors and antiretroviral therapy.* Antivir Ther, 2009. **14**(8): p. 1075-9.
529. Koblavi-Deme, S., et al., *Changes in levels of immune activation and reconstitution markers among HIV-1-infected Africans receiving antiretroviral therapy.* AIDS, 2003. **17 Suppl 3**: p. S17-22.
530. Hsu, D.C. and I. Sereti, *Serious Non-AIDS Events: Therapeutic Targets of Immune Activation and Chronic Inflammation in HIV Infection.* Drugs, 2016. **76**(5): p. 533-49.
531. Friedland, G., G.J. Churchyard, and E. Nardell, *Tuberculosis and HIV coinfection: current state of knowledge and research priorities.* J Infect Dis, 2007. **196 Suppl 1**: p. S1-3.
532. Yukl, S.A., et al., *Effect of Raltegravir-containing Intensification on HIV Burden and T Cell Activation in Multiple Gut Sites of HIV+ Adults on Suppressive Antiretroviral Therapy.* AIDS, 2010. **24**(16): p. 2451-60.
533. Funderburg, N.T., et al., *Rosuvastatin reduces vascular inflammation and T-cell and monocyte activation in HIV-infected subjects on antiretroviral therapy.* J Acquir Immune Defic Syndr, 2015. **68**(4): p. 396-404.
534. Guerin, A.P., et al., *Impact of aortic stiffness attenuation on survival of patients in end-stage renal failure.* Circulation, 2001. **103**(7): p. 987-92.
535. Piconi, S., et al., *Hydroxychloroquine drastically reduces immune activation in HIV-infected, antiretroviral therapy-treated immunologic nonresponders.* Blood, 2011. **118**(12): p. 3263-72.

536. Tanaka, Y. and E. Martin Mola, *IL-6 targeting compared to TNF targeting in rheumatoid arthritis: studies of olokizumab, sarilumab and sirukumab.* Ann Rheum Dis, 2014. **73**(9): p. 1595-7.
537. Sznol, M. and L. Chen, *Antagonist Antibodies to PD-1 and B7-H1 (PD-L1) in the Treatment of Advanced Human Cancer.* Clin Cancer Res, 2013. **19**(5): p. 1021-1034.