

Inflammation and Immune Activation in HIV infection

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Declaration

The work presented in this thesis was completed by Dr Elizabeth Hamlyn under the supervision of Dr Sarah Fidler and Professor Myra McClure at Imperial College London. Statistical methods were carried out by Dr Elizabeth Hamlyn with advice from MRC statisticians and Imperial statistical advisory service. Blinded SPARTAC statistical analyses were performed at the MRC Clinical Trials Unit with input and discussion from Elizabeth Hamlyn. Flow cytometry was carried out as a joint project between Elizabeth Hamlyn and Stephen Hickling, PhD student at Oxford University. ELISAs for sTF, ICAM-1 and LPS activity were analysed by Kiran Palmer and Elizabeth Hamlyn at the Thrombosis and Vascular Biology Unit, St Thomas' Hospital.

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Abstract

Although widespread availability of antiretroviral therapy (ART) has markedly enhanced survival in HIV-1 infected individuals, its long-term use and cessation has been linked to an increase in non-AIDS morbidity and mortality. The pathogenesis of these complications is thought to reflect excessive immune activation and inflammation. The SMART trial showed that in chronic HIV-1 infection, inflammatory and coagulation biomarkers were predictive of all cause mortality, and that stopping ART lead to a rise in these biomarkers. The impact of interrupting ART started in primary HIV-1 infection (PHI) on these biomarkers is unknown. This thesis examines the effect of different stages of HIV-1 infection on markers of inflammation, coagulation and immune activation, and focuses on the effects of short-course ART in participants enrolled in the SPARTAC trial; a blinded RCT investigating the role of short-course ART versus no therapy in PHI.

Baseline parameters associated with biomarkers of inflammation, coagulation, immune activation, endothelial activation and microbial translocation, and the effect of starting and stopping ART on IL-6 and D-dimer levels, were examined in SPARTAC participants. Viral dynamics on treatment discontinuation were compared in PHI (SPARTAC) to chronic infection (SMART).

The majority of biomarkers were raised in HIV-1 infected individuals compared to healthy controls and highly correlated with HIV RNA levels. Viral and host factors, including age, HLA type and viral tropism, determined levels of T-cell activation. IL-6 and D-dimer fell significantly on commencing ART but rebounded to baseline levels within 4 weeks of stopping. Viral rebound after stopping ART initiated in PHI was lower than that observed in chronic infection.

This work highlights factors associated with immune activation and inflammation in PHI, which could potentially be used to identify individuals at high risk of disease progression. Immune activation and inflammation are influenced by direct effects of the virus, ART and other variables. The clinical impact of these findings warrants further investigation.

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Abbreviations used in this thesis

AIDS	Acquired Immune Deficiency Syndrome
ART	Antiretroviral therapy
APC	Antigen presenting cells <i>or</i> Allophycocyanin
BMI	Body mass index
CCL2	Chemokine (C-C motif) ligand 2
CCR5	Cysteine-cysteine receptor 5
CD4	CD4 T-lymphocyte count
CI	Confidence interval
CMV	Cytomegalovirus
CRP	C-reactive protein
CTL	Cytotoxic lymphocyte
CVD	Cardiovascular disease
CXCR4	Cysteine-X-cysteine receptor 4
EBV	Epstein-Barr Virus
EndoCAb	Endotoxin core antibody
ELISA	Enzyme-linked immunosorbant assay
GALT	Gut-associated lymphoid tissue
GI	Gastro-intestinal
gp	Glycoprotein
HAART	Highly active antiretroviral therapy
HIV-1	Human Immunodeficiency virus type 1
HIV-2	Human Immunodeficiency virus type 2
HLA	Human leukocyte antigen
hs-CRP	High-sensitivity C-reactive protein
ICAM-1	Inter-Cellular Adhesion Molecule 1
IFN- γ	Interferon-gamma
IL-6	Interleukin 6
IMT	Intima medial thickness
IQR	Interquartile range
L	Litre
LBP	Lipopolysaccharide binding protein
LCMV	Lymphocytic choriomeningitis virus
LPS	Lipopolysaccharide

LTNP	Long-term-non-progressor
mg	Milligram
MFI	Median Fluorescent Intensity
MHC	Major histocompatibility complex
ml	Millilitre
MMU	IgM median-units
MSM	Men who have sex with men
MRC	Medical Research Council
NAAT	Nucleic acid amplification test
ng	Nanogram
NRTI	Nucleoside reverse transcriptase inhibitor
NNRTI	Non-nucleoside reverse transcriptase inhibitor
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed death 1
pg	Picogram
PHI	Primary HIV-1 infection
PI	Protease inhibitor
POCT	Point-of-care test
PR	Protease
RCT	Randomised Controlled Trial
R5	CCR5 using virus
R5/X4	Dual tropic virus using R5 and X4
RT	Reverse transcriptase
sCD14	Soluble CD14
SMART	Strategies for Management of Anti-Retroviral Therapy
SPARTAC	Short Pulse AntiRetroviral Therapy at HIV-1 seroConversion
SIV	Simian Immunodeficiency virus
sTF	Soluble tissue factor
sTNFR2	Soluble tumour necrosis factor receptor- α type II
TNF- α	Tumour necrosis factor alpha
μ l	Microlitre
VCAM-1	Vascular cell adhesion molecule 1
VL	HIV Viral load
X4	CXCR4 using virus

Chapter 1: Introduction

In 2009, the United Nations estimated 33.4 million people worldwide to be living with Human Immunodeficiency Virus type-1 (HIV-1) infection, of whom 2.7 million were newly infected (UNAIDS 2009). The use of combination antiretroviral therapy (ART) has led to a dramatic increase in life expectancy and quality of life amongst HIV-1 infected individuals (Hogg 2008; Palella, et al. 1998), however, overall mortality rates for ART treated individuals are still higher than in the general population (Cockerham, et al. 2010; Lewden, et al. 2007). Where AIDS-related mortality has continued to decline, non AIDS-related morbidity and mortality has increased, resulting in heightened attention to conditions that have not traditionally been considered to be HIV-1 related, including cardiovascular disease (CVD), cancers, and liver disorders (Lau, et al. 2007).

This thesis examines factors associated with immune activation and inflammatory responses in HIV-1 infection, and discusses the role of inflammation and immune activation in driving both HIV-1 related and non-AIDS disease processes in HIV-1 infected individuals. In particular this thesis will focus on inflammation and immune activation in Primary HIV-1 infection (PHI) and the effect of short-course ART in this setting.

1.1: HIV-1 biology

Structure and genome

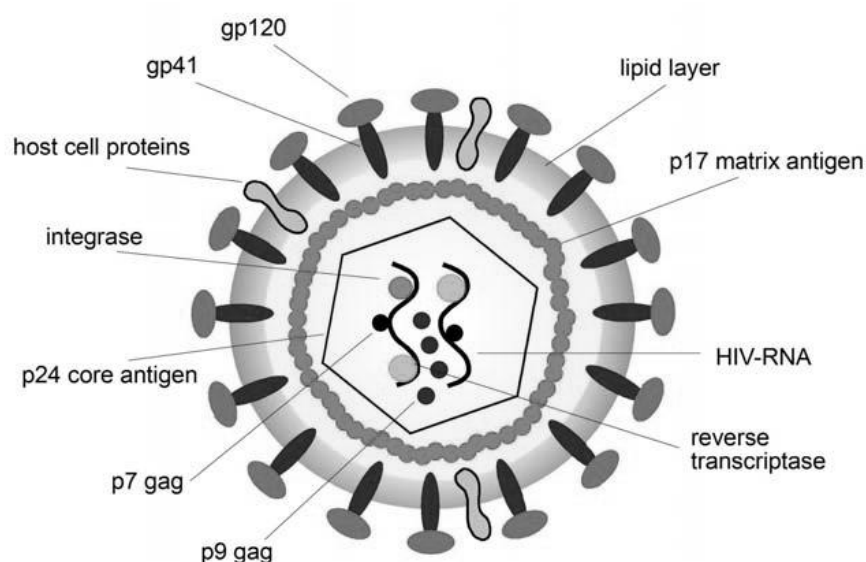
The HIV-1 virus is a retrovirus belonging to the genus Lentivirus. Lentivirus infections are typically characterised by a long latent period, a chronic course of disease and persistent viral replication. HIV-1 and HIV-2 are closely related to the Simian Immunodeficiency virus (SIV), although HIV-2 more closely resembles the strain of SIV seen in Sooty Mangabeys (SIV_{smm}), whereas HIV-1 resembles the more pathogenic strain of SIV seen in chimpanzees (SIV_{cpz}).

The structure of the HIV-1 virus is shown in Figure 1.1. HIV-1 has a diameter of 100nm and is composed of two copies of single-stranded RNA enclosed by a conical capsid composed of viral protein p24. The single-stranded RNA is part of a protein-nucleic acid complex and is tightly bound to a nucleocapsid protein, p7, and enzymes necessary for viral replication including reverse transcriptase (RT), protease (PR), and integrase. The capsid is surrounded by a matrix composed of the viral protein p17 ensuring the integrity of the virion particle. The virus is surrounded by a lipoprotein viral envelope that is composed of two phospholipid layers derived from human cell membrane during the process of viral budding from the cell. The viral envelope incorporates proteins from the host cell and 72 glycoprotein complexes, consisting of trimers of an external

glycoprotein gp120 loosely bound to a stem consisting of three gp41 molecules which span the membrane and anchor the structure into the viral envelope. This glycoprotein complex, known as Env, enables the virus to attach to and fuse with target cells to initiate viral replication (Hoffmann and Rockstroh 2009).

The RNA genome consists of nine genes encoding 16 viral proteins. Three genes are integral for viral replication: *gag* (group antigen), which codes for the nucleocapsid, *env* (envelope) coding for membrane glycoproteins, and *pol* (polymerase) coding for viral enzymes RT, PR and integrase. In addition there are two regulatory genes, *rev* and *tat*, and four accessory genes: *vif*, *vpu*, *vpr*, and *nef* (Hoffmann and Rockstroh 2009).

Figure 1.1. Structure of HIV-1



Schematic representation of an HIV-1 virion showing the nucleocapsid, containing core proteins and viral enzymes, surrounded by the lipoprotein envelope. Reproduced from HIV-1 2009, Medizin Fokus Verlag, Hamburg (Hoffmann and Rockstroh 2009)

Replication cycle

The CD4 receptor is expressed on the cell surface of T-lymphocytes, on T-cell precursors within the bone marrow and thymus, and on monocytes and macrophages, eosinophils, dendritic cells and microglial cells of the central nervous system (CNS). Binding of the viral surface protein gp120 to the CD4 receptor induces a structural change in the envelope complex also allowing interaction with a host cell chemokine coreceptor, either cysteine-cysteine receptor 5 (CCR5) or cysteine-X-cysteine receptor 4 (CXCR4), necessary for viral entry into the cell (Hoffmann and Rockstroh 2009). In early infection the majority of viral strains, termed R5 or M-tropic strains, use the CCR5 (R5) receptor, primarily expressed on activated T-cells, macrophages, dendritic cells and microglia. In the later stages of disease about 50% of individuals undergo a shift in viral tropism to CXCR4 (X4 or T-tropic) or mixed R5/X4 (dual tropic) strains of virus (Karlsson, et al. 2005). The CXCR4 receptor is expressed mainly on naïve T-cells. The binding of gp120 induces a conformational change in the envelope protein allowing gp41 to insert into the target cell membrane, resulting in fusion of the viral and cell membranes and allowing viral entry into the cell (Chan and Kim 1998). After target cell entry, the RNA genome is reverse-transcribed into unintegrated proviral DNA by the use of viral RT. The provirus is subsequently transported into the nucleus and integrated into the host cell genome by viral integrase. Following integration, production of viral proteins takes place in the cytoplasm followed by assembly of new virions at the cell surface. HIV-1 proteases cleave polyproteins into individual functional HIV-1 proteins and enzymes, allowing generation of infectious viral particles and budding through the host cell membrane. Maturation either occurs in the forming bud or in the immature virion after budding.

Viral diversification

The process of reverse transcription lacks a proof-reading mechanism and results in a high rate of nucleotide sequence changes. On average, 1-10 errors occur per genome and per round of replication (Hoffmann and Rockstroh 2009), and this, coupled with the ability to undergo recombination, leads to a high mutation rate. Escape mutants allow the virus to evade the host immune response, contributing to the long-term failure of the virus to be contained by the immune system. This results in a highly variable population of viruses (quasi-species) containing few identical sequences. Where there is selective pressure due to specific ART agents in the absence of complete viral suppression, this high mutation rate enables development of resistance to ART.

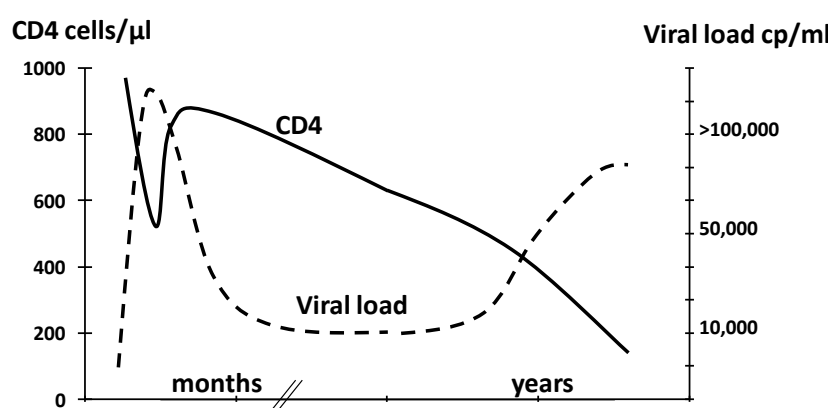
Natural history

The natural history of untreated HIV-1 infection consists of a primary phase of infection lasting up to six months and characterised by extremely high HIV-1 plasma viral load (VL) and a profound decrease in CD4 cell count, followed by a clinically latent phase usually lasting between 3-10 years. During the chronic phase of disease patients may have few clinical symptoms, despite persistent viral replication in blood and lymphoid tissue leading to progressive decline in CD4 cell count.

Progressive immunosuppression leads to susceptibility to opportunistic infection or HIV-1 related malignancy, resulting in a diagnosis of AIDS. AIDS is defined as having one or more of a list of many AIDS defining illnesses, or in the American classification system, a CD4 cell count less than 200 cells/ μ l (CDC 1993). It has been estimated that CD4 cells are lost at an average rate of 67 cells/ μ l per year (Lodi, et al. 2010). The natural history of HIV-1 is demonstrated in Figure 1.2.

The CD4 cell count is the most significant predictor of disease progression and survival. The overall risk of AIDS increases dramatically once the CD4 cell count drops below 200 cells/ μ l, however those with a CD4 cell count <350 cells/ μ l are still at increased risk compared to those with CD4 350-500 cells/ μ l. The predicted risk of developing an AIDS defining illness within a 6 month period has been estimated to be 1.3%, 2.3%, 4.1%, 8.5% and 23.1% in individuals with a CD4 cell count of 450, 350, 250, 150 and 50 cells/ μ l respectively, based on a 35 year old individual with VL of 100,000 cp/ml (Phillips and Pezzotti 2004). The level of VL is also an important predictor of disease progression (Mellors, et al. 1997; Mellors, et al. 1996; Phillips and Pezzotti 2004).

Figure 1.2. Natural history of untreated HIV-1 Infection



Schematic representation of the natural course of untreated HIV-1 infection. After an initial peak during primary infection, VL stabilizes at a set-point, before rising again in advanced disease. After an initial decline, CD4 cell count gradually declines over a period of years

Factors associated with HIV-1 disease progression

Substantial variation in HIV-1 progression occurs between individuals. Older subjects experience faster rates of CD4 cell decline and there is a significant correlation between age, higher VL and lower CD4 cell count (Phillips and Pezzotti 2004; Touloumi, et al. 1998). Approximately 3-5% of HIV-1 infected individuals are termed long-term non progressors (LTNP), usually defined as individuals who do not experience CD4 cell decline for at least 7 years after infection (Okulicz, et al. 2009). “Elite Controllers”, who naturally control viral replication to HIV RNA <50 copies/ml in the absence of ART, represent 0.1-2% of HIV-1 infected individuals in Western cohorts (Grabar, et al. 2009; Okulicz, et al. 2009; Sajadi, et al. 2009). A high proportion, but not all, elite controllers are LTNP (Grabar, et al. 2009; Sajadi, et al. 2009).

Several host and viral factors predict HIV-1 disease progression. The genetic human leukocyte antigen (HLA) background of an individual influences the quality and the efficiency of the immune response against HIV-1 infection (Altfeld, et al. 2003). Individuals with homozygous HLA types have faster HIV-1 progression than those heterozygous for HLA, possibly due to reduced host genetic diversity allowing an increased propensity for HIV-1 viral escape from cytotoxic lymphocyte (CTL) responses (Carrington and O'Brien 2003). Several individual HLA alleles have been associated with either faster or slower progression to AIDS (O'Brien, et al. 2001). HLA B*57 and B*27 are over-represented amongst cohorts of LTNP and elite controllers (Migueles, et al. 2000; Sajadi, et al. 2009), whereas HLA B*35 has been associated with fast progression (Klein, et al. 1994). Altfeld et al. demonstrated that individuals expressing HLA B*57 present significantly less frequently with symptomatic seroconversion illness, mount more numerous HIV-1 specific T-cell responses restricted by the protective HLA alleles and exhibit better control of viral replication following acute infection (Altfeld, et al. 2003; Altfeld, et al. 2006). Selection of escape variants with a significant fitness cost leads to lower VL in individuals with these HLA types (Martinez-Picado, et al. 2006; Miura, et al. 2009). Goepfert et al. (2008) showed that CTL from patients with B*57 force the virus to develop mutations in *gag* that enable it to escape from the CTL response, resulting in reduced replicative competence and lower VL. If the virus is transmitted to another individual with a different HLA type it will mutate back to the original genotype and regain its full replicative capacity (Goepfert, et al. 2008). Enhanced CTL cytolytic ability may also contribute to enhanced immunological control in individuals with protective HLA alleles (Migueles, et al. 2008).

An inherited deletion in the host CCR5 gene (delta 32 mutation) confers a significant protective effect against acquisition of HIV-1 infection and a survival advantage in those who acquire the virus (Michael, et al. 1997; Smith, et al. 1997). The majority of individuals with homozygous deletion in

CCR5 are resistant to HIV-1 infection (Liu, et al. 1996a), although transmissions with CXCR4 using viral variants have been reported (O'Brien, et al. 1997). Heterozygous individuals are frequently encountered amongst LTNP cohorts and have slower progression to AIDS (Dean, et al. 1996).

Viral factors also influence outcome. Individuals with CXCR4 or dual R5/X4 strains show more rapid clinical and immunological deterioration compared to individuals with CCR5 tropic virus (Connor, et al. 1997; Koot, et al. 1993; Schuitemaker, et al. 1992; Tersmette, et al. 1989). Deletions in the Nef gene (Deacon, et al. 1995; Kirchhoff, et al. 1995) and other viral polymorphisms (Alexander, et al. 2000), have been described amongst cohorts of LTNP and are associated with reduced disease progression.

1.2: Primary HIV-1 infection (PHI)

Immunological and virological events during PHI

Primary HIV-1 infection refers to the initial phase up to 6 months following HIV-1 acquisition and is characterised by a transient period of very high level viral replication with consequent destruction of memory CD4 T-cells. Most HIV-1 infections worldwide occur following sexual exposure, and over 75% of mucosally transmitted infections are initiated by a single virus (Keele and Derdeyn 2009). Infectious founder viruses preferentially infect CCR5+CD4 T-cells, and established infection following sexual transmission probably arises from a single focus of infected mucosal cells. Following transmission of the virus, there is a period of 10 days before HIV RNA becomes detectable in the plasma, known as the eclipse phase, where active HIV-1 replication occurs at the site of transmission (McMichael, et al. 2010). This is followed by rapid dissemination to local lymphoid tissues, and subsequent trafficking of virus throughout entire body. This "ramp-up" period is characterised by massive uncontrolled viral replication in the blood and lymphoid tissues, particularly the gut-associated lymphoid tissue (GALT), resulting in irreversible immunological damage, high levels of free HIV-1 virions circulating in the plasma, and dissemination to sanctuary reservoirs. The preferential site for HIV-1 replication occurs in the gut lamina propria and submucosa due to the predominant population of activated CCR5+CD4 memory cells providing prime targets for the virus, and over one-half of effector CD4 T-cell reserves in the GALT are irreversibly lost during acute infection (Mattapallil, et al. 2005). CD4 activation facilitates integration of HIV DNA into the host genome and lymphatic tissue provides an optimal environment for HIV-1 replication due to close cell contact between CD4 T-cells, antigen presenting cells (APC) and infectious virions in the follicular dendritic cell network, optimised by the local cytokine rich environment (Hoffmann and Rockstroh 2009).

Plasma viraemia increases to reach a peak of several million copies of HIV RNA/ml 21-28 days after initial HIV-1 infection and is accompanied by a substantial decrease in CD4 T-cell numbers, which may return later to near-normal levels in the blood but not in the GALT. The very high levels of HIV-1 viraemia are normally short lived due to the appearance of initial cellular HIV-1 specific T-cell responses which exert an immune pressure on the virus, leading to the selection of multiple escape variants and the generation of a diverse viral population (McMichael, et al. 2010; Shacklett and Anton 2010). The VL thereafter decreases over 12-20 weeks allowing the development of a viral set-point that is maintained by a balance between viral turnover and host immune responses (McMichael, et al. 2010). The viral set point, generally described as a quasi-steady state of viral replication occurring after the period of acute infection has resolved, varies between individuals and is prognostic of future disease course (de Wolf, et al. 1997; Lyles, et al. 2000; Mellors, et al. 1995).

Immune responses to PHI

Even prior to the development of detectable viraemia in PHI, increased levels of acute-phase proteins are detectable in the serum (McMichael, et al. 2010). Further acute-phase protein production is triggered by an intense cytokine storm which accompanies the rise in plasma viraemia. Elevations in plasma levels of multiple cytokines and chemokines occur, including rapid and transient elevations in interferon-alpha, a rapid and more sustained increases in tumour necrosis factor alpha (TNF- α) and Chemokine (C-C motif) ligand 2 (CCL2), more slowly-initiated elevations in additional pro-inflammatory factors including interleukin 6 (IL-6) and interferon-gamma (IFN- γ), and a late-peaking increase in the immunoregulatory cytokine IL-10 (Stacey, et al. 2009). This intense cytokine response to acute infection is much greater in magnitude to that observed in other viral infections, such as acute hepatitis B or C infections (Stacey, et al. 2009), indicating that the systemic cytokine response observed following acute infection is not a prerequisite for viral clearance, but may instead promote ongoing immune activation, viral replication and CD4 cell loss. A recent study showed that concentrations of various cytokines (IL-7, IL-12p40, IL-12p70, IFN- γ and IL-15) during acute infection better predicted viral set-point (defined as average of 3 VL measurements on 3 consecutive visits after a median of 47 weeks post-infection) than initial VL or CD4 cell count, and that plasma cytokine concentrations (IL-7, IL-1a, and eotaxin) during acute infection were associated with more rapid CD4 cell loss (Roberts, et al. 2010). The cellular sources of acute phase cytokines and chemokines have not been fully identified but include CCR5+CD4 T-cells, activated dendritic cells, monocytes, macrophages and natural killer cells, with HIV-1 specific T-cells also playing a later role (McMichael, et al. 2010)

The first T-cell responses to HIV-1 occur as viraemia approaches its peak, and peak 1-2 weeks later as the level of viraemia declines. The appearance of HIV-1 specific CD8 T-cells, and the peak in CD8 T-cell responses, is thought to be a key factor driving the development of viral immune escape variants and the decline in HIV RNA. The strong association between the rate of disease progression and different HLA class I alleles indicates that the interaction between the T-cell receptor of the CD8 T-cells and the HLA of the APC is a key element in the overall control of HIV-1 replication (Streeck and Nixon 2010). In PHI, the initial CD8 T-cell responses are directed against a narrow range of epitopes, whilst in chronic infection, an increase in the breadth and magnitude of the CTL response occurs, but is not accompanied by any increase in control of viral replication (Streeck and Nixon 2010). This suggests that the first CD8 T-cell responses are unique in their ability to efficiently suppress viral replication. Streeck et al. (2009) showed that the ability to mount a strong CD8 T-cell response during PHI is significantly associated with lower viral set-point, and that preservation of the initial CD8 T-cell immunodominance patterns from acute into the chronic phase of infection were significantly associated with slower CD4 T-cell decline (Streeck, et al. 2009).

Proliferation and activation of CTL responses are dependent on antigen specific CD4 T-cell help (Streeck and Nixon 2010). Vigorous CD4 T-cell responses in acute HIV-1 infection have been associated with subsequent control of viral replication (Rosenberg, et al. 1997). The contribution of CD4 T-helper responses against HIV-1 has been highlighted by studies demonstrating that viral escape occurs from CD4 T-cell targeted epitopes during PHI (Jones, et al. 2009; Rychert, et al. 2007). However, the exact contribution of CD4 T-cell responses to HIV-1 control is difficult to measure and the antiviral action is likely to be indirect through other effector cells.

Neutralising antibodies against autologous virus appear after the VL peak in PHI, approximately 12 weeks or longer after initial infection and, therefore, do not majorly contribute to the initial control of viral replication. Non-neutralising antibodies appear prior to this, but do not exert selective pressure or reduce VL (Frost, et al. 2008; Tomaras and Haynes 2009).

Establishment of a latent reservoir

During acute infection, the virus disseminates throughout the body and becomes trapped in the follicular dendritic cell network within lymphatic tissue, where macrophages and activated T-cells are the main targets of infection. During this time HIV-1 establishes a stable permanent viral reservoir of latently infected cells. The virus enters into quiescent T-cells where reverse transcription results in the accumulation of proviral HIV DNA. However, as cellular activation is necessary for integration of the proviral HIV DNA into the host cell genome, the virus persists in resting cells, resulting in a latent reservoir of virus. ART is unable to affect non-replicating proviruses, and latently infected memory CD4 T-cells are thought to be the major reservoir that contribute to rebound viraemia after cessation of ART (Siliciano and Siliciano 2006).

Clinical Features of PHI

After an incubation period which ranges from a few days to a few weeks, PHI presents as a transient symptomatic illness which may be manifest by a wide range of 'flu like symptoms including fever, fatigue, sore throat, maculopapular rash, myalgia, mouth ulcers, headache and lymphadenopathy. Symptoms are present in 40-90% of individuals, and typically last approximately 2 weeks, although durations of up to 10 weeks have been reported (Kassutto and Rosenberg 2004). Less common manifestations include development of opportunistic infection, and neurological presentations including aseptic meningitis, cranial nerve palsies and radiculopathy (Kassutto and Rosenberg 2004). The severity and duration of the acute illness predicts the rate of progression to AIDS (Lavreys, et al. 2006; Vanhems, et al. 1998). The diagnosis of HIV-1 infection may be easily overlooked due to the non-specific nature of symptoms, and in one UK study, approximately 50% of PHI diagnoses were missed on first presentation to a healthcare provider (Sudarshi, et al. 2008). A high degree of awareness and clinical suspicion is therefore required.

Diagnosis of PHI

Even in resource rich settings, uptake of HIV-1 testing remains disappointingly poor, with 27% of the HIV-positive population in the UK remaining unaware of their HIV-1 status in 2009 (HPA 2009). The symptomatic phase of PHI coincides with the development of HIV-1 specific immune responses, but occurs prior to development of HIV-1 specific antibodies. 1st and 2nd generation HIV-1 tests detect anti-p24 IgG antibodies, with positive result only after HIV-1 seroconversion. Third generation tests detect IgM and will give an earlier positive result (Hecht, et al. 2002). UK guidelines recommend that the first-line assay for detection of HIV-1 infection should be a 4th generation test, which simultaneously tests for HIV-1 antibody in combination with p24 antigen, reducing the time from

initial infection to testing positive to 1 month, 1–2 weeks earlier than current standard third-generation antibody tests (BHIVA, et al. 2008).

Current available 3rd generation point-of-care tests (POCT), using fingerprick or mouth swab sampling, measure detectable antibody only, and have reduced specificity and sensitivity compared to 4th generation laboratory tests (Patel 2010; Winter, et al. 2006). Preliminary data on fourth-generation antigen/antibody POCT show high specificity and increased sensitivity for early diagnosis of HIV-1 infection compared to 3rd generation POCT, however these assays are not standard care and further validation is required in clinical settings (Beelaert and Fransen 2010).

The most sensitive test for detecting PHI is by detection of plasma HIV RNA, and nucleic acid amplification tests (NAAT) with a sensitivity of 50 copies/ml typically detect HIV-1 infection approximately 7 days before a p24 antigen test, and 12 days before a sensitive HIV-1 antibody test (Fiebig, et al. 2003). Current UK HIV-1 testing guidelines do not recommend HIV RNA quantitative assays for routine screening because of the possibility of false-positive results, but advocate their use where PHI is clinically suspected (BHIVA, et al. 2008). However the use of HIV RNA testing of pooled antibody-negative samples, followed by individual NAAT of positive specimens, maybe a cost-effective and effective way of diagnosing acute infection in targeted high-risk populations (Patel, et al. 2010).

Cross-sectional incidence assays capable of accurately differentiating primary from chronic infection are essential to estimate HIV-1 incidence at a public health level and to evaluate efficacy of prevention strategies, and have also be used to identify eligible subjects for targeted prevention strategies or enrolment into clinical trials evaluating early treatment strategies. The serologic testing algorithm for recent HIV-1 seroconversion (STARHS) assay, or detuned assay, combines a high-sensitivity and a low-sensitivity enzyme immune assay at the same time, differentiating early from chronic infection. These assays have a high false incident rate in those with established HIV-1, which is reduced when combined with clinical data (Fisher, et al. 2007). A further method for identifying recently acquired HIV-1 infection is the use of avidity testing, measuring strength of antibody–antigen binding which rises with established infection, although results are affected by advanced disease and should be interpreted in the context of other clinical parameters (Chawla, et al. 2007). Novel cross-sectional incidence assays are in development.

Onward transmission in PHI

The high level plasma VL during PHI is reflected in the genital tract secretions and confers a marked enhanced risk of onward transmission to sexual partners (Morrison, et al. 2010; Pilcher, et al. 2007). Specific characteristics of recently transmitted viral variants appear to make them particularly efficient at onward transmission (Keele and Derdeyn 2009). CCR5-coreceptor utilizing variants are preferentially transmitted, corresponding with the initial cell types first infected in the genital mucosa (Margolis and Shattock 2006). Transmission of CXCR4 utilizing HIV-1 variants has been described, although this is uncommon (Brumme, et al. 2005). The enhanced transmissibility of recently acquired variants has been highlighted by recent data from primate models showing that in SIV infected Rhesus macaques, a significantly lower number of infectious virions are required to infect a new host when the source virus was isolated from an animal in the ramp-up phase of acute infection, compared with virus isolated from macaques who had reached viral set-point (Ma, et al. 2009). Data from observational studies in humans suggest that there is a similar enhanced risk of onward transmission from individuals with PHI. Population based studies indicate that the highest rate of HIV-1 transmission per coital act is during PHI, even after adjustment for VL (Wawer, et al. 2005), and mathematical models estimate that in high mixing populations, individuals with PHI disproportionately contribute to driving the HIV-1 epidemic (Miller, et al. 2010). Phylogenetic analyses demonstrate a clustering of infections during PHI (Pao, et al. 2005) and early infections account for about 50% of new sexual transmission in some populations (Brenner, et al. 2007). Thus identification and targeting of individuals with PHI to limit HIV-1 transmission could potentially confer a significant benefit at a population level.

Role of treatment in PHI and use of short-course ART

Early events following acute HIV-1 infection are likely to influence later disease course and outcome. It has been proposed that the 'die is cast' for long-term clinical outcome at the time of acute infection, and PHI therefore represents a unique window of opportunity where intervention may confer longstanding benefits (Centlivre, et al. 2007). Early treatment with ART during PHI may preserve HIV-1 specific T-cell responses, reduce the number of infected cells and limit the size of the viral reservoir, conferring enhanced control of viral replication when therapy is stopped. The aim of early ART in PHI is therefore to delay disease progression and subsequently defer the need for long-term ART. An additional goal of treatment may be to reduce onward viral transmission of the virus.

The strategic use of short-course ART during PHI with the goal of limiting long-term disease progression has been supported by initial randomised controlled trials (RCT) (Kinloch-De Loes, et al.

1995) and observational studies which have provided encouraging support for the role of early ART initiated in PHI for long-term clinical benefit (Fidler, et al. 2008). An early RCT, investigating zidovudine monotherapy for 6 months in individuals with PHI, showed beneficial rises in CD4 cell count amongst treated individuals, but no difference in VL between study groups. However, this regimen was not fully suppressive and data cannot, therefore, be extrapolated to studies of combination ART (Kinloch-De Loes, et al. 1995). Another study of 89 patients treated with 12 weeks of highly active ART (HAART) commenced an average of 1.5 months after seroconversion, compared with a matched untreated population of seroconverters from the CASCADE cohort, showed a significant decrease in the rate of CD4 cell decline in those receiving therapy (Fidler, et al. 2007).

Whether short-term treatment given at the time of early HIV-1 infection has an impact on the long-term viral set-point is unclear. Despite initial control of plasma viraemia reported in some studies (Hecht, et al. 2006; Rosenberg, et al. 2000; Steingrover, et al. 2008), durable long-term viral control following treatment in PHI was not observed in others (Desquilbet, et al. 2004; Fidler, et al. 2002; Hecht, et al. 2006; Kaufmann, et al. 2004; Koegl, et al. 2009; Streeck, et al. 2006), and few randomised studies have assessed the effect of short-term ART on viral set-point in PHI. The A5217 study, a RCT which aimed to investigate the effect of short-course ART in PHI on viral set-point, was discontinued early due to a high rate of CD4 cell decline across all treatment arms, so that the primary end-point of viral set-point could not be assessed due to initiation of long-term ART (Hogan C 2010). Lampe et al. compared data from the QUEST randomised study, where ART was given for an average of 2.6 years, with historical data from the CASCADE cohort of seroconverters. No difference in median VL was seen six months after stopping ART when compared with CASCADE 3 years following seroconversion, however 17.7% of QUEST patients had VL<1000 copies/ml compared with 10% CASCADE patients at a similar time point, suggesting some effect on lowering VL (Lampe, et al. 2007).

Studies of patients receiving short-course ART at PHI have demonstrated preservation of robust HIV-1 specific CD4 T-helper responses that are not detected during the chronic stage of HIV-1 infection, (Fidler, et al. 2002; Rosenberg, et al. 1997), however it is unknown whether these immunological observations confer a clinical benefit. To date there are no published studies which definitively address the role of ART in PHI on long-term clinical outcomes. This concept is currently under test in the Short Pulse AntiRetroviral Therapy at HIV-1 seroConversion (SPARTAC) study; an international RCT investigating the effect of short-term ART (12 or 48 weeks) versus no therapy commenced in PHI on long-term disease progression with a primary outcome measure of time to CD4 cell count <350 cells/mm³ or the initiation of clinically-indicated therapy (SPARTAC 2006).

In the absence of any demonstrated individual benefit, and pending the results of SPARTAC, ART in PHI should be given as part of a clinical trial or for clinical indications such as severe symptomatic illness, immune reconstitution in those with large CD4 cell count losses during the acute illness, development of opportunistic infection or development symptomatic CNS disease. It is hoped that the SPARTAC trial will definitively address the role of strategic use of short-course ART in PHI once it reports in mid 2011.

1.3: HIV-1 Immunopathogenesis

Mechanisms of immune dysfunction in HIV-1 infection

The mechanisms by which HIV-1 infection leads to the depletion of CD4 cells and subsequent immunodeficiency are not completely understood but include a) the direct infection of CD4 cells by the HIV-1 virus and their subsequent destruction through cytopathic and apoptotic mechanisms, b) generalised immune hyper-activation and c) immune dysfunction and exhaustion.

Direct viral Infection

HIV-1 preferentially infects activated CD4 cells, and as HIV-1 specific CD4 T-cells are among the first cells to be activated during HIV-1 infection, cells directed against the virus are at greatest risk of infection. Thus a continuous supply of target cells for the virus is maintained (Douek, et al. 2002; Grossman, et al. 2006). During acute infection, over one-half of all memory CD4 T-cells are destroyed by direct viral infection, and in particular, selective infection of CCR5 positive CD4 memory T-lymphocytes in the gut lamina propria and submucosa leads to massive depletion of CD4 T-cell reserves (Li, et al. 2005; Mattapallil, et al. 2005). Viral replication in lamina propria CD4 cells leads to direct CD4 cell death through cell lysis and through Fas-Fas-ligand mediated apoptotic pathways resulting in their preferential ablation (Li, et al. 2005).

During the chronic phase of disease, CD4 T-cell depletion continues to occur predominantly in the gut, however, at this time HIV-1 infects an apparently small proportion of CD4 T-cells directly, estimated at 1 in 100 to 1 in 1000, and thus indirect mechanisms account for the majority of T-cell losses (Brenchley, et al. 2004; Douek, et al. 2009).

HIV-associated immune activation

Chronic immune activation is a hallmark of HIV-1 infection and may be manifested by polyclonal B cell activation (Lane, et al. 1983), increased T-cell turnover (Hazenberg, et al. 2000), high levels of T-cell activation (Hazenberg, et al. 2003b), and raised levels of circulating cytokines and other pro-inflammatory mediators (Aziz, et al. 1999b; Molina, et al. 1989; Valdez and Lederman 1997).

The T-cell receptor on CD4 and CD8 T-cells cannot recognise antigen unless it is presented in association with a major histocompatibility complex (MHC) molecule, and recognises peptides bound to HLA molecules on APC. The stimulation of CD8 T-cell lymphocytes and formation of antigen specific CTL depend on presentation of an antigenic peptide together with class I MHC antigens, whereas CD4 T-cell activation depends on antigen presentation together with a class II MHC molecules. There is a strong selective pressure for pathogens that have mutated in such a way that they escape presentation by a MHC molecule. The effector functions of T-cells are determined by the array of effector molecules they produce. The CD4 effector T-cells mainly act through the production of cytokines and other proteins. They have a wide repertoire of effector activities, and after recognition of a pathogen presented by MHC class II, can differentiate into effector subsets with different immunological functions. The main subsets are Th1, Th2 and Th17 which activate their target cells and several regulatory T-cell subsets (T regs) that have inhibitory activity, limit immune activation and suppress T-cell responses. Once an effector T-cell has completed its differentiation it finds target cells displaying the MHC-peptide complex that it recognises. The CD8 CTL recognise and eliminate virus infected cells by releasing cytotoxic granules which trigger apoptosis. CTL also act by releasing cytokines such as IFN- γ and TNF- α .

Upregulation of CD38, a multifunctional transmembrane glycoprotein, occurs during early stages of T-cell activation (Giorgi and Detels 1989). Its expression correlates with other markers of cellular activation and is associated with increased cytokine production, increased CD4 cell proliferation and cell to cell adhesion (Savarino, et al. 2000). HLA-DR, a MHC class II antigen, is also upregulated on activated T-cells. Expression of HLA-DR increases on T-cells at HIV-1 seroconversion, and remains high but stable throughout disease progression (Giorgi and Detels 1989).

Although the presence of immune activation usually reflects a mounting immune response to a pathogen, it was noted early on that increased levels of T-cell activation were associated with poor prognosis in HIV-1 infected patients (Giorgi, et al. 1993). Numerous studies have now shown that the level of CD4 and CD8 T-cell activation in chronic HIV-1 infection is associated with HIV-1 disease progression (Giorgi, et al. 1993; Hazenberg, et al. 2003b; Liu, et al. 1997) and in some studies T-cell

activation has been shown to have stronger prognostic significance than CD4 cell count or VL (Giorgi, et al. 1999; Giorgi, et al. 2002; Liu, et al. 1997). The level of T-cell activation may be measured by assessing either the percentage of cells expressing activation markers of interest, or by the density per cell. Liu et al (1997) examined the prognostic value of several immunological markers in chronic HIV-1, including CD8 cell expression of CD38 and HLA-DR antigens, CD4 cell count and serum markers of immune activation, including neopterin, β 2-microglobulin and soluble tumour necrosis factor receptor- α type II (sTNFR2). Of these, elevated expression of CD38 on CD8 cells was the best predictive marker for subsequent AIDS diagnosis and death. Giorgi et al. (1999) showed that in advanced disease (CD4 cell count of <50 cells/ μ l), CD38 expression on both CD4 and CD8 T-cells was strongly predictive of progression to death, but that VL was not (Giorgi, et al. 1999). A further study showed that CD38 expression on CD8 T-cells to be an exceptionally strong predictive marker for disease progression after both short duration (median 2.8 years) and long duration (median 8.7 years) of HIV-1 infection, and that in later infection it was a stronger predictor of time to AIDS than either CD4 cell count or VL (Giorgi, et al. 2002). In a case control study comparing fast and slow progressors, increased CD38 expression on both CD4 and CD8 T-cells, and elevated proportion of CD8⁺ T-cells with dual expression of CD38 and HLA-DR, were all associated with rapid HIV-1 progression (Cao, et al. 2009). This study further showed a relative expansion of T regs identified as FoxP3⁺CD25⁺CD4⁺ T-cells during HIV-1 infection, which positively correlated with CD4⁺ T-cell activation among HIV-1 infected fast progressors and which were associated with disease progression. In contrast to the assumed role of T regs as the suppressors of activation, the authors concluded that these cells may have a negative impact on the immune control of HIV-1 infection (Cao, et al. 2009). The presence of FoxP3 expression in the CD4 T-cell population as a marker of severity of HIV-1 infection has been confirmed in other studies (Suchard, et al. 2010).

The pathogenic significance of immune activation has been highlighted in several animal models. In non-human primates, SIV infection of both the Sooty Mangabey and the Rhesus macaque results in high levels of SIV replication. However, in macaques high levels of immune activation occur resulting in progressive immunodeficiency, whereas Sooty Mangabeys display no increases in immune activation and rarely exhibit clinical disease, despite evident viral replication (Meythaler, et al. 2009; Silvestri, et al. 2003; Sumpter, et al. 2007). A similar observation comes from human studies of HIV-2 infection. HIV-2 is associated with lower levels of immune activation, and individuals with HIV-2 typically experience a more benign disease course compared to those with HIV-1 infection (Sousa, et al. 2002). The Nef protein from non-pathogenic SIV strains and from HIV-2 has a T-cell activation suppressing function which appears to have been lost in HIV-1 infection evolution (Schindler, et al. 2006).

Levels of T-cell activation correlate with plasma HIV RNA and following introduction of ART, a decline in plasma HIV RNA is accompanied by a reduction, but not normalization, in immune activation (Deeks, et al. 2004). However, several studies indicate that immune activation also occurs independently of direct antigenic stimulation resulting from HIV-1 viraemia. In transgenic mice, chronic immune activation persistently triggered through CD27-CD70 interactions, leads to the exhaustion of the naive T-cell pool, loss of immune responses, and susceptibility to opportunistic infections, indicating that this phenomenon triggers immunodeficiency in the absence of a viral infection (Tesselaar, et al. 2003). Elite controllers have higher levels of T-cell activation than those suppressed on ART, or than HIV-negative controls, and high levels of immune activation correlate with a lower CD4 cell count, suggesting that immune activation remains a pathogenic phenomenon and contributes to CD4 cell loss in the absence of detectable viraemia (Hunt, et al. 2008). In ART treated patients with sustained viral suppression, persistent T-cell activation is associated with lower CD4 T-cell gains (Hunt, et al. 2003). In keeping with this, individuals who have long-term asymptomatic HIV-1 infection despite high VLs, have significantly lower levels of activated T-cells than those with progressive disease (Choudhary, et al. 2007).

Immune activation in PHI

Few studies have focussed on levels of T-cell activation in PHI, a time when immunological events may have a marked impact on disease outcome. Hazenburg et al. (2003) assessed levels of T-cell activation, as measured by CD38% expression, HLA-DR% expression and dual expression of both markers, 12 months after seroconversion in a cohort of 97 individuals followed up for a median of 4 years. CD4 CD38% and dual CD8 CD38 HLA-DR% were predictive of progression to AIDS, however on adjusting for VL, only CD8 CD38 HLA-DR dual expression remained significant. When treated as time-dependant covariates, CD38 expression on CD8 and CD4 T-cells were predictive for disease progression, although on multivariate analysis only CD4 CD38% expression remained significant (Hazenberg, et al. 2003b). Another study by Deeks et al. (2004) assessed CD4 and CD8 CD38 expression in 68 untreated individuals within 12 months of seroconversion, and found that CD8 CD38 was independently associated with the rate of CD4 cell decline (Deeks, et al. 2004). It is clear from these studies that not all activation markers have equally predictive value, possibly reflecting their different roles in amplifying and maintaining immune responses.

Hazenburg et al. (2003) showed that levels of T-cell activation, even prior to seroconversion, predict HIV-1 disease progression. In a cohort of 56 individuals who were HIV-negative at the time of analysis but who seroconverted during a period of follow-up, higher proportions of CD70+CD4 T-cells before seroconversion were associated with a shorter AIDS free survival, and CD4 cell count <500

cells/ μ l prior to seroconversion was also associated with a significantly shorter median time to AIDS compared to those with CD4 cell count >500 cells/ μ l. The CD70 antigen is expressed on activated T-cells and controls for the magnitude and duration of T-cell responses. This study highlights that factors relating to the host immune system, even prior to HIV-1 acquisition, determine subsequent outcome upon infection with HIV-1.

Mechanisms driving HIV-1-mediated immune activation

The exact mechanism driving HIV-1 related immune activation is not fully understood. The first immune responses against HIV-1 generated in PHI are aimed at controlling the virus. The adaptive immune response to HIV-1 infection is a key driver of immune activation and persists into the chronic phase of HIV-1 infection. Chronic antigenic stimulation from HIV-1 viraemia, both directly and indirectly, leads to immune activation, but does not solely account for the full extent of immune activation seen. Antigenic stimulation from other infections may also contribute to immune activation, as demonstrated by studies showing accelerated SIV induced disease progression in macaques subjected to repeated immune stimulation via repeated injection of SIV independent antigens (Villinger, et al. 2001), and observations of reduced CD4 cell counts in association with raised levels of serum pro-inflammatory cytokines during intercurrent infections in humans (Anzala, et al. 2000). In addition, viruses such as cytomegalovirus (CMV) and Epstein-Barr virus (EBV) induce antigenic stimulation, particularly due to reactivation during the latter stages of HIV-1 infection.

A recent study showed that CD4 cell depletion results in CD4 cell proliferation, probably through the increased production of IL-7, a cytokine linked to cell cycle progression, secreted as a homeostatic response (Catalfamo, et al. 2008). It has been hypothesised that the massive depletion of mucosal CD4 cell reserves occurring during PHI creates a permanent imbalance, and that immune activation occurs as a homeostatic mechanism in order for the immune system to replace its losses (Cadogan and Dalgleish 2008).

Role of the gastro-intestinal (GI) tract

The role of the GI tract in the aetiology of HIV-associated immune activation has been highlighted in recent studies. The high level HIV-1 replication in GALT, occurring mainly during PHI, but continuing into the chronic phase of disease, is associated with permanent damage to both the structural and immunological environment of the GI tract (Centlivre, et al. 2007). Massive CD4 cell losses, in particular to the Th17 cells involved in generating a rapid response to microbial pathogens at mucosal sites, have a direct effect on the intestinal epithelial barrier as well as local innate and adaptive immune function (Dandekar, et al. 2010). In addition to CD4 cell losses, other abnormalities have been observed within the GI tract in both HIV-1 and SIV infections. Gene expression profiles from GI biopsies in HIV-1 infected individuals indicate downregulation of genes associated with cell cycle regulation, lipid metabolism and epithelial cell barrier functions (Brenchley and Douek 2008). HIV-1 infection is associated with a clinical enteropathy that can occur from acute phase through to advanced stages of disease and is associated with villous atrophy, crypt hyperplasia and villous blunting (Brenchley and Douek 2008). Brenchley et al. (2006) hypothesized that these factors result in an increase in gut permeability, permitting translocation of gut-colonising bacteria and their metabolites across the gut epithelium and into the systemic circulation. This microbial translocation results in activation of the innate immune system, with microbial products acting as toll-like receptor ligands, stimulating macrophages and dendritic cells to produce pro-inflammatory cytokines such as TNF- α and IL-6, and activating lymphocytes and further monocytes to induce systemic immune activation. This theory is supported by evidence of higher levels of circulating lipopolysaccharide (LPS), or endotoxin, in patients with chronic HIV-1 infection which correlate with the degree of both innate and adaptive immune activation (Brenchley, et al. 2006; Jiang, et al. 2009). Furthermore, in animal models, microbial translocation does not occur during non-pathogenic SIV infection of Sooty Mangabeys, whereas LPS levels are significantly increased in SIV-infected macaques (Brenchley, et al. 2006).

That LPS mainly is derived from gut sources is indicated by animal studies observing rises in LPS levels when measured before and after acute infection with SIV and dramatic reductions in LPS levels in SIV infected monkeys upon receipt of gut sterilizing antibiotics (Brenchley, et al. 2006). These data are supported by non-HIV-related disease models such as inflammatory bowel disease and graft versus host disease where gut-derived bacterial translocation is a well established factor in the pathogenic process (Caradonna, et al. 2000; Cooke, et al. 2002).

Brenchley et al. (2006) demonstrated the potential role of microbial translocation in causing immune activation in a series of experiments examining LPS in HIV-1 infection. In a cross-sectional study

looking at independent cohorts of HIV-1 infected individuals, LPS was raised in individuals with chronic HIV-1 infection, both in those with a CD4 T-cell count >200 cells/ μ l ($n=59$) and in those with a CD4 T-cell count <200 cells/ μ l ($n=39$), compared to HIV-negative individuals. When LPS was examined in relation to manifestations of immune stimulation not directly related to LPS mediated activation, a direct correlation between LPS and frequency of CD38+HLA-DR $^{+}$ CD8 T-cells, and between LPS and IFN- α , was seen. There was no correlation between LPS level and CD4 cell count or VL. LTNP had higher LPS than HIV-negative individuals and showed a trend towards lower levels than progressors. In longitudinally followed up patients who received 48 weeks ART and achieved undetectable VLs, there was a significant decrease in LPS, but levels were still raised compared to HIV-negative patients. LPS was inversely correlated with rise in CD4 cell count on ART (Brenchley, et al. 2006). Another study found that LPS was raised in HIV-1 infected patients compared to HIV-negative controls, but levels normalised after 2 years of effective ART (Troseid, et al. 2010). These findings are supported by another study which amplified a well-conserved 16S ribosomal DNA (rDNA) subunit common to most bacteria. Bacterial rDNA was higher in HIV-positive patients than HIV-negative controls and in untreated patients with chronic HIV infection compared with individuals on ART. Higher levels of bacterial DNA were associated with higher levels of T-cell activation (dual CD38 HLA-DR expression) and lower levels of immune restoration on commencing ART (Jiang, et al. 2009). A cross-sectional study in Sweden showed that levels of LPS were directly correlated with the severity of both HIV-1 and HIV-2 infection, and that LPS levels correlated with VL and inversely with CD4 cell count (Nowroozalizadeh, et al. 2010). Microbial translocation has also been associated with HIV-1 related neurological disease, as indicated by raised levels of LPS and LPS induced monocyte activation (sCD14) in individuals with HIV-associated dementia compared to other HIV-infected individuals with a CD4 cell count of <300 cell/ μ l, independent of CD4 cell count and HIV RNA levels (Ancuta, et al. 2008).

Although there are much data supporting the hypothesis that microbial translocation is a key mediator of immune activation, other groups have been unable to show a correlation between T-cell activation and LPS levels (Cassol, et al. 2010; Papasavvas, et al. 2009; Wallet, et al. 2010), and the relationship between microbial translocation, immune activation, and disease outcomes is unclear. A recent study in Ugandan patients found that LPS has little effect on rate of HIV-1 progression in the African population (Redd, et al. 2009), but showed that levels of C-reactive protein (CRP) were predictive of HIV-1 disease outcome, concluding that immune activation is associated with HIV-1 disease progression in the absence of microbial translocation (Redd, et al. 2010). Another study demonstrated increased levels of both circulating LPS and T-cell activation in HIV-1–infected South Africans with advanced disease (CD4 <200 cells/ μ l) compared with uninfected African controls, but

did not observe any direct correlations between the frequency of CD38+HLADR+ CD8 T-cells or IFN- γ and plasma LPS levels. LPS levels correlated with sCD14 and TNF- α , and remained elevated in patients receiving effective ART, suggesting that microbial translocation is a driver of inflammation. The authors conclude that the processes contributing to the increase in immune activation in an African setting are multifactorial (Cassol, et al. 2010). Few longitudinal studies have assessed the prognostic importance of microbial translocation in HIV-1 infection in Western cohorts and thus its clinical significance is not completely understood.

T-cell dysfunction and exhaustion

The preferential infection of virus specific CD4 T-cells leads to impairment of HIV-1 specific CD4 cell function, occurring very early in acute infection (Lichterfeld, et al. 2004; Rosenberg, et al. 1997), followed by a reduced ability of CD4 T-cells to respond to other antigens (Lange, et al. 2003). Impairment of CD4 T-cell responses also affects HIV-1 specific CD8 T-cells responses (Lichterfeld, et al. 2004) resulting in functional deficits ranging from loss of cytolytic activity to complete exhaustion of T-cells.

T-cell exhaustion may be defined as an inability of a CTL to produce cytokines or proliferate in response to environmental stimulus, resulting in functional CTL impairment and loss of viral control (Day, et al. 2006). Multiple studies have reported an association between expression of inhibitory molecules, such as Programmed Death-1 (PD-1) and T cell exhaustion in chronic viral infection (Barber, et al. 2006; Day, et al. 2006; Trautmann, et al. 2006). Barber et al. showed that high levels of PD-1 were expressed on CTL in chronic mouse Lymphocytic Choriomeningitis virus (LCMV) infection, and that antibody blockade of the PD-1 signaling pathway resulted in a rapid increase of effector CTL function and enhanced viral clearance, indicating that virally-mediated CTL exhaustion may be reversible and raising possibilities for novel therapies (Barber, et al. 2006).

Several studies have highlighted the role of PD-1 in negatively regulating the CTL response to HIV-1. Apoptosis of HIV-1 specific CTL is promoted by PD-1, and its expression on HIV-1 specific CTL correlates with other features of functional CTL exhaustion, such as impaired cytokine production and reduced capacity for CTL proliferation (Day, et al. 2006; Trautmann, et al. 2006). Expression of PD-1 on both HIV-1 specific CD8 and CD4 cells has been shown to correlate with predictors of disease progression, such as VL and inversely with CD4 T-cell count (D'Souza, et al. 2007; Day, et al. 2006). Blockade of PD-1 increases PD-1+ T-cell function, and in animal studies leads to reduction in plasma SIV VL (Day, et al. 2006; Petrovas, et al. 2007; Trautmann, et al. 2006).

Sauce et al. (2007) looked at PD-1 in relation to the expression of CD38 and HLA-DR in both healthy controls and chronically infected HIV-1 infected individuals and observed that activated CD8 T-cells express higher levels of PD-1, compared to resting cells. Within the PD-1 positive cell population, a consistent up-regulation of PD-1 was observed on the cell surface of CD8 T-cells with higher CD38 or HLA-DR expression. Expression of PD-1 was also related to differentiation stage, expressed by subsets of antigen-experienced CD8 T-cells and strongest on early/intermediate differentiated CD8 cells. In addition, the study observed that in individuals undergoing interruption of ART, a concomitant up-regulation of CD38 and PD-1 on antigen-experienced CD8 T-cells occurred alongside viral rebound. No direct association between the PD-1 expression and functional capacity of HIV-1 specific CD8 T-cells was seen in this study. The authors concluded that on the one hand, high PD-1 expression on HIV-1 specific CD8 T-cells may reflect cellular exhaustion and result in uncontrolled high VL, on the other hand, PD-1 is up-regulated further due to T-cell activation in the presence of high VL. Petrovas et al. showed that cells expressing high levels of PD-1 undergo high levels of spontaneous apoptosis and that manipulation of PD-1 leads to changes in the ability of the cells to survive and expand (Petrovas, et al. 2006; Petrovas, et al. 2007). Upregulation of PD-1 on activated CD8 T-cells in HIV-1 infected individuals may, therefore, be part of the homeostatic mechanism controlling T-cell proliferation and activation.

Consequences of immune activation and exhaustion

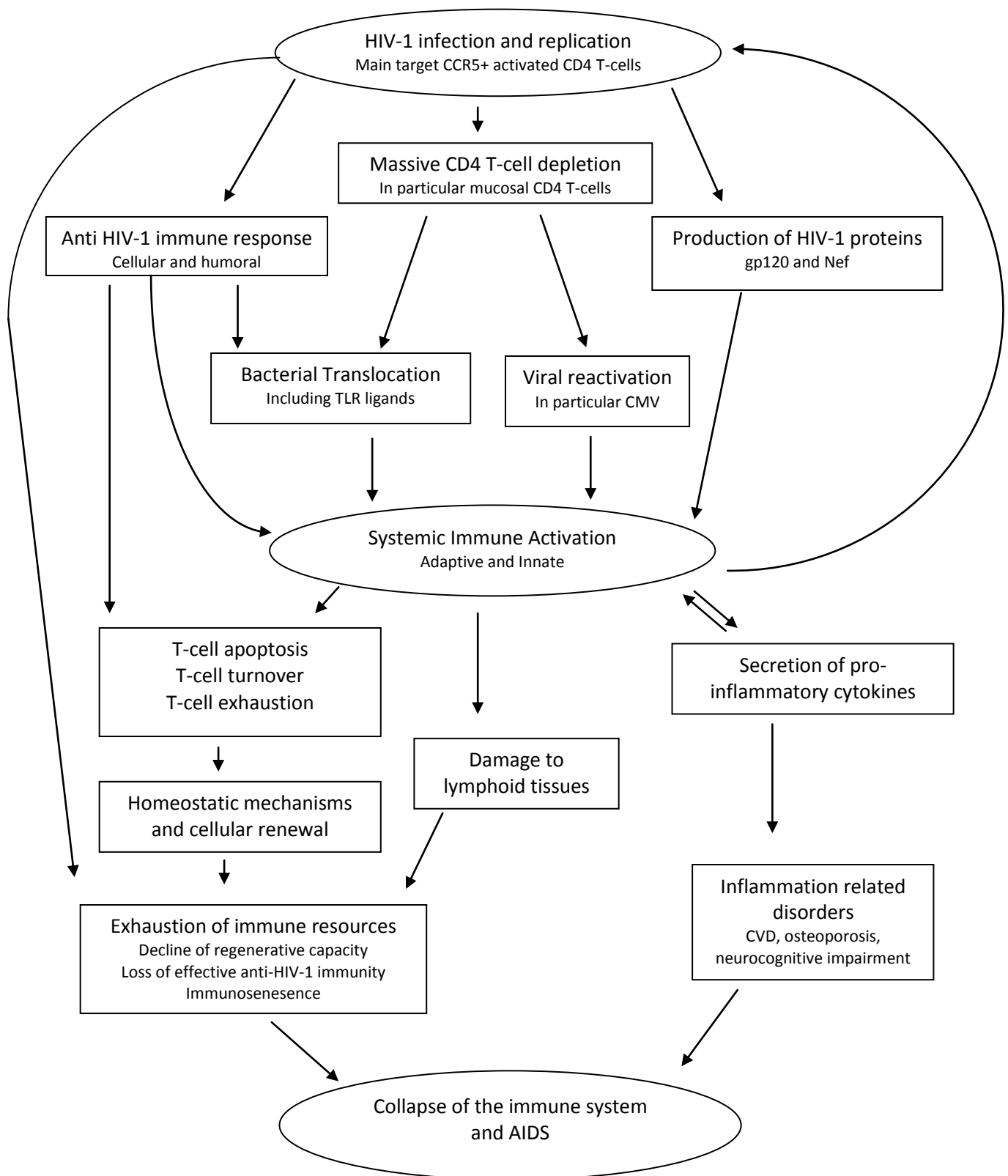
As cellular activation facilitates viral integration, T-cell activation promotes further HIV-1 replication, partly through increasing intracellular-nuclear-factor-kappa, which enhances transcription of integrated virus (Kawakami, et al. 1988). Increased levels of HIV RNA promote further immune activation thus establishing a self perpetuating vicious circle. In addition, the release of pro-inflammatory cytokines leads to ongoing T-cell activation.

Cellular activation induces T-cell proliferation and differentiation, and persistent viral replication and repeated antigenic stimulation results in exhaustion of replicative capacities and functional T-cell impairment. The dynamics of activation, expansion and apoptosis differ between CD4 and CD8 T-cells, and whereas CD8 T-cells expand rapidly on activation, establishing a pool of resting memory cells, the capacity of CD4 cells to expand and survive is lower, so that many activated CD4 cells undergo apoptosis, resulting in a decline in total CD4 T-cell numbers (Appay and Sauce 2008). Chronic immune activation results in fibrosis of the lymphatic tissue, thymic dysfunction, and a general decline in T-cell renewal capacities, meaning that the naive T-cell pool cannot be replenished and is unable to replace exhausted CD8 T-cell clones or depleted CD4 T-cells. Consequently, a shift towards differentiated antigen experienced cell populations occurs, resulting in an overall

immunosenescence similar to that seen with age. Senescent antigen experienced memory T-cells display a CD28⁻/CD57⁺ phenotype which is associated with reduced proliferative capacity and shortened telomere length, a marker of biological aging (Appay, et al. 2007).

As uncontrolled viral replication leads to depletion of the remainder of the CD4 cell pool, the immune system's ability to control pathogens is lost resulting in the clinical manifestations of immunodeficiency characteristic of HIV-1 infection (Appay and Sauce 2008). In addition, ongoing viral replication and immune activation result in a chronic pro-inflammatory state, leading to activation of pro-coagulatory pathways, endothelial activation, and accelerating non AIDS-related disease processes such as CVD. A model of HIV-1 disease pathogenesis is shown in Figure 1.3.

Figure 1.3. Model of HIV-1 pathogenesis



Causes and consequences of HIV-1-associated immune activation are shown. Figure adapted from Appay and Sauce (2008)

1.4: Inflammatory responses in chronic infection and implications for clinical disease

Inflammatory responses in chronic HIV-1 infection

Viral replication and immune activation in chronic HIV-1 infection induce a pro-inflammatory state associated with raised levels of inflammatory biomarkers, such as IL-6 and CRP, thrombotic markers, such as D-dimer and fibrinogen, and markers of endothelial activation such as Inter-Cellular Adhesion Molecule-1 (ICAM-1) and Vascular Cell Adhesion Molecule-1 (VCAM-1) (Neuhaus, et al. 2010; Tien, et al. 2010; Wolf, et al. 2002). Inflammatory and coagulation pathways are closely linked, and whilst inflammation leads to activation of coagulation, coagulation also considerably influences inflammatory activity (Levi, et al. 2004). Inflammation leads to induction of tissue factor pathways, resulting in generation of thrombin and subsequent conversion of fibrinogen to fibrin. Tissue factor, thrombin, and other activated coagulation proteases bind to specific cell receptors called protease activated receptors to induce signalling pathways that mediate further inflammatory responses (Levi, et al. 2004). Endothelial cell activation results in the formation and shedding of pro-coagulant microparticles and leukocyte adhesion molecules, triggering inflammatory responses, adhesion and transmigration of leucocytes, platelet aggregation and activation of coagulation pathways (Shantsila, et al. 2010).

In HIV-uninfected individuals, levels of IL-6, hs-CRP (high-sensitivity CRP) and ICAM-1 predict the risk of future cardiac events (Ridker, et al. 1998; Ridker, et al. 2002; Ridker, et al. 2000b). Raised D-dimer and fibrinogen are also associated with increased cardiovascular risk in apparently healthy individuals, indicating that activation of the endogenous fibrinolytic system occurs many years in advance of subsequent cardiovascular complications (Mora, et al. 2006; Ridker, et al. 1994).

Early studies showed that pro-inflammatory cytokines such as IL-6 were associated with markers of HIV-1 disease progression, such as low CD4 cell count (Aziz, et al. 1998; Lefeuvre, et al. 1991). A cross-sectional analysis of 513 patients with chronic HIV-1 infection enrolled in the US Multicenter AIDS Cohort Study (MACS) in the pre-HAART era, found that levels of hs-CRP, an acute-phase protein generated in response to proinflammatory cytokines, such as IL-6, correlated with VL and inversely with CD4 cell count. In a longitudinal analysis of 81 individuals from the same study cohort, hs-CRP levels increased over time and raised levels were significantly associated with HIV-1 progression to AIDS, independent of CD4 cell count and HIV RNA levels (Lau, et al. 2006). A recent analysis from the Fat Redistribution and Metabolic Change in HIV-1 Infection (FRAM) study showed that both fibrinogen and hs-CRP levels in HIV-1 infected patients independently predicted mortality over a five year follow-up period. When stratified by CD4 cell count, this was true even for individuals with high

CD4 cell count of >500 cells/ μ l, although the magnitude of risk was smaller (Tien, et al. 2010). A study in individuals commencing ART showed that higher pre-treatment concentrations of IL-6, as well as soluble TNF receptor-1 (sTNFR-1), soluble CD27, and soluble CD40 ligand (sCD40L), were associated with a new AIDS-defining illness or death, occurring while on ART. Soluble TNFR-1, sCD27 and sCD40L belong to the TNF superfamily of receptors that regulate lymphocyte proliferation, differentiation and survival, indicating that a heightened state of immune activation and inflammation prior to commencing ART is associated with adverse outcome while on treatment (Kalayjian, et al. 2010).

Treatment interruption and Inflammation: The SMART study

The first major indications that subclinical inflammation in chronic HIV-1 infection was associated with significant morbidity and mortality were made apparent through the results from a large-scale RCT, Strategies for Management of Anti-Retroviral Therapy (SMART), which investigated the use of strategic treatment interruption in individuals with chronic HIV-1 infection and CD4 cell counts > 350 cells/ μ l. The study was conducted to determine the effects of discontinuing successful ART in individuals with controlled infection, as a strategy to limit the complications of ART.

Although widespread availability of ART has led to a dramatic decrease in HIV-associated morbidity and mortality, ART regimens are associated with significant drug-associated toxicities, development of resistance and high costs. Early studies showed a link between ART therapy and metabolic complications, such as dyslipidaemia and insulin resistance (Grünfeld, et al. 2008), and cumulative ART exposure was shown to be associated with increased cardiovascular risk in a large prospective cohort study, the Data Collection on Adverse Events of Anti-HIV-1 Drugs (DAD) study (Friis-Møller, et al. 2003). Treatment strategies conferring sustained immunological function in the absence of these complications are, therefore, highly desirable and several studies investigated strategies minimising ART exposure with this goal in mind (Ananworanich, et al. 2006; El-Sadr, et al. 2006).

The SMART study, which included 5000 participants, compared a CD4-guided strategy of planned treatment interruptions versus continuous ART. Participants with CD4 >350 cells/ μ l were randomised to either a Drug Conservation (DC) arm or a Viral Suppression (VS) arm. In the DC arm, ART was deferred until CD4 cell count fell to <250 cells/ μ l and was discontinued when it returned to >350 cells/ μ l measured on two consecutive occasions at least two months apart. In the VS arm, ART was continued irrespective of CD4 cell count.

In contrast to expectation, interruption of ART was associated with an increased frequency of all cause mortality (Hazard ratio (HR) 1.8 95% CI, 1.2 to 2.9; $p=0.007$), and following interim analyses, an

independent data and safety monitoring board recommended premature termination of the study in January 2006. Most of the deaths that occurred were attributable to non-AIDS events, including an increased risk of cardiovascular events (HR 1.57 95% CI 1-2.46, $p=0.05$) (El-Sadr, et al. 2006).

The investigators hypothesised that the increased risk of treatment interruption in chronic infection may be due to HIV-1 induced activation of inflammatory and coagulation pathways. They therefore examined six plasma biomarkers (IL-6, D-dimer, Amyloid A, Amyloid P, hs-CRP and prothrombin fragment 1+2) in a random sample of 499 participants divided between the two study arms. Prior to randomisation, 74% of participants in this random sample were on ART and among these, 71% had a HIV RNA ≤ 400 copies/ml. Median age at baseline was 44 (IQR 38 to 51) years and 71% were male. The median baseline CD4 cell count was 540 (IQR 432 to 730) cells/ μ l. Baseline D-dimer was significantly higher in those not on ART ($p<0.001$) and in those with detectable viraemia on ART ($p=0.02$), but IL-6 did not vary significantly according to use of ART and HIV RNA at baseline. Higher D-dimer levels were also associated with higher age ($p=0.002$), black ethnicity ($p=0.01$), female gender ($p<0.001$), lower CD4 cell count ($p=0.002$), and greater body mass index (BMI) ($p<0.001$). Higher IL-6 at baseline was associated with age ($p<0.001$) and BMI ($p<0.001$) (Kuller, et al. 2008).

Treatment interruption was associated with significantly increased levels of the systemic inflammatory markers IL-6 and D-dimer coincident with recrudescence HIV-1 viraemia. One month after randomisation, there was a 16% increase in D-dimer and a 30% increase in IL-6 in those discontinuing ART. In the subgroup of participants with HIV RNA <400 copies/ml at study entry there was a median increase in D-dimer of $0.07\mu\text{g/ml}$ (a 27% increase) in those stopping ART, compared with a reduction of $0.02\mu\text{g/ml}$ in the continuous treatment arm ($p<0.0001$ for difference). Similarly, for IL-6 the median increased by 0.98pg/ml (a 43% increase) in those stopping ART and increased by 0.08pg/ml in the continuous treatment arm ($p<0.0001$). There was a significant association between the magnitude of HIV RNA rebound on discontinuing ART and the rise in both IL-6 ($p=0.003$) and D-dimer ($p=0.005$). In a case control analysis comparing those who died with those who survived, both baseline levels of IL-6 and D-dimer, and latest level prior to death, were strongly predictive of all-cause mortality ($p<0.0001$ for all). There was a weaker association between hs-CRP and mortality. Kuller et al. concluded that the adverse events seen in the SMART study were likely to be related to inflammatory and coagulatory processes induced by HIV-1 replication upon discontinuing therapy (Kuller, et al. 2008).

Further analyses of SMART data have shown that IL-6 and hs-CRP levels independently predict the development of opportunistic infection (Rodger, et al. 2009), and that sCD14, a marker of

macrophage activation secondary to LPS stimulation, is also predictive of all cause mortality (Sandler, et al. 2010).

In a cross-sectional analysis involving SMART participants, biomarkers of inflammation, coagulation and renal function in 781 SMART study participants were compared to individuals enrolled in the Multi-Ethnic Study of Atherosclerosis (MESA), and Coronary Artery Development in Young Adults (CARDIA) studies. MESA and CARDIA are two large cohort studies of healthy individuals assessing long-term cardiac risk in individuals aged 45-76 years, and 33-44 years, respectively. Levels of hs-CRP, IL-6, D-dimer and cystatin C (a marker of renal function) were all significantly higher in SMART study participants than in healthy control populations. This remained true when SMART participants receiving ART with HIV RNA levels <400 copies/ml were compared to the healthy control population. Biomarker levels in ART treated individuals were approximately 40–60% higher than matched controls from the general population (Neuhaus, et al. 2010). In a recently published SMART study analysis of a subgroup of participants who were naïve to ART or off ART for 6 months or longer at study entry, starting ART was associated with a 32% reduction in D-dimer levels at 6 months compared to those who did not initiate ART ($p<0.001$); but no significant differences were seen for hs-CRP or IL-6 levels compared to healthy controls. It should be noted that only 62% of ART treated individuals achieved HIV RNA levels <400 copies/ml in this analysis, and amongst those with VL suppression to below this level, significant reductions in IL-6 and hs-CRP were seen compared to baseline biomarker levels (Baker, et al. 2010). However, these recent analyses indicate that ART may not completely normalise levels of inflammation and risk associated with inflammation may remain even after ART is commenced.

Other treatment interruption studies assessing inflammatory responses

The SMART findings are supported by further studies indicating a rise in markers of inflammation and immune activation upon treatment interruption in chronic HIV-1 infection, which may persist even after reintroduction of ART (Calmy, et al. 2009; Papasavvas, et al. 2008; Tebas, et al. 2008). Papasavvas, et al. (2008) assessed changes in endothelial activation and T-cell markers of immune activation in individuals undergoing treatment interruption, and demonstrated that stopping ART was associated with rises in markers of endothelial activation (VCAM-1 and ICAM-1) and T-cell activation (CD8 CD38 expression). Rises in VCAM-1 correlated with both CD38 expression and HIV RNA, supporting a direct pathogenic relationship between viral replication, immune activation and endothelial stress. Tebas et al. (2008) also showed that there was an increase in T-cell activation (CD8 CD38 HLA-DR expression) on stopping ART, as well as rises in sTNFR2, and decreases in HDL cholesterol, and hypothesised that these factors may contribute to the adverse cardiovascular risk

seen in the SMART study. In the Swiss-Thai-Australia Treatment Interruption Trial (STACCATO), Calmy et al.(2009) measured a selection of biomarkers before ART was initiated, after ART had suppressed HIV RNA to < 50 copies/ml and 12 weeks after randomization to continued ART (n=48) or interrupted ART (n=97). Initiation of ART resulted in significant declines in s-VCAM-1, P-selectin (a marker of platelet activation), and D-dimer, whereas cytokines with anti-inflammatory properties, such as IL-10, increased. Twelve weeks after stopping therapy, increases in plasma RNA were associated with rises in VCAM-1 and CCL2. There was no association between treatment interruption and IL-6, however a low sensitivity assay was used for measuring IL-6 and the majority of results were under the lower limit of detectability.

HIV, endothelial dysfunction and cardiovascular disease

Chronic inflammation and immune activation are important risk factors for CVD, and in HIV-uninfected individuals, atherosclerosis is now recognised as a predominantly inflammatory disorder (Libby, et al. 2002). Chronic inflammation and immune activation associated with untreated HIV-1 disease have been associated with an increased incidence of CVD (Hsue, et al. 2009; Triant, et al. 2009). The extent to which HIV-1 itself accelerates CVD independent of its effects on traditional CVD risk factors or ART side effects is difficult to clarify. In a cohort study of 3851 HIV-1 infected and 1044589 HIV-uninfected individuals, myocardial infarction (MI) was increased in HIV-positive patients with a relative risk of 1.75 (95% CI 1.51-2.02 $p<0.0001$), adjusting for age, gender, race, hypertension, diabetes and dyslipidaemia (Triant, et al. 2007). In a recent study of internal carotid intima medial thickness (IMT), a marker of subclinical atherosclerosis predictive of CVD events, HIV-1 infected individuals had a highly significant increase in carotid IMT compared to HIV-negative controls, even after adjusting for demographics and traditional CVD risk factors, and HIV-1 was a risk factor for atherosclerosis similar in magnitude to traditional CVD risk factors, such as smoking (Grunfeld, et al. 2009).

The role of inflammatory processes in HIV-1 related cardiac disease has been investigated in several studies. In a recent study of 487 HIV-1 infected patients compared to 70,000 HIV-uninfected individuals, elevated CRP and HIV-1 were each independently associated with increased risk of MI, and patients with HIV-1 with increased CRP had a particularly increased risk of MI (OR 2.13; 95% CI 1.92 to 2.37; $p<0.0001$)(Triant, et al. 2009). In a case control study comparing HIV-1 infected individuals who had had a cardiac event with those who had not, Ford et al. demonstrated that several biomarkers (D-dimer, VCAM-1, soluble tissue factor and tissue inhibitor of metalloproteinase-1) predicted cardiac events when measured either a year or 4 months prior to the event, although traditional risk factors were also of key importance (Ford, et al. 2010). A study

comparing HIV-negative individuals to elite controllers showed that controllers had increased levels of subclinical atherosclerosis. This indicates that HIV-1 infection increases cardiac risk even in the absence of HIV-1 viraemia or ART (Hsue, et al. 2009). Controllers have higher levels of hs-CRP, and T-cell activation compared to HIV-negative individuals, indicating that premature atherosclerosis in HIV-1 may be due to HIV-1-induced inflammation and immune activation independent of VL, and highlighting that although controllers have improved outcomes compared to viraemic patients, elite control is not a completely benign condition (Hsue, et al. 2009; Hunt, et al. 2008).

Biological markers of endothelial and platelet activation are increased in patients with arterial disease and are associated with progression of atherosclerosis. A study of 52 HIV-1 infected individuals showed that ICAM-1 and VCAM-1 were both raised compared to HIV-negative controls, and that on multivariable analysis these endothelial activation markers correlated with sTNFR2, suggesting that endothelial activation is related to inflammation in the setting of HIV-1 infection (Melendez, et al., 2008). A study of 41 HIV-1 infected individuals showed that levels of endothelial markers VCAM-1, ICAM-1, as well as D-dimer and von Willebrand Factor (vWF), reduced significantly on commencing ART. VCAM-1 and vWF significantly correlated with HIV RNA levels (Wolf, et al. 2002). Another recent study compared endothelial and platelet activation markers in 56 HIV-1 infected individuals starting ART with HIV-negative controls and HIV-1 infected patients not starting ART. VCAM-1, CCL2, vWF and P-selectin, were all raised in HIV-1 infected patients, and VCAM-1, vWF and P-selectin reduced on commencing ART (Francisci, et al. 2009). The authors concluded that HIV-1 itself, rather than ART, causes endothelial dysfunction. Another study showed that CCL2 levels correlated with HIV RNA levels, and found an independent positive association between these factors and aortic vessel wall area and vessel wall thickness, measured by magnetic resonance imaging, supporting the link between HIV-1 infection, inflammation and atherosclerotic burden (Floris-Moore, et al. 2009).

A study of patients commencing ART reported a significant improvement in endothelial function after starting therapy, as measured by brachial artery flow-mediated dilation, indicating that controlling HIV-1 viraemia with ART may be a successful approach to reducing cardiac risk (Torriani, et al. 2008). However, few studies have assessed the effects of starting ART on non HIV-1 related disease outcomes. ART itself is associated with some degree of risk and as described above does not completely normalise inflammatory and immune activation parameters compared to HIV-negative individuals (Neuhaus, et al. 2010).

ART and cardiovascular disease

Prolonged exposure to ART is associated with dyslipidaemia and insulin resistance, which may potentially accelerate the onset of CVD (Grunfeld, et al. 2008). The DAD study demonstrated cumulative exposure to protease inhibitors (PI) as a risk factor (Friis-Moller, et al. 2007), partly explained by the effects of this drug class on lipid profile. Further analyses showed that the nucleoside reverse transcriptase inhibitors (NRTIs), abacavir and didanosine, were also associated with an increased risk of MI (Sabin, et al. 2008). The mechanism by which this occurs is unclear, as the risk was independent of the effects of ART on lipid and metabolic profiles, and cardiovascular events were limited to patients currently receiving, or within 6 months of receipt of the drug, rather than cumulative exposure.

A sub-analysis of the SMART study confirmed that current use of abacavir was associated with an excess risk of CVD, and showed that subjects on abacavir had significantly higher levels of the inflammatory markers, IL-6 and hs-CRP, than subjects not receiving abacavir (Lundgren 2008). A prospective study described hyper-reactive platelets in patients receiving abacavir, compared to those not receiving abacavir (Satchell C 2009). Hence, platelet activity or other inflammatory mechanisms may be responsible for the adverse risk seen. The association between abacavir and cardiovascular risk has been confirmed in some further studies (Durand 2009; Lang 2009; Martin, et al. 2009) but not in others (Bedimo R 2009; Benson 2009; Brothers, et al. 2009). In the Simplification with Fixed-dose Tenofovir/Emtricitabine or Abacavir/Lamivudine in Adults (STEAL) study, a randomized simplification study in which treatment-experienced patients with controlled HIV-1 infection were switched to simplify their regimen with either fixed-dose combination of tenofovir/emtricitabine or abacavir/lamivudine, there was a statistically significant increase in cardiac events in the abacavir arm, although the study was limited by small overall numbers of events (Martin, et al. 2009). However, no difference in circulating levels of inflammatory or coagulatory biomarkers were seen between the study arms (Martin, et al. 2010). In a review of compiled HIV-1 clinical trial safety data from clinical trials sponsored by the abacavir manufacturer, GSK, there was no evidence of excess risk related to abacavir (Brothers, et al. 2009). The role of abacavir in causing CVD remains controversial.

In the FRAM study, fibrinogen levels were 11% higher for participants taking a protease inhibitor, compared to those not using a protease inhibitor, further suggesting that specific ART components may have different effects on inflammatory or pro-thrombotic processes that influence cardiovascular risk (Madden, et al. 2008).

As HIV-1 infected patients live longer due to availability of ART, the impact of non-opportunistic disease becomes apparent. The aforementioned studies indicate that accelerated onset of CVD in HIV-1 infection may be related not only to the metabolic complications of ART and a high incidence of traditional CVD risk factors, but also to inflammatory responses triggered by HIV-1 itself, even in the absence of virus, or by specific components of ART. The exact role of immune activation and inflammation in the pathogenesis of CVD in HIV-1 infected individuals remain to be clarified.

1.5: Antiretroviral therapy and when to start

There are currently 5 classes of licensed ART drugs that act at different stages of the HIV-1 life cycle. The most effective means of establishing durable suppression of HIV-1 replication is the simultaneous initiation of a combination of usually 3 or more effective agents. The goal of combination ART is to suppress HIV-1 replication to below the limits of detection of current plasma HIV RNA assays (usually <50 copies HIV RNA/ml), thereby delaying disease progression and also limiting potential for selection of ART resistant HIV-1 variants.

Despite undetectable HIV RNA levels of <50 copies/ml on standard clinical assays, numerous studies have demonstrated ongoing viral replication on ultrasensitive assays in the plasma of ART-treated individuals, persisting even in patients who have been treated for several years (Bonora, et al. 2009; Havlir, et al. 2003; Palmer, et al. 2008). Treatment intensification with additional ART agents in subjects receiving suppressive regimens does not reduce this residual HIV-1 viraemia (Dinso, et al. 2009; McMahon, et al. 2010). As ART agents inhibit new cycles of viral replication, but do not block virus production from cells that are already infected, ongoing replication may reflect long-lived reservoirs of chronically-infected cells or sanctuary sites with poor ART penetration (McMahon, et al. 2010). The clinical significance of this lower level residual viraemia during successful ART remains unclear.

The use of ART has remarkably altered the clinical course of HIV-1 infection; however, the optimum timing for treatment initiation remains unknown. It is possible that early ART, by reducing HIV-1 related inflammation and immune activation, could profoundly impact non-HIV-1 related disease outcomes, although no data are available from randomised studies addressing this question. In the SMART study, treatment interruption was associated with higher rates of liver, kidney and cardiovascular disease, however the CD4 T-cell thresholds for starting and discontinuing ART were relatively low (200 and 350 cells/μl, respectively). Current UK and European guidelines suggest commencing long-term ART at a CD4 T-cell threshold of 350 cells/μl (EACS Version 5.2; Gazzard, et

al. 2008) whereas US guidelines recommend ART is commenced at a threshold of 500 cells/ μ l (DHHS 2009).

Two large cohort studies have addressed the question of starting ART at higher CD4 cells counts. The North American AIDS Cohort Collaboration on Research and Design (NA-ACCORD) study, found that mortality was significantly reduced in individuals starting treatment at above 500 cells/ μ l (Kitahata, et al. 2009). However, European data from a collaboration of cohorts (ART Cohort Collaboration) found little evidence of a significant improvement in survival if treatment is started above a CD4 cell count of 350 cells/ μ l (Sterne 2009). It is possible that confounding factors such as socioeconomic group may have impacted on results of the NA-ACCORD study, for example, those with poor access to care and greater likelihood of mental health issues would be less likely to start ART early and other factors may influence their health. An international RCT of early (CD4 cell count >500 cells/ μ l) vs. deferred ART (current threshold of CD4 cells count 350 cells/ μ l), the Strategic Timing of ART (START) study, began recruitment in April 2009, and has been designed to answer the question of the best time to start ART. The study has been powered to assess the effect of early ART on the development of AIDS-related outcomes and non-AIDS morbidity and mortality including cardiovascular disease. Pending the results of the START study, it is important that the potential benefits of starting early ART are weighed against potential adverse effects such as toxicity and high costs.

The use of ART in PHI has already been discussed (section 1.2). The use of short-course ART during PHI is under investigation in the SPARTAC trial.

1.6: Hypotheses and aims of this thesis

The role of immune activation and inflammation in chronic HIV-1 infection is becoming increasingly apparent; however there are few available data on inflammation and immune activation in PHI, a time when early events are likely to impact on long-term disease outcomes. Little is known about which factors determine the extent of immune activation and inflammation in HIV-1 infection, and the mechanisms surrounding these processes are not completely understood.

There are currently no data on whether treatment with ART in PHI impacts on levels of inflammatory and coagulation biomarkers. It is possible that the use of short-course ART in PHI, by influencing the immunological and virological events surrounding this phase of infection, will have a long-term impact even once ART is discontinued. This could be through several mechanisms: short-course ART may limit the size of the viral reservoir, lowering viral set-point and thereby reducing subsequent inflammatory responses, or damage to the GALT may be limited by early ART, and therefore reduce long-term consequences such as microbial translocation and immune activation.

There are no data examining the effects of stopping ART that has been started in PHI on inflammatory processes. It is not known whether the findings of the SMART study can be extrapolated to the setting of PHI. In the SPARTAC study, all participants who were randomised to ART underwent a protocol-indicated treatment discontinuation after either 12 or 48 weeks of therapy. PHI represents a very different immunological stage of HIV-1 mediated disease to chronic infection, and biomarker changes seen in PHI may therefore differ.

Overall aims:

- i. To explore the relationship between surrogate markers of immune activation, inflammation, coagulation and microbial translocation in individuals with PHI (within six months of seroconversion). Markers examined in this thesis are detailed in Table 1.1
- ii. To examine host and viral factors associated with these markers
- iii. To assess the impact of commencing short-course ART in PHI on IL-6 and D-dimer levels
- iv. To assess whether the effects of discontinuing ART differ for ART commenced in PHI versus chronic HIV-1 infection in terms of 1) biomarker changes and 2) virological rebound

Table 1.1. Biomarkers examined in this thesis

a) Inflammatory and endothelial biomarkers

Marker		Function and significance in non HIV-1 infected cohorts	HIV-1 related studies
Inflammatory biomarkers	IL-6	<ul style="list-style-type: none"> Pro-inflammatory cytokine produced by monocytes, endothelial cells, and lymphocytes. Strongly associated with future cardiac events in healthy individuals. 38% increased MI risk for each quartile increase in IL-6 ($p=0.001$) where quartile 1 <1.04, quartile 2: 1.04 –1.46, quartile 3: 1.47–2.28, quartile 4: >2.28 pg/ml) (Ridker, et al. 2000b) 	<ul style="list-style-type: none"> Raised in HIV-1 infection in both ART treated and untreated individuals (Aziz, et al. 1998; Neuhaus, et al. 2010). Increased levels 4 weeks after stopping ART initiated in chronic infection (SMART study) and correlated with VL. Baseline level associated with all cause death (OR 12.6, $p<0.0001$) and CVD death (OR 2.8, $p=0.003$) (Kuller, et al. 2008) . Also associated with future opportunistic infection (Rodger, et al. 2009)
	hs-CRP	<ul style="list-style-type: none"> Acute-phase protein secreted by liver in response to inflammatory cytokines including IL-6. Pro-inflammatory function, activates complement cascade and enhances phagocytosis. Strong predictor of 1st CVS events (Ridker, et al. 2000a) levels of <1 mg/L low, 1-3 mg/l moderate, and >3mg/L high risk (Ridker, et al. 2002) 	<ul style="list-style-type: none"> Raised in HIV-1 infection in both ART treated and untreated individuals (Neuhaus, et al. 2010) SMART study: baseline level associated with all cause death (OR 2.4, $p=0.003$). No change 4 weeks after stopping ART. (Kuller, et al. 2008). Associated with future opportunistic infection (Rodger, et al. 2009) Correlates with VL and inversely with CD4 cell count. Baseline levels in chronic infection predict progression to AIDS (Lau, et al. 2006). Independent predictor of mortality in HIV-1 infected individuals, even in individuals with high CD4 cell counts (Tien, et al. 2010)
Endothelial function	ICAM-1 (CD54)	<ul style="list-style-type: none"> Intercellular adhesion molecule, expressed on leukocytes and endothelial cell membranes, mediate the adhesion and transmigration of leukocytes to vascular endothelium & promotes T-cell proliferation and cytokine release(Springer 1990) Raised levels associated with future CVD events (Ridker, et al. 2000a; Ridker, et al. 1998). Risk factor for atherosclerotic disease progression (Blann, et al. 1998). 	<ul style="list-style-type: none"> Elevated in HIV-1, decreases with ART, no correlation with VL but correlates with another marker of endothelial activation, VCAM-1, which correlates with VL (Wolf, et al. 2002) ICAM-1 and VCAM-1 increase on treatment interruption in chronic infection. Change in VCAM-1 and T-cell activation correlate with change in VL on treatment interruption (Papasavvas E 2008)

b) Coagulation biomarkers

Marker		Function and significance in non HIV-1 infected cohorts	HIV-1 related studies
Coagulation Biomarkers	D-dimer	<ul style="list-style-type: none"> Fibrin degradation product and marker of haemostatic activation Becomes elevated when cross-linked fibrin is produced as a result of coagulation activation followed by secondary fibrinolysis Elevated levels associated with increased risks of future cardiac events (Lowe, et al. 2004; Ridker, et al. 1994) 	<ul style="list-style-type: none"> Raised in HIV-1 infection in both ART treated and untreated individuals (Neuhaus, et al. 2010). Decreases with ART commenced in chronic HIV-1 infection (Baker, et al. 2010; Calmy, et al. 2009; Wolf, et al. 2002). Correlates with VL (Calmy, et al. 2009; Wolf, et al. 2002) SMART study: increases 4 weeks after stopping ART initiated in chronic infection. Baseline levels predict all cause death (OR per quartile increase 13.3, $p<0.0001$) and CVS death (OR 2, $p=0.06$)(Kuller, et al. 2008) Independent association with cardiac risk in chronic HIV-1 infection (Ford, et al. 2010)
	Fibrinogen	<ul style="list-style-type: none"> Soluble plasma glycoprotein, synthesised by the liver. Converted into fibrin by action of thrombin. Acute-phase protein, increases with inflammation Elevated fibrinogen in persons aged 25-37 is independently associated with subclinical CVD in the subsequent decade (Green, et al. 2008). 	<ul style="list-style-type: none"> HIV-1 infected patients have higher levels of fibrinogen compared with controls. Higher fibrinogen associated with protease inhibitor based regimens (Madden, et al. 2008). Independent predictor of mortality in chronic HIV-1 infection, even in individuals with higher CD4 cell counts (Tien, et al. 2010)
	Tissue Factor	<ul style="list-style-type: none"> Glycoprotein expressed on a variety of surfaces, including platelets, monocytes, and sub-endothelium of blood vessel wall. Activated when vascular endothelium is damaged to initiate the extrinsic coagulation pathway(Breitenstein, et al. 2010). 	<ul style="list-style-type: none"> Monocyte tissue factor expression and TF activity higher in HIV-positive compared to HIV-negative individuals. Production stimulated by LPS exposure. Monocyte tissue factor expression correlates with viraemia and inversely with CD4 cell count (Funderburg, et al. 2010b) Soluble tissue factor predictive of cardiac events in HIV-1 infected individuals when measured 4 months prior to the event (Ford, et al. 2010).

c) Surrogate markers of T-cell activation and microbial translocation

Marker		Function and significance in non HIV-1 infected cohorts	HIV-1 related studies
Cellular markers of T-cell activation & exhaustion	CD38	<ul style="list-style-type: none"> Multifunctional transmembrane glycoprotein, upregulated on activated T-cells, with role in increasing cytokine production, CD4 cell proliferation and cell to cell adhesion 	<ul style="list-style-type: none"> Stronger predictor of HIV-1 disease progression than either CD4 or VL load when measured in chronic HIV-1 infection (Giorgi, et al. 1999; Giorgi, et al. 2002; Liu, et al. 1997). CD8 CD38 expression independently predictive of CD4 decline when measured within 12 months of seroconversion (Deeks, et al. 2004), and CD4 CD38 expression independently predictive of progression to AIDS if measured at one year after seroconversion (Hazenbergh, et al. 2003b)
	HLA-DR	<ul style="list-style-type: none"> MHC class II cell surface receptor, upregulated on activated T-cells 	<ul style="list-style-type: none"> Predictive of progression to AIDS in chronic HIV-1 infection (Hazenbergh, et al. 2003b). Dual expression of CD38 and HLA-DR also predictive of progression to AIDS when measured in chronic infection (Cao, et al. 2009) or one year after seroconversion (Hazenbergh, et al. 2003b)
	PD-1	<ul style="list-style-type: none"> Marker of T-cell exhaustion and upregulated on activated T-cells. Negatively regulates immune responses. High levels of PD-1 expressed on CTL in LCMV infection. Antibody blockade of the PD-1 signalling pathway results in rapid increase in CTL function and enhanced viral clearance (Barber, et al., 2006) 	<ul style="list-style-type: none"> Correlates with VL and inversely with CD4 T-cell count (Day, et al., 2006). Upregulated on activated T-cells in chronic HIV-1 infection (Sauce, et al. 2007). Promotes apoptosis of HIV-1 specific CTL. Expression on HIV-1 specific CTL correlates with other features of functional CTL exhaustion, such as impaired cytokine production and reduced proliferative capacity (Day, et al. 2006; Trautmann, et al. 2006).
Surrogate markers of microbial translocation	sCD14	<ul style="list-style-type: none"> Upregulated on activated macrophages in the presence of endotoxin. Mediates the interaction of LPS with cells. Good indicator of LPS bioactivity <i>in vivo</i> and is less subject to contamination than LPS assays. Binds LPS via LBP, and triggers production of inflammatory cytokines. 	<ul style="list-style-type: none"> Raised in patients with both acute and chronic HIV-1 infection compared with HIV-negative individuals. Directly correlates with both LPS and LBP (Brenchley, et al. 2006). sCD14 correlates with HIV RNA in chronic infection and inversely correlates with CD4 cell count. Longitudinal measurement of sCD14 in advanced HIV-1 (n=26) showed increasing levels associated with death (Lien, et al. 1998). In the SMART study, plasma levels of sCD14 also predicted mortality. LPS levels themselves were not associated with mortality (Sandler, et al. 2010).
	LBP	<ul style="list-style-type: none"> Acute-phase protein produced by GI and hepatic endothelial cells in response to LPS, and required for CD14 to bind LPS. 	<ul style="list-style-type: none"> As with LPS, LBP is raised in chronic infection but not in those with hyper-acute infection, when compared to HIV-negative individuals and correlates with sCD14 (Brenchley, et al., 2006).
	Endotoxin core Ab	<ul style="list-style-type: none"> In acutely unwell individuals with sepsis, EndoCAb bind LPS to clear it from the circulation and titres decrease. Increased levels in chronic conditions of microbial translocation (Barclay 1995) 	<ul style="list-style-type: none"> IgM EndoCAb levels inversely correlate with LPS levels in HIV-1 infected individuals (Ancuta, et al. 2008; Brenchley, et al. 2006; Papasavvas, et al. 2009). Significantly reduced IgM levels seen in acute HIV-1 compared to HIV-negative, and lower in chronic infection compared to both acutely infected patients and HIV-negatives (Brenchley, et al., 2006)

Chapter 2: Materials and Methods

2.1. Ethical approval

Ethical approval was obtained for the studies described in this thesis, and included approval for studies of immunological and inflammatory function from participants of the St Mary's Hospital Seroconverter study (REC 99/1A/161E), the SPARTAC study (MREC/04/2/025) and the Darunavir/r Raltegravir Pharmacokinetic study (REC 08/H0718/56). Written informed consent was obtained from all participants. Healthy controls at St Mary's hospital were recruited to the Communicable Diseases Research Tissue Bank, Wright-Fleming Institute of Imperial College London (MREC 09/H0606/106).

2.2. Study cohorts

Subjects with PHI

St Mary's Hospital Seroconverter pilot study

One-hundred-and-five patients with PHI were recruited into a pilot study at St Mary's Hospital, London, between November 1999 and October 2003. In this non-randomized open-label study patients were offered 3-months short-course ART initiated immediately following diagnosis of PHI. PHI was defined by using one of the following criteria:

1. positive HIV-1 antibody test result within 6 months of a previous documented negative HIV-1 antibody test;
2. acute symptomatic seroconversion illness in the presence of an evolving HIV-1 specific antibody response by ELISA;
3. positive HIV DNA PCR or positive HIV RNA quantification in the absence of an antibody response.
4. HIV-positive antibody test result with an 'incident' detuned assay result (Optical density (OD) < 0.6).

Fifteen participants declined therapy and the remaining 90 were prescribed ART. Patients choosing ART were prescribed either fixed dose NRTI Combivir™ (zidovudine/lamivudine) with non-nucleoside reverse transcriptase inhibitor (NNRTI) nevirapine (n=29); or fixed dose NRTI combination Trizivir™ (zidovudine/lamivudine/abacavir) with NNRTI efavirenz (n=33); or protease inhibitor (PI) based

regimen Combivir and Kaletra™ (lopinavir/ritonavir)(n=17). Eleven individuals received alternate regimens for reasons of patient choice, resistance or toxicity.

Both treated and untreated subjects were followed monthly for two years and monitored for evidence of disease progression. Follow-up was then 3 monthly or according to clinical need. Fifty-six participants remained in clinical care at St Mary's Hospital in July 2009 with available clinical and laboratory follow-up data. Frozen plasma samples from 30 participants for whom samples were available were included in pilot inflammatory biomarker work for this thesis (Chapter 3).

HIV-1 specific CD4+ and CD8+ T-cell immune responses were measured as the primary study outcome. Secondary outcomes included CD4 cell count and VL over time, development of resistance, and development of opportunistic infection. Study findings have been previously published (Fidler, et al. 2007; Fidler, et al. 2006; Fidler, et al. 2002). The study demonstrated preserved HIV-1 specific CD4 T-helper responses in study participants and that short-course ART in PHI may delay CD4 cell decline, and concluded that larger RCT are needed to establish if short-course ART in PHI results in long-term clinical benefit.

SPARTAC study

The SPARTAC study is an international blinded RCT assessing the effect of short-course ART in PHI (MRC 2007). The primary end-point of the study is time taken to reach CD4 < 350 cells/ μ l on more than one occasion. Clinical data, VL data and analysis of PBMC and plasma samples from SPARTAC participants for studies on markers of immune activation, inflammation, coagulation and microbial translocation are included in this thesis (chapters 4-7).

SPARTAC participants were randomized to one of three arms: 12 weeks short-course ART at PHI diagnosis, 48 weeks short-course ART at PHI diagnosis, or no treatment. Patients randomised to ART were recommended to receive PI based regimens with 2 NRTIs. Three-hundred-and-sixty-six patients were recruited between 2003 and 2007, across 7 countries: UK, Spain, Italy, Brazil, Australia, South Africa and Uganda. Overall baseline characteristics of study participants, a breakdown of study sites and details of ART regimens started are shown in Table 2.1.

Table 2.1. Baseline characteristics of participants enrolled in the SPARTAC trial (n=366)

		Number or Median	(%) or [IQR]
Sex:	Male	220	(60)
	Female	146	(40)
Risk group:	Sex between men (MSM)	205	(56)
	Heterosexual	158	(43)
	Not known	3	(1)
Age (years)		32	[25, 40]
CD4 (cells/ μ l) overall		559	435, 700]
HIV RNA (copies/ml)		34,068	[4702, 150,000]
Seroconversion-type illness reported		215	(59)
Subtype*	B	210	(58)
	Non-B	154	(42)
Baseline resistance*	none	321	(88)
	Stanford 3-5	33	(9)
	Stanford 5	12	(3)
Time from est. SC to baseline visit (days)		79	[58, 103]
BMI (kg/m ²)		24	[22, 27]
Hepatitis B +ve		12	(3)
Hepatitis C +ve		4	(1)
Study site	UK	149	(40.7)
	Ireland	2	(0.6)
	Australia	36	(9.8)
	Brazil	17	(4.6)
	Italy	23	(6.2)
	Spain	2	(0.6)
	South Africa	118	(32.2)
	Uganda	19	(5.2)
Randomised to and received ART		238	(65)
ART regimen	2 NRTI + 1 (boosted) PI	217	(91)
	2 NRTI + 1 NNRTI	15	(6)
	3 NRTI + 1 NNRTI	3	(1)
	Other	3	(2)

Baseline characteristics of the 366 participants enrolled in the SPARTAC trial of short-course ART in PHI are shown. *available in 364 participants

Identification of PHI in SPARTAC

Methods of PHI diagnosis used in SPARTAC are shown in Table 2.2. All patients were estimated to be enrolled into the study within a maximum of 6 months from documented seroconversion. The majority of study participants (67%) were identified as having PHI by an HIV-positive antibody test within 6 months of a negative HIV-1 antibody test. The estimated date of seroconversion was calculated according to method of PHI diagnosis as shown in Table 2.2.

Table 2.2. Diagnosis of PHI and estimated date of seroconversion in SPARTAC study participants (n=366)

Method of PHI diagnosis	Estimated date of seroconversion	No of participants (%)
HIV-positive antibody test within 6 months of a negative HIV-1 antibody test	Midpoint between negative and positive test dates	246 (67)
HIV-1 antibody negative with positive RT-PCR	PCR date	9 (2)
“Incident” test at low level (<0.6) using detuned assay (for subtype B virus only)	Assay date	72 (20)
Equivocal HIV-1 antibody test supported by a repeat test within 2 weeks showing a rising optical density	Midpoint between equivocal and positive test dates	1 (0)
Clinical manifestations of symptomatic HIV-1 seroconversion illness supported by P24 antigen positivity and greater than 4 positive bands on Western Blot	Date of Western Blot / antigen test	38 (10)

Patients were diagnosed with PHI by one of 5 methods. The method of calculating the estimated date of seroconversion, and number of patients diagnosed by each method, is shown. All patients were estimated to be within six months of seroconversion at study entry.

Clinical assessments of SPARTAC participants

Participants attended for clinical follow-up at weeks 4, 12, 16, 24, 36, 48, 52 and 60, and then every 12 weeks or according to clinical need. Clinical history since last assessment, including adherence assessment of trial medication, HIV-1 related and non HIV-1 related adverse events, AIDS events, use of concomitant medication and occurrence of pregnancy, were documented.

Physical examination according to patient history including weight and blood pressure was undertaken. To standardise measurements a British Hypertension Society approved, semi-automatic device, the OMRON 705-CP was used to measure the patients' blood pressure. At each scheduled visit 3 seated measurements were made and recorded, and average calculated. Blood for biochemistry, FBC, CD4 cell count and HIV RNA were taken as well as trial specific samples for plasma and PBMC isolation (section 2.4). Genotypic ART resistance tests were taken at baseline, 4 weeks after discontinuing the study drugs and then annually or according to clinical need.

Primary End-points

The primary end-point of the study is the time from randomisation to reaching a CD4 cell count of <350 cells/ μ l measured on two consecutive occasions not more than 4 weeks apart, or time to the initiation of late treatment. Secondary outcomes include HIV-1 specific CD4+ and CD8+ T-cell responses at week 60, time from randomisation to virological failure of first regimen, development of new drug resistance not present at baseline, development of an AIDS defining illness or death and differences in blood pressure from randomisation at week 12 and week 48. The study will report in the second quarter of 2011. The study remains blinded to clinical outcome and hence all data in this thesis referring to samples collected as part of the SPARTAC study remain blinded to randomisation arm and to CD4 end-point.

A summary of the SPARTAC trial design is shown in Appendix 1.

Subjects with Chronic HIV-1 infection

Darunavir/r Raltegravir Pharmacokinetic study

Plasma samples were obtained from 15 participants with chronic HIV-1 infection enrolled in a prospective study, which investigated the steady state pharmacokinetic profile of 3 consecutive ART regimes: phase 1 - darunavir/ritonavir dosed once daily with tenofovir/emtricitabine; phase 2 - darunavir/ritonavir/tenofovir/emtricitabine and the integrase inhibitor raltegravir; and phase 3- darunavir/ritonavir/raltegravir alone. Patients receiving boosted PI based regimens were eligible for study entry. Four participants were female (27%) and the median age was 42 years (IQR 35.5, 49.5). All patients had undetectable plasma HIV-1 VLs at time of enrolment and were healthy with no current health complaints other than asymptomatic controlled HIV-1 infection.

Chapter 3 examines the effect of freeze thawing on plasma levels of IL-6 and D-dimer obtained from 20 samples taken from 15 patients over the 3 study phases (15, 3 and 2 samples from study phases 1, 2 and 3 respectively).

Healthy controls

Healthy controls for IL-6 and D-dimer plasma studies (chapter 4, n=11) were recruited from the clinical trials centre, St Mary's Hospital, and invited to donate blood samples to the Communicable Diseases Research Tissue Bank, Wright-Fleming Institute of Imperial College London. Informed consent was taken for venepuncture, sample storage and analysis of samples. Healthy controls for plasma studies of LPS surrogate marker studies, ICAM-1 and sTF (chapter 7, n=30) were recruited from the Thrombosis & Vascular Biology Group, Rayne Institute, St Thomas' Hospital, in line with departmental procedures for recruitment of healthy controls. Informed consent for venepuncture, sample storage and analysis was taken and samples were taken according to institution health and safety procedures. Healthy controls for PBMC samples (chapter 6, n=10) were recruited from The Peter Medawar Building, Oxford, in line with departmental policy for recruitment of healthy controls. Volunteers were recruited following informed consent and samples were taken in line with the Oxford University Health and Safety guidelines.

Healthy controls were not known to be HIV-positive but were not formally tested for HIV-1 infection.

2.3 Statistical methods

Data were analysed using SPSS version 17.0, except for blinded SPARTAC data analyses which were carried out using Stata statistical software in collaboration with SPARTAC statisticians (Medical Research Council). Biomarker results were $\log_{10}$ transformed where necessary to normalize distributions for analysis purposes. Baseline variables were compared between different populations using Mann-Whitney U tests and T Tests. Changes in biomarkers were assessed using Wilcoxon signed rank test and T tests. All p-values were reported from two-sided tests and significance inferred if $p < 0.05$. The association of baseline characteristics with biomarker results were examined using linear regression. For multivariate models, variables were included if the p-value was < 0.1 in univariate models and backwards selection was used to find significant variables. For linear regression analyses the regression coefficient (B) is reported and represents the rate of change of one variable as a function of changes in the other, i.e. the slope of the regression line. Further individual statistical analyses are described in each chapter.

Power calculations for SPARTAC IL-6 and D-dimer studies (Chapter 4) were based on future planned analyses which will take place once the SPARTAC trial is unblinded, and which will compare biomarker levels across the 3 arms of the study. The sample size was calculated to power the study to detect a difference in biomarker levels equivalent to a one-third change in risk of death, based on data from the SMART study which estimated that the rises in biomarkers observed one month after discontinuing ART would be associated with an increased risk of death of 16% and 24% for IL-6 and D-dimer, respectively (Kuller, et al. 2008). The differences in biomarker levels equivalent to this risk and their standard errors were taken from published data from the SMART study (Kuller, et al. 2008) and the sample sizes corrected for the multiple comparisons across the 3 study arms. Based on SMART study data, 75 patients in each arm are required to demonstrate an increase in IL-6 level equivalent to an increased risk of death of one-third, based on 80% power, 5% alpha and allowing for multiple comparisons across the arms of the trial. For D-dimer, 41 participants in each arm are required.

2.4. Laboratory Methods

HIV-1 parameters

CD4 cell count measurement

For pilot seroconverter patients, pharmacokinetic study patients, and SPARTAC patients recruited at St Mary's hospital, CD4 cell count was measured at the Department of Immunology, St Mary's Hospital. Samples were processed using an ST1000 automated processor and analysed by flow cytometry using SC500 or Navios flow cytometers. The Becton Coulter tetra CXP panel was used. For SPARTAC participants at other sites, CD4 cell count was measured locally using validated QC approved assays.

Plasma HIV RNA assays

Quantification of HIV RNA for pilot seroconverter patients, PK study patients, and SPARTAC patients recruited at St Mary's hospital was measured at the Department of Virology, St Mary's Hospital using the VERSANT HIV RNA 3.0 Assay (bDNA) kit (Bayer Siemens, previously Chiron). This has a lower limit of detectability of 50 copies/ml and upper limit of quantification of 500,000 copies/ml. HIV RNA for SPARTAC participants from other sites was quantified according to local procedure in diagnostic laboratories, using validated QC approved assays. The most sensitive assay available at the local laboratory was used; at some sites (eg Brazil) this had a lower limit of detection of 400 copies/ml.

HIV-1 co-receptor usage

Genotypic tropism was determined by the molecular diagnostics unit of the Jefferiss Trust laboratories, Imperial College London. RNA was extracted from stored plasma samples, reversed transcribed and PCR amplified using primers adjacent to the V3 loop of *env*. PCR products were sequenced in both directions and edited sequences submitted to the Geno2pheno [coreceptor] algorithm (www.geno2pheno.org). The false positive rate (FPR) was set at 5.7%.

Viral genotyping for HIV-1 resistance

HIV-1 genotyping for UK SPARTAC participants was performed by the Molecular Diagnostics Unit in the Jefferiss Trust laboratories, Imperial College London. HIV-1 drug resistance was determined by sequencing of the PR and RT genes followed by submission of sequences to the Stanford Algorithm <http://HIV-1db.stanford.edu>

HLA typing

HLA type was determined for UK SPARTAC participants only at the ASHI (American Society for Histocompatibility and Immunogenetics) accredited HLA typing laboratory, University of Oklahoma Health Sciences Centre, USA. HLA type was determined using reverse sequence-specific oligonucleotide kits for the HLA-A, HLA-B, and HLA-C loci (Dynal Biotech). To obtain four-digit typing, Dynal Biotech sequence-specific priming kits were used, in conjunction with the Sequence-Specific Oligonucleotide type.

Collection and storage of blood samples

Collection of plasma

Plasma was separated on the day of HIV-1 clinic attendance and processed in a category 3 laboratory. Whole venous blood was collected using vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) for plasma samples. Blood collection tubes were centrifuged at 400 x g for 10 minutes. Using a sterile transfer pipette, plasma was aliquoted into 1ml cryotube vials and stored at -80°C.

Separation and cryopreservation of peripheral blood mononuclear cells

Whole venous blood for peripheral blood mononuclear cells (PBMCs) was collected using vacutainer tubes containing anticoagulant citrate dextrose (ACD) and processed on the day on clinic attendance in a category 3 laboratory. The PBMCs were collected by density gradient centrifugation. In brief, blood was diluted 1:1 in phosphate buffered saline (PBS). The diluted blood was layered onto Histopaque-1077 solution (Sigma-Aldrich) in a 1:1 ratio in a 50ml centrifuge tube and centrifuged at 800 x g for 30 minutes with no brake. The white blood cell fraction was transferred to a new 50 ml tube using a sterile Pasteur pipette, made up to 50ml in PBS and centrifuged at 400 x g for 5 minutes, brake 3. The supernatant was discarded and cells re-suspended in 50ml PBS and centrifuged at 400 x g for 5 minutes. The cell pellet was resuspended in 2ml of Roswell Park Memorial Institute solution (RPMI, Sigma-Aldrich) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 0.5% penicillin and streptomycin. PBMCs were counted using Trypan blue staining and a haemocytometer. After counting, the cells were centrifuged at 400 x g for 5 minutes, and resuspended in freezing medium, a solution of 50% heat-inactivated FCS, 40% RPMI and 10% Dimethyl sulfoxide (DMSO) at a volume of 3×10^6 cell per ml. One ml aliquots were placed in

cryotube vials (Sarsted). The PBMCs were frozen overnight at -80°C in Mr Frosty cryocontainers (Nalgene, UK) and then transferred to liquid nitrogen or -150°C freezers.

Cell revival

Cryopreserved cells were rapidly defrosted in a water bath at 37°C, diluted in RPMI with 10% FBS and 0.5% penicillin and streptomycin, and supplemented with 10µl of deoxyribonuclease (DNase, Qiagen). DNase was added to breakdown DNA from dead cells to prevent live/dead cells clumping together. The cells were twice washed by centrifuging at 400 x g for 10 minutes, and resuspended in RPMI/FBS solution, and counted. Cells were re-suspended in RPMI/FCS and rested 12-16h in an incubator at 37°C, 5% CO₂ (Sanyo, CO₂ incubator) before use for flow cytometry.

Cell counting

Fifty microlitres of cell suspension were added to 5µl 37% formaldehyde and 45µl Trypan Blue (Sigma) in a 1.5ml tube. The Trypan Blue cell mixture was placed in a well of plastic haemocytometer (Immune Systems) and cells in 4 squares were counted. The number of cells was calculated as follows: average cell count x 10⁴ x 2 (dilution factor) x 2 (resuspended volume) = total number of cells/ml.

Measurement of markers of Inflammation, coagulation and endothelial activation

IL-6

IL-6 was measured using Quantikine HS IL-6 immunoassay (R&D systems), an ultrasensitive quantitative sandwich enzyme immunoassay with a range 0.02–10 pg/ml. A highly sensitive assay was chosen as up to 75% of the patients with chronic HIV-1 have values below the detection limit of less sensitive kits used in some studies (Calmy, et al. 2009). All samples and standards were assayed in duplicate and co-efficient of variation (CV) calculated for each pair. If CV was >10% the reason for this was reviewed and the sample repeated if necessary. A standard curve was repeated for each ELISA plate.

Manufacturer's instructions were followed throughout. Frozen plasma was thawed at room temperature. A standard curve was prepared by making a 2-fold dilution series from the IL-6 standard provided (10 pg/ml). Standards or samples (volume 100µl) were pipetted into the wells of a microplate pre-coated with a monoclonal antibody specific for IL-6. The plate was incubated for 2 hours at room temperature on a horizontal microplate shaker and then washed 6 times using the

wash buffer provided. A conjugate solution, containing an alkaline phosphatase linked polyclonal antibody specific for IL-6, was added to the wells in 200µl aliquots. The plate was incubated for a further 2 hours on the microplate shaker and washed 6 times. The provided substrate solution was added to the wells in 50µl aliquots and the plate incubated on the bench top for 60 minutes. An amplifier solution was added, and colour development stopped with 50µl sulphuric acid (1M). The absorbance of each well was immediately determined using a microplate reader (Biotrak 2, Amersham sciences) set to 490nm, with background wavelength correction set to 620nm.

Hs-CRP

Hs-CRP levels were evaluated using a chemiluminescent immunoassay (Immulite 2500 Hs-CRP, Siemens Healthcare Diagnostics) at St George's Hospital NHS trust laboratories. An automated analyser was used (Immulite 2500).

D-dimer

D-dimer was measured in the diagnostic haematology laboratory at St Mary's Hospital, Imperial College Healthcare NHS Trust using an immuno-turbidimetric assay (Innovance D-dimer, Siemens Healthcare Diagnostics). An automated analyser was used (Sysmex CS2100i)

Fibrinogen

Fibrinogen was measured in the diagnostic haematology laboratory at St Mary's Hospital, Imperial College Healthcare NHS Trust. Clotting time after addition of bovine thrombin (Thrombin reagent, Siemens) to diluted plasma was compared to a calibration curve using reference plasma (Standard Human Plasma, Siemens). An automated analyser was used (Sysmex CS2100i)

Soluble tissue factor

Soluble tissue factor (TF) was measured using Quantikine Human Coagulation Factor III / Tissue Factor Immunoassay R&D systems. All samples and standards were assayed in duplicate and manufacturer's instructions were followed throughout. Frozen plasma was thawed at room temperature. Samples were diluted 2-fold by diluting 150µl plasma in to 150µl of provided diluent.

A standard curve was prepared by making a 2-fold dilution series from the standard provided (500 pg/ml). Standards or samples (volume 100µl) were pipetted into the wells of a micro plate pre-coated with a monoclonal antibody specific for TF. The plate was incubated for 2 hours at room

temperature on a horizontal microplate shaker and then washed 4 times using the wash buffer provided. The provided conjugate solution, containing a polyclonal antibody against TF, conjugated to horseradish peroxidase, was added to the wells in 200µl aliquots. The plate was incubated for a further 2 hours on the microplate shaker and washed 4 times. The provided substrate solution was added to the wells in 200µl aliquots and the plate incubated on the bench top for 30 minutes. Colour development stopped with 50µl sulphuric acid (1M). The absorbance of each well was immediately determined using a microplate reader (Dynax MRX) set to 450 nm, with background wavelength correction set to 540 nm. Results were analysed using Revelation software Version 4.06 (Dynax).

Soluble ICAM-1

Soluble ICAM-1 was measured using Quantikine sICAM-1 / CD54 Immunoassay (R &D systems). All samples and standards were assayed in duplicate and manufacturer's instructions were followed throughout. Frozen plasma was thawed at room temperature. Samples were diluted 20-fold by diluting 20µl sample into 380µl diluent. A standard curve was prepared by making a 2-fold dilution series from the standard provided (50ng/ml). The provided conjugate solution (containing a monoclonal antibody against sICAM-1 conjugated to horseradish peroxidase) was first added to the wells of a micro plate pre-coated with a monoclonal antibody specific for sICAM-1 in 100µl aliquots. Standards or samples (volume 100µl) were then added. The plate was incubated for 1.5 hours at room temperature on a horizontal microplate shaker and then washed 4 times using the wash buffer provided. The provided substrate solution was added to the wells in 200µl aliquots and the plate incubated on the bench top for 30 minutes. Colour development stopped with 50µl sulphuric acid (1M). The absorbance of each well was immediately determined using a microplate reader (Dynax MRX) set to 450 nm, with background wavelength correction set to 540 nm. Results were analysed using Revelation software Version 4.06 (Dynax).

Surrogate markers of microbial translocation

Assays measuring LPS are highly subject to contamination and require specific procedures when collecting and storing plasma in order to ensure accurate results (Redl, et al. 1992). Blood should be collected, and plasma stored in pyrogen-free containers, and subjects should be fasting when undergoing venepuncture (Personal communication Daniel Douek 2009). As SPARTAC samples have not been collected specifically for this purpose, surrogate markers of LPS activity, including soluble CD14, LPS-Binding Protein and Endotoxin Core Antibody, were measured in this study. In particular,

no information on dietary status at the time of venepuncture was available from SPARTAC participants.

sCD14

Soluble CD14 was measured using Quantikine Human sCD14 Immunoassay (R&D systems). All samples and standards were assayed in duplicate and manufacturer's instructions were followed throughout. Frozen plasma was thawed at room temperature. Plasma was diluted 200-fold by diluting 10µl plasma into 1990 µl of provided diluent. A standard curve was prepared by making a 2-fold dilution series using the standard provided (16000 pg/ml). Standards or samples (volume 100µl) were pipetted into the wells of a microplate pre-coated with a monoclonal antibody specific for sCD14. The plate was incubated for 3 hours at room temperature on the bench top and then washed 4 times using the wash buffer provided. The provided conjugate solution containing polyclonal antibodies against sCD14 conjugated to horseradish peroxidase, was added to the wells in 200µl aliquots and the plate incubated for a further hour. After washing 4 times, the substrate solution provided was added to the wells in 200µl aliquots and the plate incubated for 30 minutes whilst protecting from light. Colour development stopped with 50µl sulphuric acid (1M). The absorbance of each well was immediately determined using a microplate reader (Dynax MRX) set to 450nm, with background wavelength correction set to 540nm. Results were analysed using Revelation software Version 4.06 (Dynax).

LPS-Binding Protein (LBP)

LBP was measured using LBP Human ELISA test kit (Hycult biosciences). All samples and standards were assayed in duplicate and manufacturer's instructions were followed throughout. Plasma was diluted 1000 fold by diluting 10µl in 90µl of provided diluents, and then further diluting 10µl of resulting solution in 990 µl of diluent. A standard curve was prepared by making a 1.5-fold dilution series using the standard provided (50 ng/ml). The provided microplate pre-coated with antibody to LBP was prewashed 4 times before adding 100ul standard or sample. The plate was incubated on the benchtop at room temperature for 1 hour and further washed 4 times. One-hundred microlitres of biotinylated tracer antibody solution was then added and the plate incubated a further hour at room temperature. After washing 4 times 100ul provided conjugate solution containing streptavidin peroxidase was then added. Following further one hour incubation and washing 4 times, 100ul of substrate solution was added. Colour development was stopped after 30 minutes by adding 100ul

oxalic acid, and the absorbance of each well determined using a microplate reader (Dynax MRX) set to 450nm. Results were analysed using Revelation software Version 4.06 (Dynax).

Endotoxin-core antibodies (EndoCAb)

Endotoxin-core IgM antibodies were measured using EndoCAb ELISA kit (Hycult biosciences). All samples and standards were assayed in duplicate and manufacturer's instructions were followed throughout. Samples were diluted 100 fold by diluting 10µl in 990µl of diluent. A standard curve was prepared by making a 2 fold dilution series using the standard provided (3.50 MMU/ml). 100µl standard or sample was added to the provided microplate pre-coated with endotoxins. The plate was incubated at 37°C (Dynax MRX) for 1 hour and further washed 4 times. 100µl of horseradish peroxidase conjugated antibody solution was then added to the wells and the plate incubated a further hour at 37°C. After washing 4 times 100µl provided substrate solution was added. Colour development was stopped after 30 minutes at room temperature by adding 100µl oxalic acid. The absorbance of each well was determined using a microplate reader (Dynax MRX) set to 450nm. Results were analysed using Revelation software Version 4.06 (Dynax).

Flow cytometry techniques and markers of T-cell immune activation

Cells were stained with a panel of antibodies for CD3, CD4, CD8, immune activation markers HLA-DR and CD38 and T-cell exhaustion markers PD-1, TIM-3 and LAG-3. TIM-3 and LAG-3 were added as part of another project in order to maximise the use of the SPARTAC PBMC resource, and results for these markers are not described in this thesis. Antibodies used and corresponding fluorochromes are shown in Table 2.3

Antibody optimisation

The optimal antibody concentration for each marker was determined by performing titrations of different antibody concentrations as demonstrated in Appendix 2. For activation markers CD38 and HLA-DR, PBMCs from healthy individuals were stimulated overnight at 37°C with 5µg/ml Phytohaemagglutinin in RPMI/FCS in order to induce T-cell activation.

Cell staining

An antibody solution was made up by adding a 2µl aliquot of each antibody per patient sample (dilution 1:50 from stock) to 83.5µl PBS per sample, with the exception for CD38/QDot 605 where 0.5µl was used (dilution 1:200). This resulted in a total volume of 100µl per patient sample. Final

antibody concentrations are shown in Table 2.3. A separate solution of isotype control and PBS was made up for negative samples.

For each patient, 250,000 live cells were used in each positive FACS reaction and 100,000 cells used for the negative control. PBMCs were centrifuged at 500 x g (1600 rpm, ALC multispeed centrifuge PK131) for 10 minutes and supernatant removed. One-hundred microlitres of the antibody solution were added to the positive samples and 100µl of isotype control solution to the negative control sample. The solution was gently mixed to re-suspend the cell pellet and incubated at 4°C for 30 minutes. Following the 30 minute incubation period, 1ml of PBS was added to the cell/antibody mix, and the cells centrifuged for 10min at 500 x g. The supernatant was then poured off, and the cells fixed using 200µl 1% paraformaldehyde solution in PBS.

Compensation controls

Positive compensation beads, polystyrene microparticles which bind immunoglobulin, and negative beads which have no binding but are used detect background fluorescence, were used to optimize fluorescent compensation settings by providing distinct positive and negative stained populations. Compensation controls were set up for each colour. One drop of positive compensation beads (BD Biosciences) were added to individual FACS tubes each assigned a colour and one drop of negative beads (BD Biosciences) was added to a negative control tube. Two microlitres of the antibody used for each colour was added to individual positive tubes. The solution was incubated at 4°C for 10-20 min, then 1ml PBS added, the solution centrifuged at 500 x g for 10 minutes and fixed with 1% paraformaldehyde in PBS.

Determination of negative gates

Negative gates for analysis were established using Fluorescence minus 1 (F-1). Due to a paucity of cells from SPARTAC patients' samples, cells from healthy controls were used for F-1. 250,000 cells were placed into each tube and the cell antibody solution was prepared as described above. One antibody was omitted from each tube in order to establish negative gates for analysis of that particular antibody.

Sample Analysis

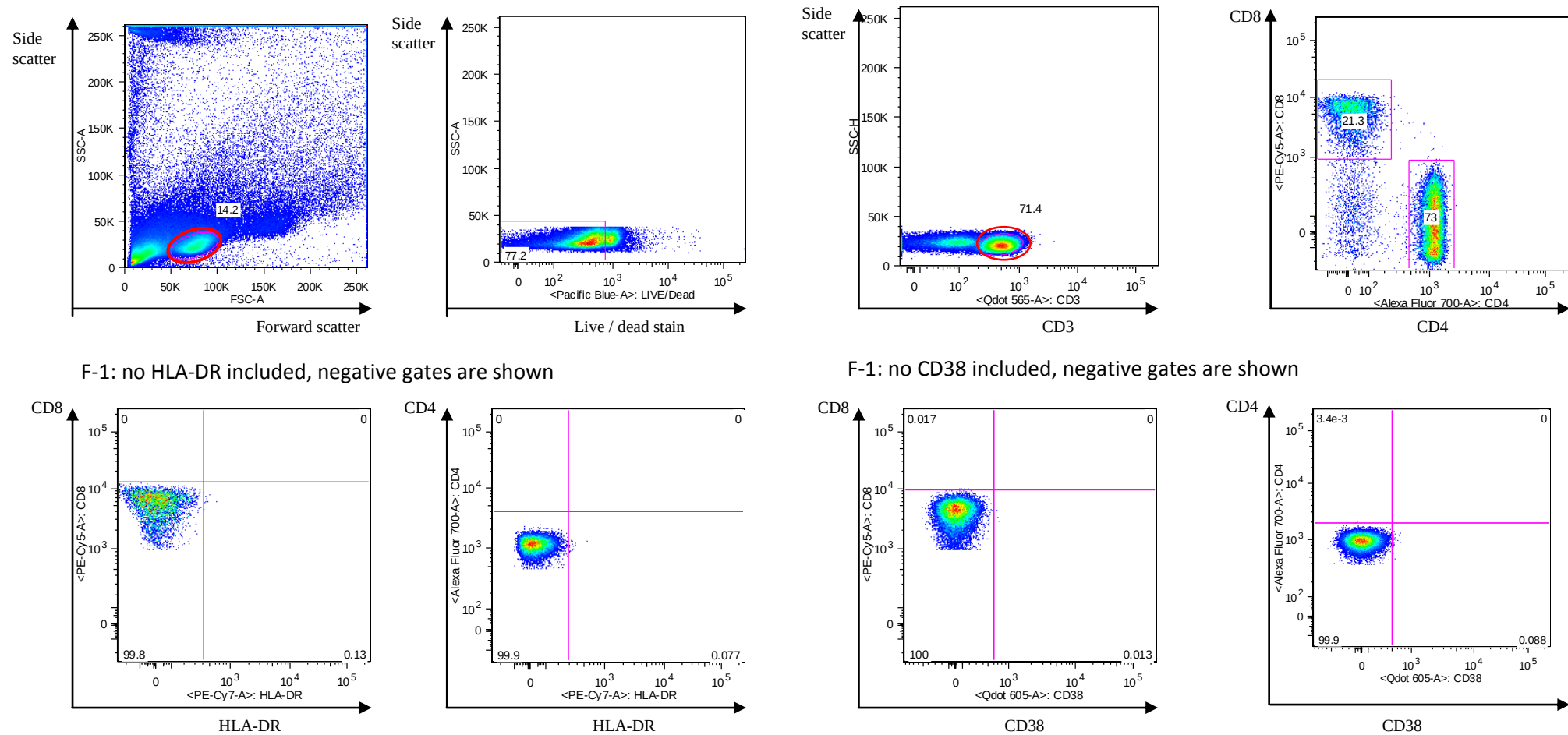
Samples were analysed within 6h of fixation. Flow cytometry was carried out using a four-laser, 18-color benchtop flow cytometer, LSR 11 equipped with a blue, red and violet lasers; (BD biosciences). The LSR11 was standardised on a daily basis using cytometry setup and tracking beads (BD biosciences) to ensure reproducibility of data. Compensation between fluorochromes was regularly updated using compensation beads prepared as described above. Data were analysed using FloJo software version 8.8.6 (TreeStar, Inc.). T-cell analysis was performed on a gated population of lymphocytes as defined from forward versus side scatter 2 dimensional dot-plots for each sample. An example of the gating strategy and the placement of negative gates are shown in Figure 2. 1. The percentage of CD8 and CD4 cells expressing CD38 and HLA-DR were calculated, in addition to the median fluorescent intensity (MFI). In addition the percentage of CD8 or CD4 cells with dual CD38 HLA-DR expression, and percentage expressing PD-1, was calculated.

Table 2.3. Antibodies and fluorochromes used for T-cell activation study

Antibody	Fluorochrome	Clone	Company	Final Concentration ($\mu\text{g}/100\mu\text{l}$)
CD3	Pacific Orange	UCHT1	BD Biosciences	0.4
CD4	Alexa 700	RPA-T4	BD Biosciences	0.4
CD8	PE-Cy5 (PerCP)	SK1	BD Bioscience	0.4
Live/Dead	Pacific Blue	n/a (Dye)	Invitrogen	2 $\mu\text{l}/100\mu\text{l}$
CD38	Qdot 605	HIT-2	Invitrogen	0.4
HLA-DR	PE-Cy7	LN3	eBiosciences	0.4
PD-1	APC	MIH4	eBiosciences	0.5
LAG-3*	FITC	17B4	LifeSpan Biosc	2
TIM-3*	PE	344823	R and D	0.05

Antibodies used, fluorochromes, and final concentration of antibody used for flow cytometry are shown. *TIM-3 and LAG-3 were added as part of another project in order to maximise the use of the SPARTAC PBMC resource and results are not described in this thesis. PerCP -Peridinin chlorophyll protein; PE – Phycoerythrin; FITC - Fluorescein isothiocyanate; APC - Allophycocyanin

Figure 2. 1. Examples of gating strategy and placement of negative gates



Gating strategy and Fluorescence minus one (F-1) results are demonstrated. For F-1, the antibody of interest was omitted from the cell antibody mix in order to establish the placement of negative gates for that particular antibody during analysis

Chapter 3: Pilot study assessing inflammatory and pro-coagulatory biomarkers in PHI and effects of freeze thawing

Introduction

Uncontrolled viral replication in chronic HIV-1 infection is associated with raised levels of inflammatory and pro-coagulatory biomarkers which are reduced upon commencing ART (Baker, et al. 2010; Calmy, et al. 2009). Data from the SMART trial indicates that subclinical inflammation after stopping ART in chronic HIV-1 infection may be associated with significant morbidity and mortality (Kuller, et al. 2008; Rodger, et al. 2009). Whether these findings can be applied to PHI warrants further investigation. The full extent of the inflammatory response in PHI, and effect of starting and stopping ART in this setting are unknown. We investigated the levels of pro-inflammatory and coagulatory biomarkers in stored samples obtained from patients with PHI, and assessed feasibility of analysing frozen samples, with a plan to expanding this pilot study to the SPARTAC trial participants, if successful.

The aims of this chapter were to:

- i. explore the feasibility of testing plasma for inflammatory and pro-coagulatory biomarkers after longer term freezing
- ii. measure levels of inflammatory and pro-coagulatory biomarkers in PHI, to assess the effect of ART in these patients and to assess the relationship between inflammatory markers and long-term clinical outcome in a pilot study

Results

3.1. Pilot work to assess the effect of long-term freeze-thawing on IL-6 and D-dimer assays.

To assess the potential impact of freezing for a one year period on IL-6 and D-dimer assays, 20 plasma samples were obtained from 15 patients with chronic HIV-1 infection enrolled into a pharmacokinetic study, on separate clinic visits. The patient cohort enrolled in this study is described in methods chapter 2.2. All patients had HIV RNA levels <50 copies/ml at enrolment and were in good health.

For IL-6, EDTA plasma samples were collected according to the methods described in chapter 2.4, aliquoted into more than one vial, and immediately frozen at -20°C. Samples were batched and assayed within one month. A further aliquot was analysed after a one year period. For D-dimer a fresh citrate sample was taken and sent straight to the haematology laboratory according to routine NHS procedure for assessing D-dimer. A further EDTA plasma sample was collected and

immediately frozen at -20°C. This sample was assayed after a one year period. Details of the assays used for IL-6 and D-dimer are described in methods chapter 2.4.

Table 3.1 shows median IL-6 and D-dimer levels and Spearman rank correlation between initial samples and those stored for one year. For IL-6, there was a highly significant correlation between results from initial samples and those assayed after one year of freezing (Rank correlation coefficient 0.94). Scatter plots of biomarker results measured within one month of sampling and then repeated after a 1 year period are shown in Figure 3.1a and demonstrated the relationship between initial and subsequent assay results. Agreement between samples was further assessed by constructing Bland Altman plots (Bland and Altman 1986). Figure 3.2a shows a Bland Altman plot for IL-6. The mean difference between the two IL-6 measurements is -0.1pg/ml (95% CI -0.25, 0.03). IL-6 measurements after one year fall within 0.50pg/ml below or 0.72pg/ml above the measurement within one month of freezing, indicating good agreement between initial and subsequent assays.

For D-dimer, there was a strong correlation between fresh sample results and those repeated after one year, but the strength of relation between the ranks was weaker (Rank correlation coefficient 0.79). The relationship between initial biomarker results on fresh samples and those repeated after one year is shown Figure 3.1b. Initial D-dimer results tended to be higher than those from samples that had been frozen for a year. A Bland Altman plot for D-dimer is shown in Figure 3.2b. The mean difference between the measurements is 0.04 mg/L (95% CI 0.01, 0.07). From the calculated limits of agreement, the majority of D-dimer measurements after one year are within 0.18 mg/L below and 0.09 mg/L above the measurement taken one month after freezing.

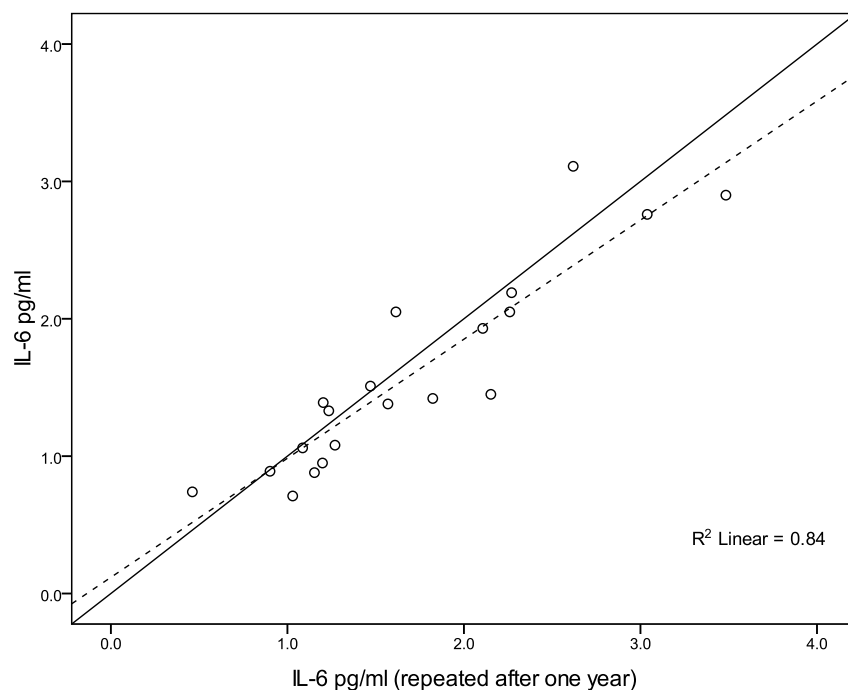
Table 3.1. IL-6 and D-dimer results measured within one month and after one year of freezing (n=20)

	Initial biomarker result Median (IQR)	Repeated after 1 year Median (IQR)	Spearman rank Coefficient	p-value for correlation
IL-6 pg/ml	1.41 (1.01, 2.05)	1.52 (1.18, 2.21)	0.94	<0.0001
D-dimer mg/L	0.19 (0.16, 0.38)	0.13 (0.11, 0.29)	0.79	<0.0001

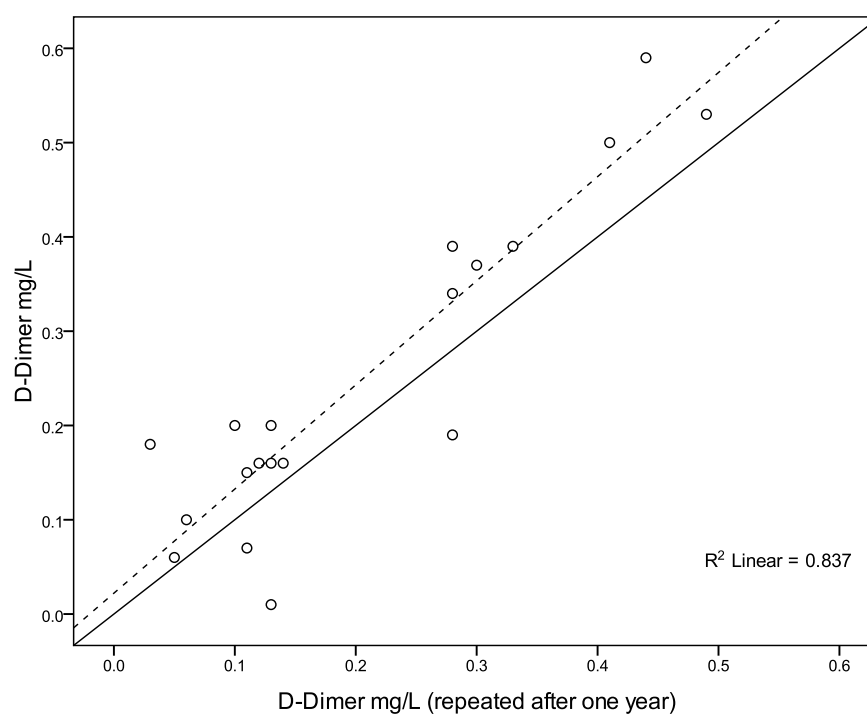
20 EDTA plasma samples for IL-6 were batched and frozen at -20°C. The initial sample was measured within one month of sample being taken and second sample measured after one year. For D-dimer a fresh sample was assayed within hours of being taken and a second sample frozen at -20°C and assayed after a year. Spearman's rank correlation coefficient for agreement between initial and subsequent samples is shown alongside median values and IQR.

Figure 3.1. Scatter plot showing the relationship between initial biomarkers levels and assays repeated after one year (a) IL-6 and (b) D-dimer

(a)

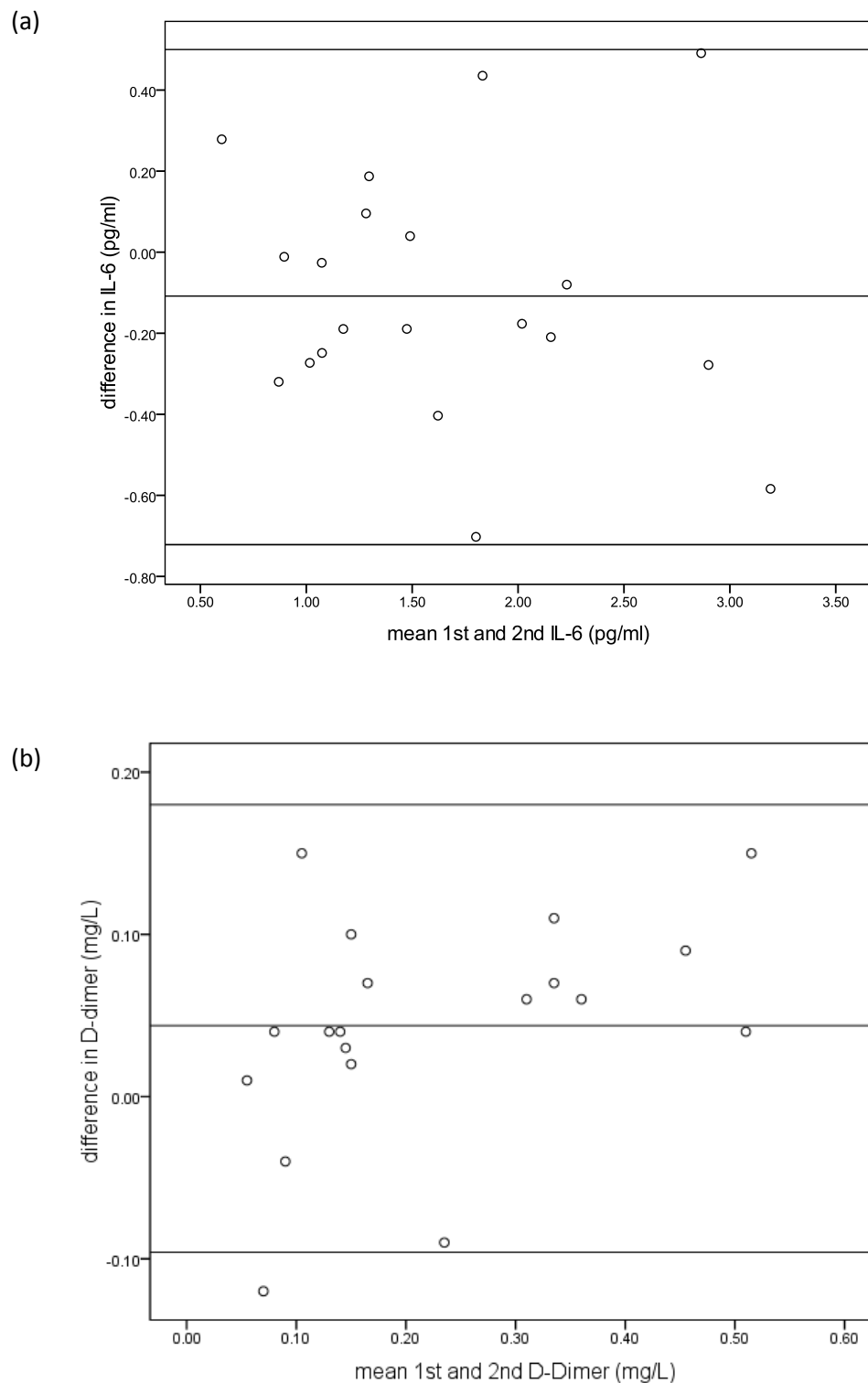


(b)



Scatter plot of biomarker results measured within one month of sampling and then repeated after a 1 year period, frozen at minus 20°C. The line of equality ($x=y$, solid line), indicating exact agreement between the two measurements, and best fit line showing relationship between the 2 measurements (dashed line) are shown.

Figure 3.2. Bland-Altman plot showing level of agreement between frozen samples for (a) IL-6 and (b) D-dimer



Bland Altman plots were constructed to assess agreement between first and second biomarker measurements. The mean of the 2 measurements (x axis) is plotted against the difference between the 2 measurements (y axis). The horizontal lines demonstrate the mean difference and limits of agreement (2 Standard Deviations of mean difference).

For IL-6, the limits of agreement are -0.72 and 0.50pg/ml therefore initial IL-6 measurement may be up to 0.5 pg/ml above or 0.72 pg/ml below the measurement after one year of freezing. For D-dimer, the limits of agreement are -0.09 and 0.18, and initial D-dimer measurements can be expected to be within 0.18 mg/L above and 0.09 mg/L below the measurement taken after 1 year after freezing.

Summary of freeze thaw analysis

These data confirm that analysis of stored frozen plasma samples for IL-6 and D-dimer is a valid methodology to estimate these biomarkers in HIV-1 infected cohorts. Freezing at -20°C for a one year period has little effect on IL-6 results. It should be noted that D-dimer results after one year of freezing are slightly lower than results from fresh citrated samples.

3.2. Pilot study of inflammatory and pro-coagulatory biomarkers in PHI

Prior to use of stored samples from the SPARTAC study, evaluation of the baseline and subsequent levels of biomarkers in PHI were assessed using stored plasma samples from a previous seroconverter study, described in methods chapter 2.2. Frozen plasma samples from 30 participants for whom serial samples were available were included in this analysis. Baseline samples had been stored a median of 6.3 years (IQR 5.7, 6.7 years; range 5.4, 7.2 years).

In this non-randomized open-label study patients were offered 3-months short-course ART initiated immediately following diagnosis of PHI. Plasma levels of IL-6, hs-CRP, D-dimer and fibrinogen were measured at 4 time points: baseline, or the nearest time point to PHI diagnosis, 12 weeks, or just before stopping therapy in those who received treatment, a median of 8 weeks after stopping (range 4 to 12 weeks after stopping), and a median of 9 months after stopping (range 8 to 10 months). Samples at equivalent time points from seroconversion were analyzed in those who did not receive therapy.

Baseline characteristics

Baseline characteristics and antiretroviral regimen of the 30 included participants are shown in Table 3.2. Overall the median age was 34 years (range 27-65) and 87% were male. Twenty-five subjects received ART in PHI and 5 declined. Patients who opted to receive therapy had significantly higher baseline HIV RNA than those who declined therapy ($p=0.04$, 95% CI 2.3, 92.8), and had a higher incidence of seroconversion symptoms, although this was not statistically significant ($p=0.07$, 95% CI-0.8, 0.03). The ART regimen started reflected prescribing practice at the time of the study. Over half of patients were commenced on quadruple therapy consisting of Trizivir™ and efavirenz. Nearly one third commenced a PI based regimen comprising of Combivir™ and Kaletra™.

Table 3.2. Baseline characteristics and ART regimen started in 30 patients diagnosed with PHI

	Received ART n=25	Declined ART n=5
Median age (range)	33 (27,65)	40 (29,56)
Male (%)	23 (92)	3 (60)
Median CD4 cells/ μ l (IQR)	530 (310, 570)	448 (420, 630)
Median VL copies/ml (IQR)	122534 (9980, 381860)	5577 (1317, 34948)
Symptomatic at presentation	20 (80)	3 (60)
<i>ART regimen started:</i>		
Combivir™ / Nevirapine (%)	4 (16)	
Trizivir™ / Efavirenz (%)	14 (56)	N/A
Combivir™ / Kaletra™ (%)	7 (28)	

Baseline characteristics, incidence of symptoms related to seroconversion, and ART regimen started in 30 patients diagnosed with PHI with available serial plasma samples.

Assessment of biomarker levels in pilot study participants

Samples were available for 27/30 patients at time point 1, for 100% patients at time points 2-4. Assay failure occurred for 7/27 fibrinogen samples and 1/27 D-dimer samples at time point 1. This was due to the automated analyser being unable to recognize the sample. One fibrinogen sample failed at each of time points 2 and 3, and one D-dimer assay failed at time point 4. All IL-6 and hs-CRP assays were successful. No assays failures occurred in the 5 control patients.

Median levels and interquartile range (IQR) of IL-6, hs-CRP, D-dimer and fibrinogen at each time point are shown for ART treated patients in Table 3.3 alongside VL and CD4 cell count data. At baseline, there was a significant correlation between $\log_{10}$ transformed IL-6 and D-dimer (Pearson correlation coefficient 0.4, $p=0.04$), and between IL-6 and fibrinogen levels (both $\log_{10}$ transformed, Pearson correlation 0.5, $p=0.02$). hs-CRP was significantly correlated with fibrinogen (both $\log_{10}$ transformed, Pearson correlation 0.9, $p=0.001$). There was no association between biomarker levels at baseline and CD4 cell count or VL in this analysis.

Table 3.3. Inflammatory biomarkers and HIV-1 parameters in 25 patients with PHI who received 3 months ART at diagnosis

Time point		Week 0 (1 st clinic visit)	Week 12 (on ART)	Week 20 (8 weeks after stopping ART)	Week 48 (9 months after stopping ART)
IL-6 pg/ml	median	2.17	1.13	0.76	1.15
	(IQR)	(1.01,10.9)	(0.64,1.76)	(0.48,1.37)	(0.72,1.67)
D-dimer mg/L	median	0.38	0.24	0.24	0.32
	(IQR)	(0.17, 1.87)	(0.12, 0.39)	(0.16, 0.48)	(0.12,0.49)
hs-CRP mg/L	median	0.70	0.70	0.80	1.00
	(IQR)	(0.2, 2.1)	(0.25, 2.0)	(0.50, 1.75)	(0.33,1.90)
Fibrinogen g/L	median	1.83	1.71	2.1	2.0
	(IQR)	(1.53, 2.17)	(1.48, 2.30)	(1.97, 2.31)	(1.8, 2.33)
VL copies/ml	median	122,534	50	10946	20582
	(IQR)	(9980, 381859)	(50-50)	(3223,36758)	(3734, 116969)
CD4 cells/ μ l	median	420	670	650	445
	(IQR)	(310, 570)	(550,765)	(450,730)	(342,690)

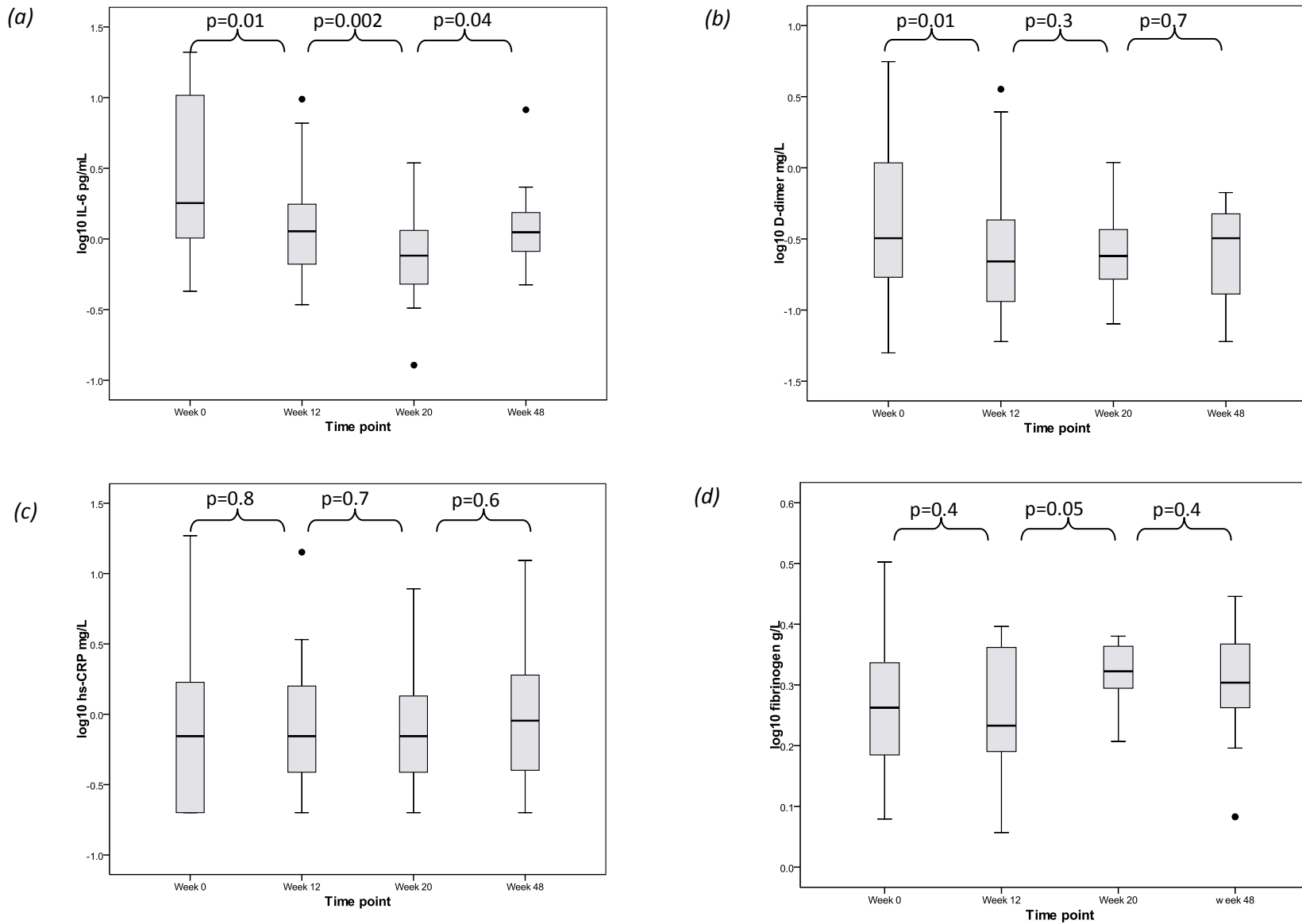
Median levels and IQR of IL-6, D-dimer, CD4 cell count and VL (VL) in 25 individuals diagnosed with PHI are shown across 4 study time points. Patients were started on ART at week 0 and discontinued at week 12. Plasma samples were available for 22 patients at week 0 and for 25 patients all other time points. Assay failure occurred for 7/27 fibrinogen samples and 1/27 D-dimer samples at time point 1. One fibrinogen sample failed at each of time points 2 and 3, and one D-dimer assay failed at time point 4. IL-6: n=22 week 0, n=25 weeks 12-48; D-dimer: n=21 week 0, n=25 weeks 12 and 20, n=24 week 48; hs-CRP: n=22 week 0, n=25 weeks 12-48; fibrinogen: n=15 week 0, n=24 week 12 and 20, n=25 week 48

Changes over time in patients receiving ART (n=25)

All patients receiving ART achieved a plasma HIV RNA of <100 copies/ml by 12 weeks of ART, and 23 (92%) achieved an HIV RNA of <50 copies/ml by this time point. Figure 3.3 demonstrates biomarker changes for log₁₀ transformed results across the 4 study time points in patients receiving ART.

Changes in biomarkers were assessed using Wilcoxon signed rank test. There was a statistically significant decrease in plasma IL-6 concentration in treated patients corresponding with viral suppression 12 weeks after starting ART ($p=0.01$). Following interruption of ART, at week 20, there was a further decline in IL-6 ($p=0.002$) despite a rebound in plasma HIV RNA. At one year, or 9 months after stopping ART, IL-6 levels had rebounded when compared to week 20 ($p=0.04$), but were not significantly higher than those whilst virally suppressed ($p=0.4$) and remained significantly lower than baseline values ($p=0.01$). There was a significant reduction in D-dimer after 12 weeks of ART ($p=0.01$). Once ART was stopped D-dimer levels appeared static at 8 weeks ($p=0.3$). At 9 months there was no significant rise in D-dimer when compared to week 20 ($p=0.7$) and levels remained significantly lower than baseline ($p=0.02$). There was no significant change in hs-CRP across the 4 time points in treated individuals (change on starting ART $p=0.8$, change on stopping ART $p=0.7$, change at 9 months compared to week 20 $p=0.6$). There was no change in fibrinogen on starting ART ($p=0.4$), however there was a rise on stopping ART ($p=0.046$). At 9 months there was no further significant changes compared to week 20 ($p=0.4$) although levels remained significant higher than while suppressed on ART ($p=0.005$) and similar to baseline levels ($p=0.3$).

Figure 3.3. Pro-inflammatory and coagulatory biomarkers in patients receiving 3 months ART at PHI diagnosis



Log₁₀ transformed biomarkers (median, IQR and upper and lower adjacent values) in 25 patients treated with ART for 3 months at Primary HIV-1 diagnosis are shown across 4 study time points. ART was prescribed at HIV-1 diagnosis (week 0) and discontinued at week 12. Changes in biomarkers were assessed using Wilcoxon signed rank tests.

(a) IL-6; n=22 week 0, n=25 weeks 12-48 (b) D-dimer; n=21 week 0, n=25 weeks 12 and 20, n=24 week 48 (c) hs-CRP; n=22 week 0, n=25 weeks 12-48 (d) fibrinogen; n=15 week 0, n=24 week 12 and 20, n=25 week 48

Changes over time in untreated participants with PHI (n=5)

There were no significant changes in IL-6, D-dimer, hs-CRP or fibrinogen levels across the time points in the 5 patients who declined ART. Although not significant, median levels of biomarkers levels did decrease at week 12 compared to baseline (Table 3.4). It was not possible to compare biomarker changes in those receiving therapy to those who declined due to small numbers of untreated patients.

Table 3.4. Inflammatory biomarkers and HIV-1 parameters in untreated patients with PHI

Time point		Week 0 (1 st clinic visit)	Week 12	Week 20	Week 48
IL-6 pg/ml	median	1.21	0.64	1.38	1.42
	(IQR)	(0.52, 2.09)	(0.47, 1.81)	(0.55, 3.85)	(0.50, 1.92)
D-dimer mg/L	median	0.24	0.23	0.14	0.13
	(IQR)	(0.12, 0.35)	(0.15, 0.31)	(0.1, 0.39)	(0.12, 0.42)
hs-CRP mg/L	median	1.0	0.50	0.30	1.50
	(IQR)	(0.30, 2.9)	(0.25, 1.70)	(0.20, 1.05)	(0.60, 1.70)
Fibrinogen g/L	median	2.13	1.88	1.91	2.27
	(IQR)	(1.86, 2.21)	(1.79, 2.01)	1.81, 1.93)	(1.90, 2.37)
VL copies/ml	median	5577	11935	15767	35823
	(IQR)	(1317, 34948)	(8326, 47135)	(4170, 53144)	(3661, 37484)
CD4 cells/ μ l	median	530	630	510	500
	(IQR)	(448, 630)	(540, 720)	(405, 665)	(395, 670)

Median levels and IQR of IL-6, D-dimer, CD4 cell count and VL (VL) in 5 individuals with PHI who declined therapy are shown across 4 study time points

Long-term follow-up

Subjects were followed up for a median 5.35 years (IQR 4.9, 5.4 years); total 160.5 person years follow-up. 24/27 (88%) of subjects with baseline biomarker levels commenced on long-term ART. The median time to starting ART was 2.9 years (IQR 1.6, 5.1 years). Cox proportional hazards regression was used to assess whether baseline biomarkers, CD4 cell count and VL were associated with time to starting long-term ART (Table 3.5). Variables were $\log_{10}$ transformed for analysis purposes. A one log increase in VL was associated with a 36% increase risk of starting ART (Hazard Ratio 1.36, 95% CI 1.12, 1.64 $p=0.002$). There were no association between biomarker levels at baseline and starting ART in this analysis, which is limited by small numbers.

Table 3.5. Relationship between baseline parameters and time to starting long-term ART in 27 participants with PHI

Variable	Hazard Ratio	95% CI	p-value
VL	1.36	1.12, 1.64	0.002
CD4	0.48	0.17, 1.34	0.16
IL6	0.99	0.93, 1.06	0.8
D-dimer	1.03	0.87, 1.25	0.4
Hs-CRP	0.98	0.9, 1.04	0.6
Fibrinogen	0.66	0.25, 1.74	0.4

Hazard ratio, 95% CI and p-value are shown for effect of baseline parameters at time of PHI diagnosis (VL, CD4 cell count and biomarker levels) on time to starting long-term ART. 27 participants with available results at baseline were included.

Summary of pilot seroconverter study

Frozen plasma samples obtained from participants enrolled in a pilot study of short-course ART in PHI and stored for a median of 6.3 years, were successfully analysed for inflammatory and coagulation biomarkers. Levels of IL-6 and D-dimer significantly declined in parallel with starting ART in PHI, however a decline was also observed in untreated individuals at week 12. On discontinuing ART there was a delay in rebound of plasma biomarkers. Hs-CRP levels did not change significantly over the course of the study. A high proportion of fibrinogen assays failed. There was a significant rise in fibrinogen on stopping ART. Baseline HIV RNA was the only variable associated with time to starting long-term ART in this pilot study.

Discussion

Several factors have been shown to influence the outcome of assays measuring cytokine and coagulation markers. Differences in methods of sample collection and processing of samples, including type of anticoagulant used, may affect assay results and limit comparability between laboratories (Aziz, et al. 1998; De Jongh, et al. 1997). Although multiple freeze-thaw cycles should be avoided, long-term freezing is thought to be a reliable method of sample storage (Aziz, et al. 1999a). Aziz et al. (1999) demonstrated that many cytokine and immune activation analytes, measured in HIV-1 infected samples, were stable under several different storage conditions, and concluded that batch testing of frozen stored samples for cytokine assessment was feasible. A study measuring short and long-term stability of D-dimer assays in citrated plasma samples showed short-term stability for at least 24h at room temperature and at 4–8°C as well as long-term stability for samples stored up to 3 years at $\leq -60^{\circ}\text{C}$ (Bohm-Weigert, et al. 2010). However, the effects of long-term freezing (up to 7 years) on plasma biomarker assays for IL-6 and D-dimer, and the effects of shorter term freezing on specific assays used in this thesis are unknown.

This study demonstrates the feasibility of measuring biomarker levels from frozen plasma that has been stored for up to 7 years. Firstly, we assessed the effect of freezing for a one year period on IL-6 and D-dimer results. Samples were assayed within one month of collection for IL-6 and repeated after one year of sampling. Results were highly correlated and showed good agreement. D-dimer was measured immediately using fresh citrated plasma and repeated after 1 year from frozen EDTA plasma. Although there was a highly significant correlation between the measurements, D-dimer results were lower after one year. This could reflect a difference in anticoagulant between 1st and 2nd measurements in this analysis or be related to the temperature of storage (-20°C versus -60°C compared to the study by Bohn-Weigert et al.). This potential difference should be taken into account when comparing D-dimer results between studies using fresh citrated plasma, commonly used for D-dimer measurement in routine NHS care, and frozen plasma collected by other methods for research purposes.

This analysis is limited by several factors. For logistical reasons, samples were stored at -20°C and may be better preserved at colder temperatures. In addition we were only able to assess the effects of freezing over a one year period due to time constraints, although samples assessed in this thesis were stored for longer time periods. For D-dimer a different anticoagulant was used for the 1st and second assays, and measuring initial D-dimer levels from EDTA plasma may have been preferable. Due to cost and logistic restraints associated with measuring individual IL-6 samples by ELISA, assays were batched and measured within one month. We were therefore unable to measure this

biomarker from fresh plasma. However, this study provides reassuring data that although these biomarkers may be moderately affected by freezing and storage conditions, results are highly correlated and generally comparable.

We next analysed samples obtained from a pilot study where ART was offered to individuals with PHI. This pilot biomarker study demonstrates the feasibility of measuring biomarkers from EDTA plasma samples which have been frozen for a longer time period (median 6.3 years). Results were obtained for the majority of samples for D-dimer, IL-6 and hs-CRP. A high proportion of samples measuring fibrinogen failed, in particular samples at the earliest time point, indicating that this assay is less amenable to such long-term storage. Following this pilot work, the effects of short-course ART in PHI on IL-6 and D-dimer were further investigated in a larger powered study, SPARTAC (chapter 4). Fibrinogen analysis was not undertaken in the SPARTAC population based on the problems identified in this pilot study. Hs-CRP was not analysed in SPARTAC samples due to cost constraints.

This pilot study shows significant changes in IL-6 and D-dimer after 12 weeks on therapy. This work is underpowered to give a clear evaluation of the effects of treatment in PHI; hence it is not possible to draw firm conclusions from these data. Although there were no significant changes over time in untreated patients, it appears that reductions in biomarkers levels also occur in untreated patients at 12 weeks. The small numbers of untreated subjects mean that it is not possible to evaluate the natural history of biomarker changes in untreated PHI or infer whether the changes seen in this study in treated patients are due to ART or due to the natural history of HIV-1 infection.

In this pilot study, levels of IL-6 and D-dimer do not immediately rebound following interruption of short-course ART commenced in PHI, although they did begin to rise by week 48. Biomarkers did not rebound to pre-treatment levels and this may reflect high levels of IL-6 and D-dimer at the time of PHI associated with intense viral replication and immune activation. Larger studies are needed to assess the natural history of these biomarkers in PHI. The unexpected delayed rise observed in IL-6 and D-dimer compared with the reported change in these markers from the SMART study may be confounded by the small numbers in the study. In addition, the ART regimens used in the pilot study may impact on delayed biomarker changes: nearly 60% of pilot study participants were started on NNRTI based regimens which have longer plasma half life, compared to PI based regimens. It is possible that there may be a prolonged effect of the drug, even on ART discontinuation. In later studies, such as SPARTAC, NNRTI based regimens were not the preferred regimen due to the risk of developing resistance associated with essential monotherapy during the NNRTI washout period, as well as known toxicities associated with nevirapine in patients with higher CD4 cells counts, and higher numbers of female patients particularly at African sites, meaning efavirenz was not

recommended. We observed a significant rise in fibrinogen levels on discontinuing ART, in keeping with the SMART study findings of raised biomarkers on stopping ART, although fibrinogen was not specifically measured in SMART.

No significant changes were seen in hs-CRP over time in this study. This may be due to small numbers in the study, the short duration of treatment or due to a lack of hs-CRP response to ART. Calmy et al.(2009) saw no change in hs-CRP on starting and stopping ART. In the SMART study there was a decrease in hs-CRP, compared to baseline, in individuals commencing ART who achieved HIV RNA<400 copies/ml at six months (Baker, et al. 2010), but no difference in hs-CRP was seen at one month in individuals undergoing treatment interruption compared to those on continuous therapy (Kuller, et al. 2008) .

The level of T-cell activation in PHI is predictive of disease progression (Deeks, et al. 2004) , and a recent study of 40 South African women found a small panel of plasma cytokines (IL-7, eotaxin and IL-1 α) in PHI to be associated with rate of CD4 cell loss (Roberts, et al. 2010). We assessed the impact of HIV-1 parameters and baseline biomarker values on long-term disease progression as assessed by time to starting ART. It is known that HIV RNA levels in PHI and at viral set point predict disease progression (Lyles, et al. 2000; Mellors, et al. 1995) and in this analysis HIV RNA in PHI was significantly associated with time to starting ART. There was no association between baseline biomarker levels and time to starting ART. However, this analysis is limited by small numbers and will be expanded to a larger cohort of individuals with PHI (SPARTAC) once long-term follow-up data from these individuals are available.

In this non randomized study, clear baseline differences between those who decline and accept therapy are observed, despite the small number of patients who declined therapy. Patients who declined therapy had lower plasma VL at baseline and had a trend towards fewer symptoms. Many ART studies in PHI are in non-randomized cohorts, and these data demonstrate the importance of a randomized study to assess effects of short-course ART in PHI, in order to eliminate bias associated with reason for starting or stopping therapy. Therefore, in order to gain a clear understanding of the effects of PHI on IL-6 and D-dimer, and to assess the effects of stopping and starting ART in this study, this work was expanded to the SPARTAC study in Chapter 4. SPARTAC has the largest existing cohort of randomized participants recruited in early infection, and its randomized design represents an ideal setting in which to investigate effects of treatment in PHI on immunological mechanisms and outcomes. This presents an opportunity to investigate inflammatory responses associated with PHI and the effects of short-course ART on pro-inflammatory and coagulatory biomarkers.

Chapter 4: Interleukin-6 and D-dimer in PHI and the effect of ART

Introduction

Individuals with chronic HIV-1 infection, both treated and untreated, have raised levels of IL-6 and D-dimer compared with HIV-negative controls (Neuhaus, et al. 2010). Previous studies in HIV-1 infected and HIV-negative individuals demonstrate that raised levels of these biomarkers are associated with considerable morbidity and mortality (Ford, et al. 2010; Kuller, et al. 2008; Ridker, et al. 1994; Ridker, et al. 2000b; Rodger, et al. 2009).

The SMART trial investigated the effect of treatment interruption in chronic HIV-1 infection. Episodic use of ART, guided by CD4 cell count, was compared to the current practice of continuous therapy. The study found that risk of opportunistic disease or death was more than twice as great (HR 2.6, $p < 0.001$) in those who discontinued ART (drug conservation arm) compared to those who continued therapy (viral suppression arm). The drug conservation arm was associated with an 84% ($p = 0.007$) increased risk of all-cause mortality including an increased risk of fatal cardiovascular events (El-Sadr, et al. 2006). Interruption of ART in chronic HIV-1 infection was associated with significant rises in IL-6 and D-dimer four weeks following ART discontinuation (Kuller, et al. 2008). Kuller et al. hypothesized that the observed adverse outcome following ART interruption was due to activation of inflammatory and fibrinolytic pathways, secondary to increases in HIV RNA. Increases in IL-6 and D-dimer were significantly associated with magnitude of VL rebound and in a case control analysis comparing those who survived to those who died, baseline biomarker levels were predictive of all cause mortality. These findings have led to a recommendation that ART is not discontinued in chronic HIV-1 disease (Gazzard, et al. 2008).

It is unknown whether interruption of ART initiated in PHI elicits the same response in IL-6 and D-dimer levels as seen in SMART. Acute HIV-1 infection is associated with an intense cytokine storm which may promote ongoing immune activation, viral replication and CD4 cell loss (Stacey, et al. 2009). This stage of infection is associated with massive viral replication, predominantly in the gut, where it is estimated that over one-half of the body's effector CD4 T-cell reserves are irreversibly lost (Mattapallil, et al. 2005). In chronic HIV-1 infection, long-term immune activation, in addition to viral replication and / or ART exposure have been linked to ongoing inflammation, with clinical implications including an increased risk of CVD (Grunfeld, et al. 2009). This has been demonstrated in studies linking increased carotid IMT in HIV-1 infected individuals to high CRP levels (Hsue, et al. 2009). PHI represents a very different stage of HIV-1 mediated disease to chronic infection, and the absence of long-term immunological damage means that biomarker changes seen in PHI may differ to those seen in chronic disease. IL-6 and D-dimer are known to confer an increased risk of mortality

in chronic HIV-1 infection; however there are no data examining factors associated with these biomarkers in early HIV-1 infection, or assessing the impact of short-course ART initiated in PHI. It is possible that interrupting therapy following treatment in PHI is a safe intervention due to the short duration of HIV-1 infection and short exposure to ART, and that short-course ART itself in PHI has a beneficial effect attenuating levels of immune activation and inflammation following discontinuation of therapy. Conversely, it may be that high levels of immune activation and inflammation triggered by PHI confer an increased risk of biomarker rebound when treatment is discontinued.

The SPARTAC trial is currently investigating the impact of short-term ART in PHI on long-term disease progression. Three-hundred-and-sixty-six participants identified as having PHI were randomised to one of three arms: no treatment, 12 weeks ART or 48 weeks ART. Therefore all subjects who were randomised to one of the 2 treatment arms (n=238) underwent protocol-indicated treatment interruption following completion of short-course therapy.

The aims of this chapter were to:

- i. summarise baseline IL-6 and D-dimer levels in PHI, and to compare biomarker levels in PHI to those at SPARTAC week 60 and to HIV-negative individuals
- ii. assess factors associated with IL-6 and D-dimer in PHI
- iii. evaluate changes in biomarker levels after 12 weeks on ART commenced in PHI and assess factors associated with change in biomarker on starting ART
- iv. evaluate changes in biomarker levels after stopping therapy initiated in PHI and assess factors associated with change in biomarker on cessation of ART
- v. compare biomarker changes associated with stopping ART commenced in PHI to those seen in the SMART trial.

Results

4.1. IL-6 and D-dimer in PHI

Stored plasma samples from the baseline SPARTAC study visit were used to assess levels of IL-6 and D-dimer in PHI. 225 participants were enrolled into SPARTAC outside Africa and data from trial participants in Europe, Australia, and Brazil, were included. No data from African sites were included due to constraints in shipping samples in time for this analysis, and in addition, to focus inclusion towards MSM cohorts with clade B HIV-1 infection in order to enable more accurate comparison with the SMART population. Baseline samples from 201 participants were available.

Baseline characteristics of SPARTAC participants included in biomarker analysis

Baseline characteristics of 201 individuals with available plasma for biomarker analysis are shown in Table 4.1. Subjects were predominantly MSM with subtype B virus. The median estimated time from seroconversion at randomisation, calculated by randomisation date minus the estimated seroconversion date, was 71.5 days. The median CD4 cell count at baseline was 550 cells/ μ l and median VL was 44,407 copies/ml. Of note, 13 (6.5%) participants had VL <400 copies/ml prior to initiation of ART. The majority of individuals (64%) were recruited from UK sites with 18%, 9.5% and 8.5% recruited from Australia, Italy and Brazil respectively.

Table 4.1. Baseline characteristics in 201 SPARTAC participants

		Number or Median	(%) or [IQR]
Sex:	Male	192	(96)
	Female	9	(4)
Risk group:	MSM	181	(90)
	Heterosexual	18	(9)
	Not known	2	(1)
Age (years)		34.7	[29.4,42.6] [range 19.6, 62.9]
Subtype	B	178	(92)
	Non-B	16	(8)
Time from est. SC to baseline visit (days)		71.5	[49, 94]
BMI (kg/m ²)		23.5	[21.7, 25.4]
Systolic blood pressure (mmHg)		125	[117, 135]
Diastolic blood pressure (mmHg)		76	[68, 82]
CD4 (cells/ μ l) overall		550	[432, 711]
<350		19	(9.5)
350-499		53	(26.5)
500+		128	(64)
HIV RNA (copies/ml)	Overall	44407	[6371, 165855]
	<400	13	(6.5)
	400-9,999	44	(22)
	10,000-99,999	72	(36)
	$\geq$ 100,000	71	(35.5)
Log ₁₀ HIV RNA (copies/ml)		4.7	[3.8, 5.2]
SPARTAC site	UK	129	(64)
	Brazil	17	(8.5)
	Australia	36	(18)
	Italy	19	(9.5)

Baseline demographics of 201 SPARTAC participants are shown alongside estimated time from seroconversion, CD4 cell count and VL. The time from seroconversion at randomisation was estimated by randomisation date minus the estimated seroconversion date. Subtype unavailable for 7 participants, CD4 cell count and VL unavailable in 1 participant

IL-6 and D-dimer at baseline SPARTAC visit (week 0), week 60 and in healthy controls

In order to investigate longitudinal changes in IL-6 and D-dimer in SPARTAC participants, a comparison of biomarker levels between week 0 to week 60 was undertaken. Baseline plasma samples at week 0 of the study were available from 201 individuals and from 193 individuals at week 60. SPARTAC participants were randomised to either no treatment, 12 weeks ART or 48 weeks ART; therefore all individuals had discontinued the ART given as a trial intervention by week 60. Due to the blinded nature of the SPARTAC trial, week 60 results were analysed together regardless of randomisation arm, and the impact of different lengths of ART used in the SPARTAC study (12 or 48 weeks therapy) will be addressed at the end of the trial once the data is unblinded. Biomarker levels in 11 healthy individuals were analysed to assess whether these biomarkers are higher in HIV-1 infected individuals compared to HIV-negative controls.

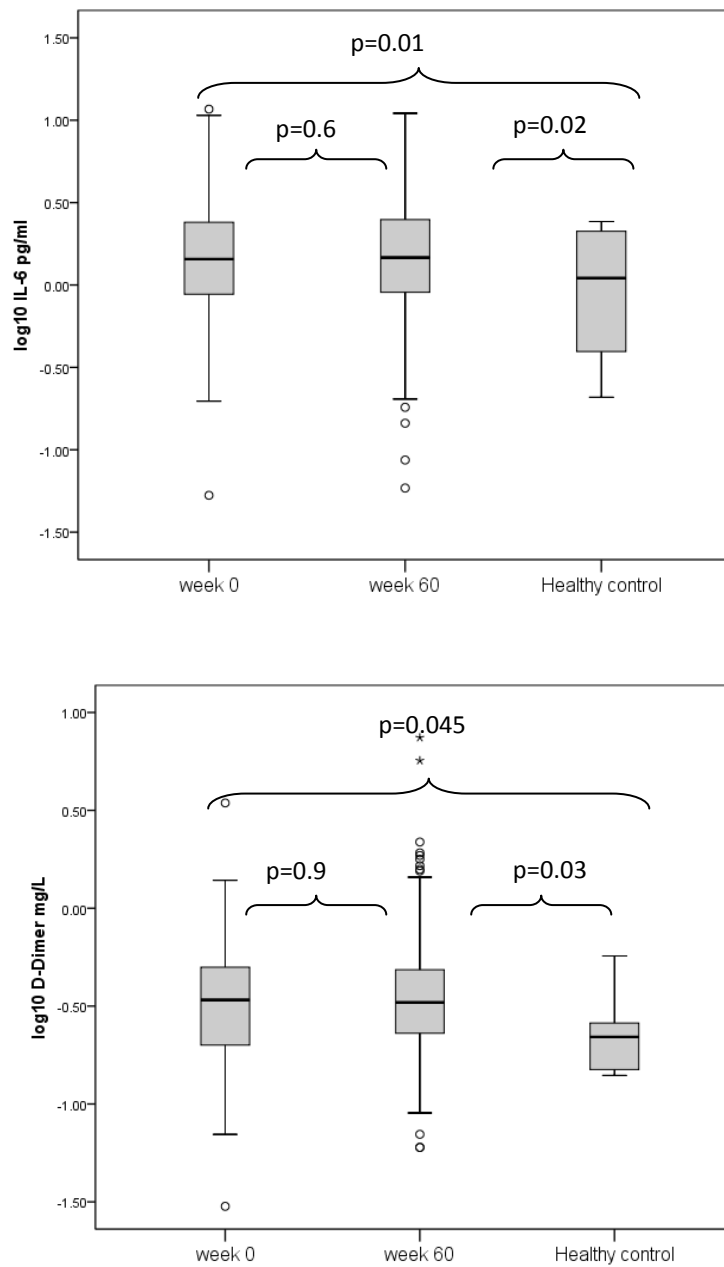
Median levels and IQR are shown in Table 4.2 and $\log_{10}$ transformed data are demonstrated visually by box-plot graphs in Figure 4.1. The median levels of IL-6 and D-dimer were 1.43 pg/ml and 0.34 mg/L respectively at PHI (baseline) and 1.45 pg/ml and 0.33mg/L respectively at week 60. Differences in biomarkers at week 60 were compared to week 0 using the Wilcoxon signed rank test. There was no difference in either IL-6 or D-dimer levels between week 0 and week 60. IL-6 and D-dimer at week 0 and week 60 were compared to healthy controls using Mann-Whitney U tests. IL-6 was significantly higher at both week 0 ($p=0.01$) and week 60 ($p=0.02$) than in healthy controls. Levels of D-dimer were also significantly higher at both week 0 and week 60 than in healthy controls (week 0 $p=0.045$; week 60 $p=0.03$).

Table 4.2. IL-6 and D-dimer at SPARTAC week 0, week 60 and healthy controls

	Week 0			Week 60			Healthy controls		
	N	Median	[IQR]	N	Median	[IQR]	N	Median	[IQR]
IL-6 pg/ml	201	1.43	0.9, 2.4	193	1.45	0.9, 2.5	11	0.54	0.2, 1.7
D-dimer mg/L	192	0.34	0.2, 0.5	192	0.33	0.2, 0.5	11	0.2	0.15, 0.3

Median values and IQR are shown for IL-6 and D-dimer at week 0, week 60 and in healthy controls. Plasma samples were available for 201 individuals at week 0 and 193 individuals at week 60. D-dimer assay failure occurred in 9 samples at week 0 and 1 sample at week 60.

Figure 4.1. IL-6 and D-dimer levels at SPARTAC week 0 (PHI), week 60 and in healthy controls



Box-plots showing median, IQR and upper and lower adjacent values for IL-6 and D-dimer in SPARTAC participants at week 0 and week 60, and in HIV-negative controls. Week 0 was compared to week 60 using Wilcoxon signed ranks test for paired samples. Mann-Whitney U tests were used to compare healthy controls to SPARTAC participants

4.2 Factors associated with IL-6 and D-dimer in PHI (SPARTAC week 0)

The association between baseline IL-6 and D-dimer and baseline characteristics (age, sex, duration of infection, CD4 cell count, HIV RNA, systolic blood pressure, diastolic blood pressure and BMI) was examined using linear regression. Due to skewed distributions, both IL-6 and D-dimer were $\log_{10}$ transformed to achieve normality for analysis purposes. The association between baseline $\log_{10}$ IL-6 and $\log_{10}$ D-dimer and other baseline variables is shown in Table 4.3.

Baseline $\log_{10}$ IL-6 was significantly associated with age and HIV RNA levels. A 1 log increase VL was associated with a 16% increase in IL-6 and a 10 year increase in age associated with a 16% increase in IL-6. Baseline $\log_{10}$ D-dimer was associated with HIV RNA and age on univariate analysis, with a trend towards lower levels in female participants, individuals with higher CD4 cell counts, and higher levels closer to seroconversion. On multivariate analysis HIV RNA and age remained significant. A 17% rise in D-dimer was seen for every 10 year increase in age and a 31% increase in D-dimer was seen for every 1 log increase in HIV RNA.

To further explore the relationship between plasma VL at baseline and biomarker levels, analysis of a further breakdown of the biomarkers by baseline VL was undertaken. IL-6 and D-dimer were assessed according to VL strata as shown in Figure 4.2. For IL-6, patients with HIV RNA >400 copies/ml had increased biomarker levels with increasing VL strata. Thirteen individuals had HIV RNA <400copies/ml at baseline, in the absence of ART, and these individuals had higher than expected IL-6 levels. For D-dimer there was a linear relationship between increasing D-dimer and increasing VL strata.

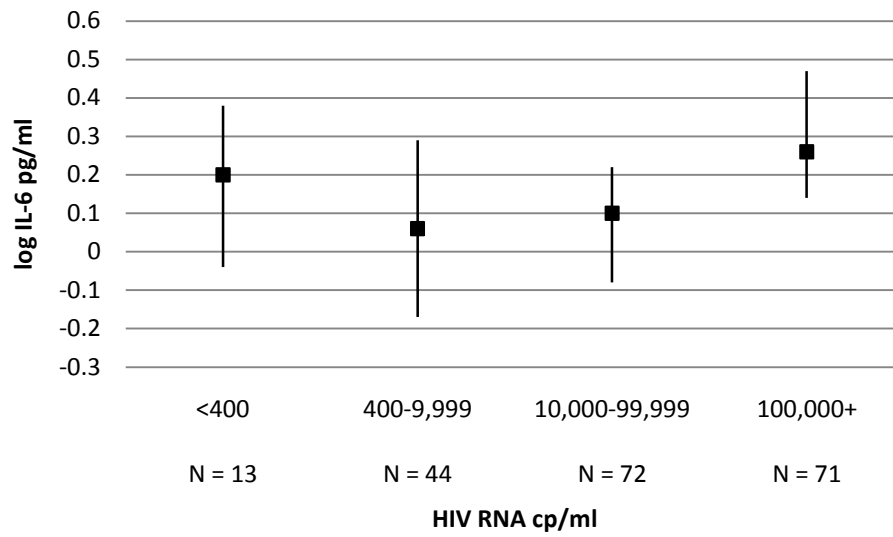
Table 4.3. Linear regression models for baseline IL-6 and D-dimer

Marker	Baseline variable	Univariate			Multivariate		
		B Coefficient	p value	95% CI	B Coefficient	p value	95% CI
Log ₁₀ IL-6 (n=201)	Age (+10 years)	0.07	0.01	0.01, 0.12	0.06	0.03	0.004, 0.11
	Sex (female)	-0.028	0.8	-0.27, 0.22			
	BMI (+1 kg/m ²)	-0.005	0.6	-0.02, 0.01			
	Time from SC (+1 week)	0.002	0.8	-0.009, 0.01			
	Systolic BP (+ 1 mmHg)	0.000	0.9	-0.004, 0.004			
	Diastolic Bp (+ 1 mmHg)	0.001	0.7	-0.004, 0.006			
	CD4 (+100 cells)	-0.02	0.1	-0.04, 0.004			
	Log₁₀ HIV RNA (+ 1 log)	0.07	0.007	0.02, 0.12	0.06	0.01	0.004, 0.11
Log ₁₀ D-dimer (n=192)	Age (+10 years)	0.07	0.001	0.03, 0.14	0.05	0.02	0.01, 0.09
	Sex (female)	-0.19	0.06	-0.39, 0.01			
	BMI (+1 kg/m ²)	0.001	0.9	-0.1, 0.02			
	Time from SC (+1 week)	-0.008	0.09	-0.02, 0.001			
	Systolic BP (+ 1 mmHg)	0.002	0.17	-0.001, 0.006			
	Diastolic Bp (+ 1 mmHg)	0.001	0.6	-0.003, 0.005			
	CD4 (+100 cells)	-0.02	0.09	-0.04, 0.003			
	Log₁₀ HIV RNA (+ 1 log)	0.12	<0.001	0.08, 0.15	0.1	<0.001	0.07, 0.15

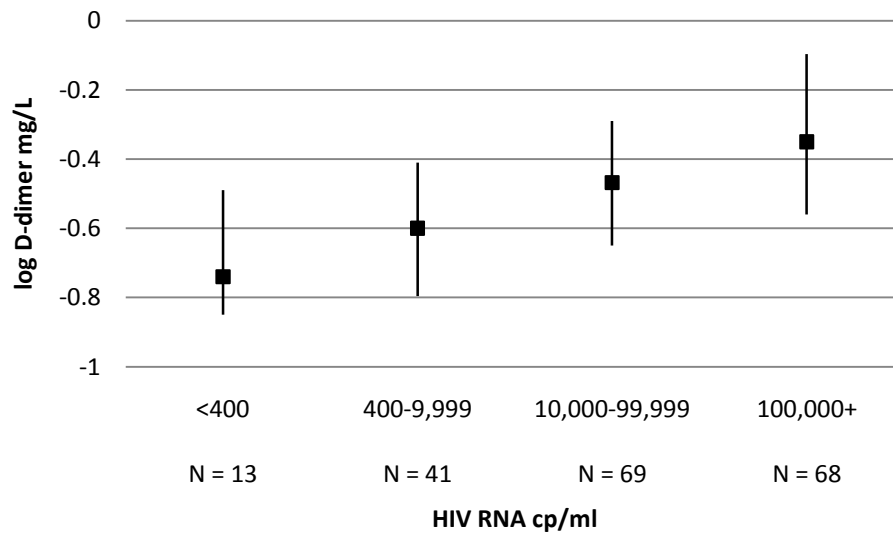
Factors associated with IL-6 and D-dimer at baseline study visit are shown. IL-6 and D-dimer were log₁₀ transformed to achieve normality. B (regression coefficient), 95% confidence interval and p-value are given. For multivariate models, variables were included if the p-value was <0.1 in univariate models.

Figure 4.2. IL-6 and D-dimer levels by VL strata in individuals with PHI

a) IL-6



b) D-dimer



IL-6 and D-dimer levels (median and IQR) are shown for each VL strata. IL-6 and D-dimer are $\log_{10}$ transformed.

4.3 Effect of ART on IL-6 and D-dimer levels

In order to investigate the effects of short-course ART on IL-6 and D-dimer levels in PHI, data from the two treatment arms of SPARTAC were combined and analysed. Participants were included in this analysis if they were randomised to start therapy for either 12 or 48 weeks as per the SPARTAC study protocol, if it was confirmed that they received ART and if available longitudinal stored plasma was available for biomarker analysis. IL-6 and D-dimer levels were analysed from stored EDTA plasma samples at baseline, 12 weeks after starting ART, at ART cessation (combined week 12 and week 48 data according to arm), and 4 weeks after cessation (combined week 16 and week 52 data according to arm). The time point of 4 weeks after stopping therapy was chosen in order to allow comparison with the published SMART trial data of treatment interruption in chronic HIV-1 infected subjects.

Effect of 12 weeks ART on IL-6 and D-dimer levels

Excluding African sites, 150 participants were randomised to start therapy for either 12 or 48 weeks as per the SPARTAC study protocol and were confirmed to have received ART. Of these, plasma samples were available at both week 0 and week 12 in 115 individuals. Paired baseline and week 12 biomarker levels for these individuals are presented in Table 4.4.

103 (90%) of participants suppressed HIV RNA <400 copies/ml at week 12. Differences in biomarkers at week 12 were compared to zero using the Wilcoxon signed rank test. On commencing ART, there was significant decline of 0.22pg/ml in the median IL-6 and a significant decline of 0.05 mg/L in the median D-dimer from baseline to week 12.

The association of change in biomarkers after 12 weeks on ART with baseline covariates (age, sex, risk group, duration of infection, CD4 cell count, log₁₀ HIV RNA, systolic blood pressure, diastolic blood pressure, change in CD4 cell count, HIV RNA suppression at week 12, and change in HIV RNA) was studied using linear regression to adjust for baseline and time dependant variables. Table 4.5 shows factors associated with change in IL-6 and D-dimer after 12 weeks of ART.

On univariate analysis, there was a trend toward an association between change in HIV RNA and change in IL-6 with larger change in HIV RNA being associated with larger fall in IL-6 levels. On multivariate analysis, a higher baseline CD4 cell count was associated with a larger decrease in Log₁₀ IL-6 at week 12. Baseline IL-6 levels were strongly associated with change in IL-6, with an increase of 1 log₁₀ IL-6 being associated with a -0.07pg/ml greater decrease in log₁₀ IL-6 after 12 weeks (an additional 17% decrease in absolute IL-6).

For D-dimer, on univariate analysis, HIV RNA, BMI and both systolic and diastolic blood pressure were significantly associated with change in D-dimer level on commencing ART. Higher HIV RNA levels were associated with a greater reduction in HIV RNA, and those with higher blood pressure and higher BMI had smaller reductions in D-dimer levels. In addition baseline D-dimer values were strongly associated with change in D-dimer, and there was a trend towards an association between change in D-dimer and change in HIV RNA levels. On multivariate analysis, BMI remained significant with a higher baseline BMI being associated with a smaller decrease in $\log_{10}$ D-dimer. In addition baseline levels of D-dimer remained highly significant predictors of change in D-dimer on ART. An increase of 1 $\log_{10}$ in baseline D-dimer was associated with a -0.06pg/ml greater decrease in $\log_{10}$ D-dimer after 12 weeks (an additional 15% decrease in absolute D-dimer). The changes in both $\log_{10}$ IL-6 and $\log_{10}$ D-dimer from baseline to week 12 were very strongly associated with baseline values with higher baseline values resulting in a greater decrease than lower values.

Table 4.4. IL-6 and D-dimer after 12 weeks on ART (n=115)

Marker	N	Baseline		+ 12 weeks		Change from baseline		p-value
		Med	IQR	Med	IQR	Med	IQR	
IL-6	115	1.39	[0.83, 2.39]	1.17	[0.67, 1.75]	-0.22	[-0.99, 0.35]	0.01
$\log_{10}$ IL-6	115	0.14	[-0.08, 0.38]	0.07	[-0.17, 0.24]	-0.14	[-0.30, 0.18]	0.01
D-dimer	112	0.34	[0.20, 0.51]	0.31	[0.17, 0.43]	-0.05	[-0.16, 0.06]	0.003
$\log_{10}$ D-dimer	112	-0.47	[-0.69, -0.29]	-0.51	[-0.76, -0.37]	-0.08	[-0.22, 0.09]	0.003

$\log_{10}$ transformed and absolute levels of IL-6 and D-dimer (median and IQR), and median change after 12 weeks ART, are shown. Participants from both SPARTAC treatment arms are included (patients randomised to either 12 or 48 weeks ART). Note D-dimer assay failure occurred in 3 participants

Table 4.5. Linear regression models for change in IL-6 and D-dimer after 12 weeks on ART

	Baseline variable (or change at week 12)	Univariate			Multivariate		
		B	p-value	95% CI	B	p-value	95% CI
Log ₁₀ IL-6	Baseline Log₁₀ IL-6 (+ 0.1)	-0.07	<0.001	[-0.08, -0.05]	-0.07	<0.001	[-0.08,-0.05]
	Sex (Female)	0.19	0.40	[-0.27, 0.65]			
	Risk group (MSW)	0.14	0.29	[-0.12, 0.40]			
	(Not known)	0.23	0.42	[-0.33, 0.79]			
	Age (+10 years)	0.00	0.61	[-0.01, 0.01]			
	BMI (+1 kg/m ²)	0.01	0.32	[-0.01, 0.04]			
	Time from SC (+1 week)	0.00	0.73	[-0.02, 0.01]			
	Systolic BP (+ 1 mmHg)	0.00	0.85	[-0.01, 0.01]			
	Diastolic BP (+ 1 mmHg)	0.00	0.31	[-0.01, 0.00]			
	CD4 (+100 cells)	-0.03	0.10	[-0.07, 0.01]	-0.04	0.009	[-0.07,-0.01]
	Log ₁₀ HIV RNA (+ 1 log)	0.06	0.10	[-0.01, 0.14]			
	Change CD4 (+100)	0.00	0.83	[-0.04, 0.04]			
	Change Log ₁₀ HIV RNA (+1)	-0.07	0.07	[-0.14, 0.01]			
	HIV RNA < 400 wk 12	-0.01	0.91	[-0.25, 0.23]			
Log ₁₀ D-dimer	Baseline Log₁₀ D-dimer (+0. 1)	-0.06	<0.001	[-0.08, -0.04]	-0.06	<0.001	[-0.07,-0.04]
	Sex (Female)	0.18	0.37	[-0.22, 0.59]			
	Risk group (MSW)	-0.02	0.88	[-0.24, 0.21]			
	(Not known)	0.21	0.40	[-0.29, 0.71]			
	Age (+10 years)	0.00	0.28	[0.00, 0.01]			
	BMI (+1 kg/m²)	0.04	0.01	[0.01, 0.06]	0.03	0.01	[0.01, 0.05]
	Time from SC (+1 week)	0.01	0.21	[-0.01, 0.02]			
	Systolic BP (+ 1 mmHg)	0.01	0.04	[0.00, 0.01]			
	Diastolic BP (+ 1 mmHg)	0.01	0.04	[0.00, 0.01]			
	CD4 (+100 cells)	0.02	0.39	[-0.02, 0.05]			
	Log ₁₀ HIV RNA (+ 1 log)	-0.07	0.03	[-0.14, -0.01]			
	Change CD4 (+100)	0.00	0.95	[-0.03, 0.04]			
	Change Log ₁₀ RNA (+1)	0.06	0.07	[0.00, 0.12]			
	HIV RNA < 400 wk 12	0.17	0.11	[-0.04, 0.38]			

Results from linear regression models looking at the change in Log₁₀ IL-6 and Log₁₀ D-dimer from baseline to week 12, and their associations with baseline variables, are shown. IL-6 and D-dimer were log₁₀ transformed to achieve normality. B (regression coefficient), 95% confidence interval and p-value are given. For multivariate models, variables were included if the p-value was <0.1 in univariate models.

4.4 IL-6 and D-dimer after the cessation of ART in individuals achieving VL<400 copies/ml on treatment

To assess the effect of stopping ART on IL-6 and D-dimer data from the two treatment arms were blinded and analysed together. IL-6 and D-dimer were assessed at end of treatment (either week 12 or 48 depending on arm) and 4 weeks after stopping ART. 104 participants had available plasma at the end of ART for biomarker analyses. In order to standardise comparisons only those participants with HIV RNA < 400 copies /ml on treatment were included in this analysis. 96 (92%) participants had suppressed HIV RNA <400 copies/ml at the end of therapy and analyses were restricted to these individuals. Of these, 88/96 (92%) had plasma available for analysis 4 weeks after stopping ART.

Table 4.6 presents paired biomarker data for the individuals included in this analysis and demonstrates the effects of starting and stopping ART in these participants. Median (IQR) biomarker levels are shown at beginning of therapy, after 12 weeks of ART, at the end of therapy and 4 weeks after stopping. Differences in biomarkers at 12 weeks compared to start of ART, and at 4 weeks after the end of treatment compared to end of treatment, were assessed using the Wilcoxon signed rank test. As previously demonstrated, there was a significant decline in IL-6 and D-dimer from baseline to week 12 of therapy. It should be noted that although the median values for D-dimer are the same at baseline and week 12, there was a reduction in mean d-dimer levels at week 12, indicating a skewed distribution of these data (mean [SD] D-dimer 0.43 [0.42] mg/L at baseline versus 0.41[0.75] mg/L at week 12; mean log₁₀ D-dimer -0.48 [0.29] mg/L at baseline vs. -0.54 (0.32)mg/L at week 12).

There was a significant increase of 0.23pg/ml [IQR -0.18, 0.91] in IL-6 from the end of ART to 4 weeks after treatment cessation and a significant increase of 0.04mg/L [IQR -0.06, 0.15] in the D-dimer over the same period. The median [IQR] increase 4 weeks after stopping compared to baseline levels prior to starting therapy was 0.02 [-0.94, 0.78] for IL-6 (p=0.88) and -0.01 [-0.19, 0.12] for D-dimer (p=0.36) i.e. overall for the population tested biomarker levels for IL-6 and D-dimer returned to baseline levels by 4 weeks after stopping treatment.

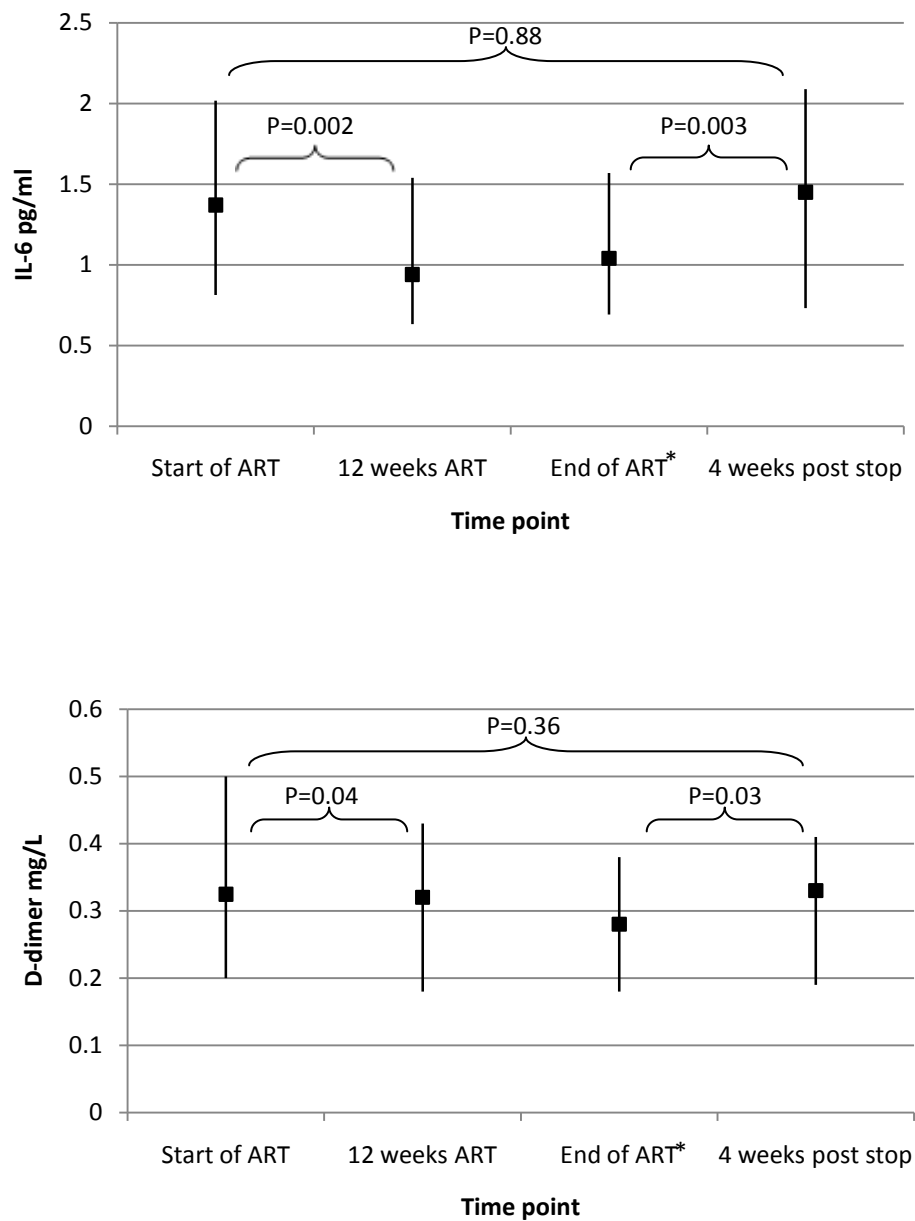
Median biomarker changes on stopping and starting ART in these individuals are further demonstrated in Figure 4.3.

Table 4.6. IL-6 and D-dimer on starting and stopping ART in individuals with HIV RNA <400 copies/ml at time of stopping ART

Marker	Start of ART (Baseline)		12 weeks after starting ART		p-value ¹	End of ART		4 weeks after stopping ART		p-value ²
	Med	IQR	Med	IQR		Med	IQR	Med	IQR	
IL-6	1.37	[0.82, 2.01]	0.94	[0.64, 1.53]	0.002	1.04	[0.70, 1.56]	1.45	[0.74, 2.08]	0.003
Log ₁₀ IL-6	0.14	[-0.09, 0.30]	-0.03	[-0.19, 0.18]	0.004	0.02	[-0.16, 0.19]	0.17	[-0.12, 0.32]	0.001
D-dimer	0.32	[0.20, 0.50]	0.32	[0.18, 0.43]	0.04	0.28	[0.18, 0.38]	0.33	[0.19, 0.41]	0.03
Log ₁₀ D-dimer	-0.49	[-0.71, -0.30]	-0.49	[-0.74, -0.37]	0.03	-0.55	[-0.74, -0.42]	-0.48	[-0.72, -0.39]	0.06

Median biomarker levels on stopping and starting ART are shown in individuals achieving VL<400 copies/ml on therapy with available plasma samples. Biomarkers were measured at baseline (n=96), 12 weeks after starting ART (n=94), at end of ART (either week 12 or week 48 depending on arm (n=96), and 4 weeks after stopping therapy (n=88). ¹ P-value for difference between baseline and week 12. ² P-value for difference between end of ART and + 4 weeks. D-dimer assays failed in 2 participants at end of ART and 2 failed at 4 weeks after stop.

Figure 4.3. The effect of starting and stopping ART in PHI on IL-6 and D-dimer*



Plasma IL-6 and D-dimer levels on starting and stopping ART are shown in 88 individuals who achieved HIV RNA<400 copies/ml by end of ART. Biomarkers were measured at baseline (PHI), 12 weeks after starting ART, at end of ART and 4 weeks after stopping therapy. P-values were derived using Wilcoxon signed rank tests. *Note end of ART data includes results from either week 12 or week 48 depending on randomisation arm.

Factors associated with change in IL-6 and D-dimer on stopping ART

Factors associated with change in biomarker 4 weeks after stopping ART were assessed using linear regression. Table 4.7 presents results from univariate and multivariate regression analyses for change in IL-6 and D-dimer on stopping ART, based on covariates at the end of treatment. The increase in biomarkers does not appear to be associated with any variables at cessation of ART other than the biomarker value at the end of ART. A lower value of IL-6 or D-dimer at the end of ART is associated with a larger increase after cessation. BMI was associated with change in D-dimer on univariate analysis, with a lower BMI at the cessation of ART being associated with a larger increase in D-dimer after cessation of therapy, but this lost significance in the multivariate model. The association between the change in $\log_{10}$ IL-6 and $\log_{10}$ D-dimer with the change in $\log_{10}$ HIV RNA and CD4 cell count is also shown. From the models there does not appear to be any association with the change in VL and the increase in either biomarker. It should be noted that the change in VL is equal to absolute VL at 4 weeks, as all participants included in this analysis were suppressed at the end of ART. The relationship between VL strata and change in biomarker is investigated further in Figure 4.4. It appears that there may be an upwards trend across lower VLs for $\log_{10}$ IL-6 but that those who virologically rebounded to 50,000 copies/ml had smaller than expected increases in $\log_{10}$ IL-6. For D-dimer those with low levels of virological rebound (<400 copies/ml) on stopping ART had larger than expected rebound in D-dimer.

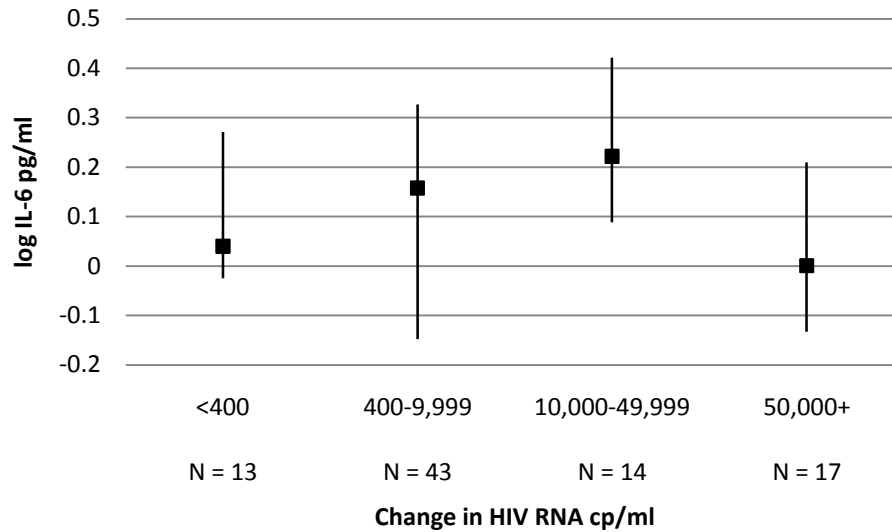
Table 4.7. Linear regression models for change in IL-6 and D-dimer 4 weeks after cessation of ART compared to end of ART

Change in marker	End of ART variable (or change 4 weeks after stop)	Univariate			Multivariate		
		B	p-value	95% CI	B	p-value	95% CI
Log ₁₀ IL-6	Log₁₀ IL-6 (+ 0.1)	-0.05	<0.001	[-0.07,-0.03]	-0.05	<0.001	[-0.07,-0.03]
	Sex (Female)	-0.05	0.82	[-0.45, 0.36]			
	Risk group (MSW)	0.04	0.77	[-0.21, 0.29]			
	(Not known)	-0.09	0.71	[-0.59, 0.41]			
	Age (+10 years)	0.00	0.86	[-0.01, 0.01]			
	BMI (+1 kg/m ²)	-0.01	0.46	[-0.04, 0.02]			
	Time from SC (+1 week)	-0.10	0.29	[-0.29, 0.09]			
	CD4 (+100 cells)	0.00	0.96	[-0.03, 0.03]			
	Change in CD4 (+100)	0.00	0.71	[-0.04, 0.02]			
	Change in log ₁₀ HIV RNA (+1)	-0.03	0.44	[-0.10, 0.04]			
Log ₁₀ D-dimer	Log₁₀ D-dimer (+ 0.1)	-0.05	<0.001	[-0.07,-0.03]	-0.05	<0.001	[-0.07,-0.02]
	Sex (Female)	0.11	0.56	[-0.28, 0.51]			
	Risk group (MSW)	0.11	0.38	[-0.13, 0.34]			
	(Not known)	-0.15	0.54	[-0.63, 0.33]			
	Age (+10 years)	0.00	0.64	[-0.01, 0.01]			
	BMI (+1 kg/m²)	-0.03	0.04	[-0.06, 0.00]	-0.02	0.17	[-0.05, 0.01]
	Time from SC (+1 week)	0.07	0.47	[-0.12, 0.25]			
	CD4 (+100 cells)	-0.01	0.27	[-0.04, 0.01]			
	Change in CD4 (+100)	0.00	0.72	[-0.03, 0.02]			
	Change in Log ₁₀ HIV RNA (+1)	0.05	0.18	[-0.13, 0.31]			

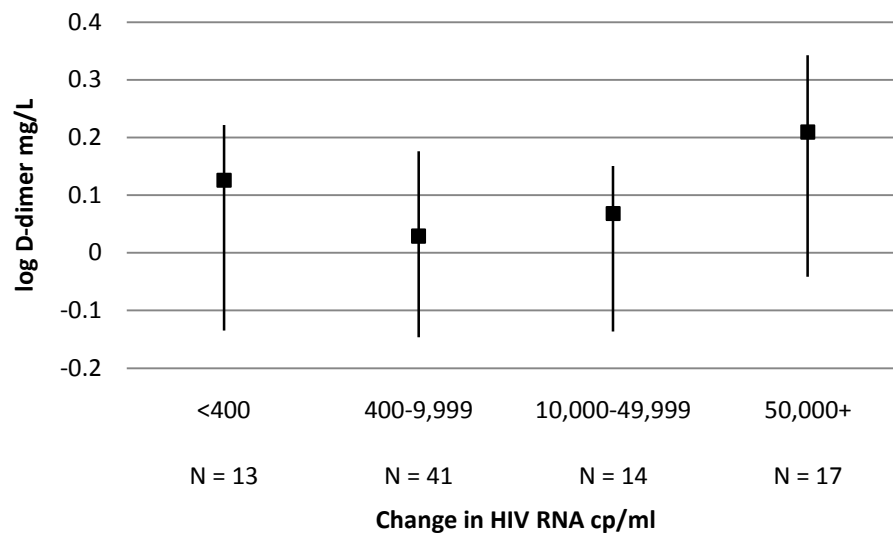
Linear regression analysis of factors associated with change in IL-6 and D-dimer 4 weeks after stopping ART. IL-6 and D-dimer were log₁₀ transformed to achieve normality. B (regression coefficient), 95% confidence interval and p-value are given. For multivariate models, variables were included if the p-value was <0.1 in univariate models.

Figure 4.4. Change in IL-6 and D-dimer 4 weeks after stopping ART by change in VL

a) Change in Log_{10} IL-6



b) Change in Log_{10} D-dimer



Change in IL-6 and D-dimer levels on discontinuing ART (median and IQR) are shown for each VL strata. Although there was no overall association in this cohort between change in biomarkers and change in HIV RNA, there does appear to be a relationship for IL-6 for all except the highest VL strata, and for D-dimer in all but the lowest VL strata.

4.5. Comparison with the SMART study

Comparison of techniques used to measure IL-6 and D-dimer assays in SPARTAC and SMART.

Prior to performing a comparison of biomarker changes associated with stopping ART in the SMART versus the SPARTAC study, a comparison of the techniques used in the two studies was undertaken in order to evaluate the potential inter-laboratory variation and to assess differences between the commercial assays used. Samples were identified from 10 SPARTAC patients where 2 or more vials of EDTA plasma were available at the same time point. Samples from SPARTAC week 252 were used due to the large availability of plasma at this time point. Plasma from one vial was analysed by the methods described in methods chapter 2.4 (IL-6 ELISA, R&D systems HS600B; D-dimer immunoturbidimetric assay Innovance, Dade Behring). The second vial was sent in dry shippers containing liquid nitrogen to Laboratory for Clinical Biochemistry Research, University of Vermont, the laboratory that had undertaken the biomarker evaluations in the SMART trial, where a chemiluminescent assay was used for IL-6 (R&D systems Q6000B) and an immunoturbidimetric assay was used for D-dimer (STA-R analyzer, Stago, Parsippany, NJ).

Table 4.8 demonstrates median results for IL-6 and D-dimer when measured in SPARTAC and SMART study laboratories. Spearman rank coefficient for correlation between the samples is also shown. IL-6 assays results were highly correlated (Spearman Rank coefficient 0.83, $p=0.003$), however correlation between D-dimer assays was poor (Spearman Rank coefficient 0.52, $p=0.11$). On parametric testing, Pearson correlation for $\log_{10}$ transformed results showed highly significant correlation between IL-6 assays measured at the two sites (coefficient 0.97, $p<0.0001$, and a less strong but still significant correlation between D-dimer assays (coefficient 0.77, $p=0.014$).

The relationship between the results from the 2 laboratories is shown in Figure 4.5. Despite the high correlation between IL-6 results from the 2 laboratories, all IL-6 results measured by SPARTAC methodology fall below the line of equality indicating that results from samples measured for the SPARTAC study were consistently lower than those measured in the SMART laboratory. For D-dimer, all SPARTAC laboratory measurements lie above the line of equality indicating that results were consistently higher. Bland Altman plots were constructed to further assess agreement between biomarker measurements at SPARTAC and SMART laboratories (Figure 4.6). For IL-6, the limits of agreement are -2.30 and -0.36 pg/ml, indicating that IL-6 results for SPARTAC are consistently lower than when measured in the SMART laboratory. For D-dimer, the limits of agreement are -0.01 and 0.35, indicating that D-dimer measurements may be between -0.01 mg/L lower and 0.35 mg/L higher in the SPARTAC laboratory. Thus lack of agreement is demonstrated for both biomarkers.

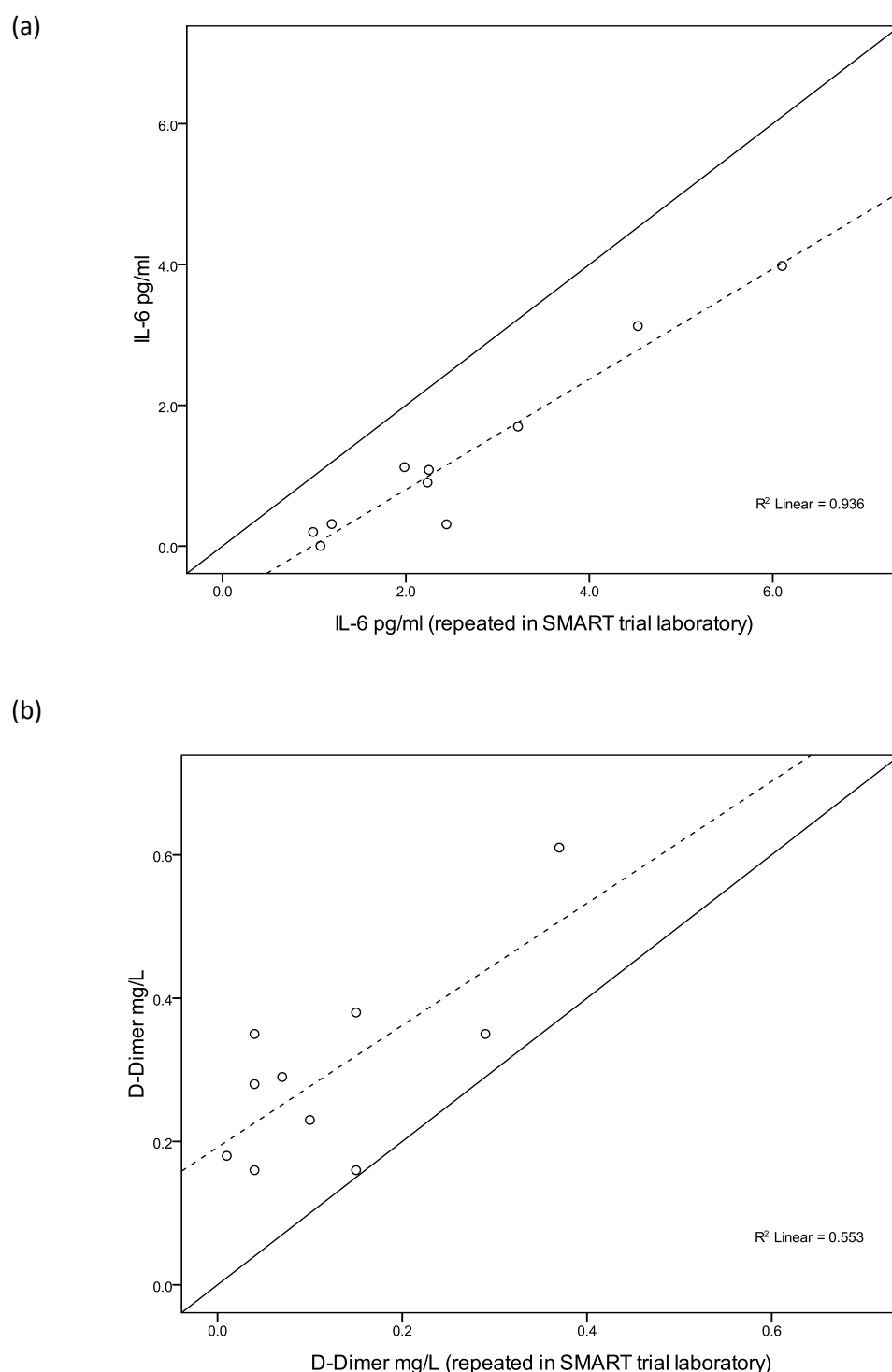
It was therefore not possible to undertake a direct comparison between absolute levels of biomarkers between the two study populations as there was a significant difference between the assays employed.

Table 4.8. IL-6 and D-dimer results measured from same samples in SPARTAC and SMART laboratories

	SPARTAC biomarker result Median (IQR)	SPARTAC sample processed in SMART lab Median (IQR)	Spearman rank Coefficient	p-value for correlation
IL-6 pg/ml	0.99 (0.28, 2.05)	2.24 (1.19, 3.22)	0.83	0.003
D-dimer mg/L	0.29 (0.18, 0.36)	0.08 (0.04, 0.15)	0.52	0.11

IL-6 and D-dimer were measured in SPARTAC and SMART laboratories. Spearman's rank correlation coefficient is shown alongside median values and IQR.

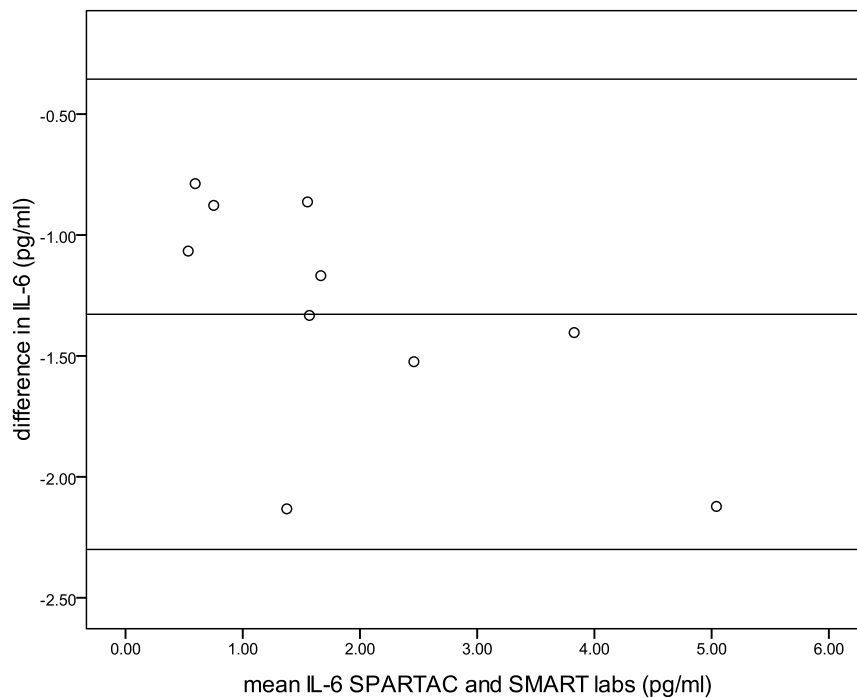
Figure 4.5. Scatter plot showing the relationship between biomarkers levels in SPARTAC samples measured at Imperial College, London and repeated in the SMART study laboratory, Vermont USA



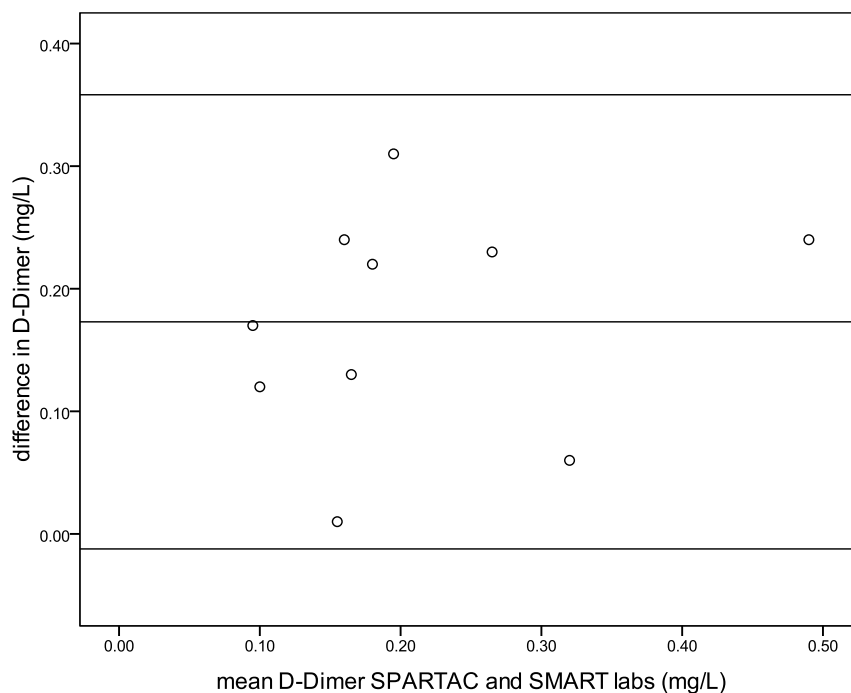
Scatter plot of biomarker results measured in SPARTAC and SMART laboratories. The line of equality ($x=y$, solid line), indicating exact agreement between the two measurements, and best fit line showing relationship between the 2 measurements (dashed line) are shown.

Figure 4.6. Bland-Altman plot showing level of agreement between SPARTAC and SMART laboratories measuring (a) IL-6 (b) D-dimer

(a)



(b)



Bland Altman plots were constructed to assess agreement between biomarker measurements at SPARTAC and SMART laboratories. The mean of the 2 measurements (x axis) is plotted against the difference between the 2 measurements (y axis). The horizontal lines demonstrate the mean difference and limits of agreement (2 Standard Deviations of mean difference). For IL-6, the limits of agreement are -2.30 and -0.36 pg/ml, indicating that IL-6 measurement in the SPARTAC laboratory is consistently lower than in the SMART laboratory and demonstrating lack of agreement. For D-dimer, the limits of agreement are -0.01 and 0.35, indicating that that D-dimer measurements may be between -0.01 mg/L lower and 0.35 mg/L higher in the SPARTAC laboratory, again demonstrating lack of agreement.

Comparison between SPARTAC and SMART study findings

In view of the lack of agreement between assays used in the SPARTAC versus SMART trials, it was not possible to perform a direct comparison of individual patient data between SPARTAC and SMART studies. However, proportional change in biomarker values on stopping therapy in SPARTAC were compared with published results from the SMART study. Standard errors and 95% CI were calculated using bootstrapping techniques and two-sided p-values using t-tests.

In published manuscripts the SMART study reported a 43% and 27% rebound in IL-6 and D-dimer, respectively, 4 weeks after stopping ART. Corresponding figures from SPARTAC were 22% (95% CI: 0, 46%, p-value for difference from 43%: 0.09) for IL-6 and 14% (95% CI: 0, 29%, p-value for difference from 27%: 0.10) for D-dimer. Thus rebound for both biomarkers appears to be lower when treatment was stopped in the SPARTAC study compared to the SMART, although this was not statistically significant. For IL-6 this should be interpreted with caution due to SPARTAC values being lower than SMART values in the comparison between laboratories.

4.6 Summary of results in this chapter

In this chapter we demonstrate that levels of IL-6 and D-dimer are raised in HIV-1 infected individuals in both PHI and at week 60, compared to healthy controls. In PHI, these biomarkers correlate with HIV RNA levels and age. There was a significant reduction in both biomarkers on commencing ART, however levels rebounded to baseline within 4 weeks of stopping ART. Due to differences in the assays used, a direct comparison with individual patient data from the SMART study was not possible. Comparing proportional change in biomarkers on stopping ART between the two studies, there was a trend towards lower biomarker rebound in SPARTAC compared with SMART ($p \leq 0.1$).

Discussion

This is the first study to examine levels of IL-6 and D-dimer in a large cohort of individuals with PHI and to assess the effect of starting and stopping ART commenced in this setting on these parameters. Individuals with chronic HIV-1 infection have raised levels of IL-6 and D-dimer compared with HIV-negative controls (Neuhaus, et al. 2010), and stopping ART is associated with rises in these biomarkers (Kuller, et al. 2008); however there are few data at the time of PHI. In view of the adverse events demonstrated in the SMART trial, it is important to demonstrate the impact of stopping ART commenced in PHI on biomarker levels. As all SPARTAC participants randomised to receive ART undergo protocol-indicated cessation of therapy after 12 or 48 weeks, this cohort circumvents any biases that may be introduced by either clinical or patient-clinician led choice to start or stop ART initiated at PHI.

IL-6 and D-dimer levels were significantly higher at both week 0 (PHI) and week 60 than in healthy controls. This is consistent with results from previous studies showing raised IL-6 and D-dimer in chronic HIV-1 infection compared to HIV-negative individuals (Neuhaus, et al. 2010), and another study of 14 South African women with available plasma samples before and after HIV acquisition, showing rises in IL-6 during acute infection (Roberts, et al. 2010). Baseline levels of IL-6 and D-dimer in PHI were comparable to those seen at week 60 and in individuals on chronic suppressed ART (chapter 3.1 freeze thaw analysis), and slightly lower than those seen at baseline in individuals choosing to take ART in the pilot study (chapter 3.2 seroconverter study) although higher than those who declined ART in that study. Acute HIV-1 infection is associated with an intense cytokine storm (Stacey, et al. 2009), however participants recruited to SPARTAC represent later stages of PHI up to 6 months after seroconversion and may have missed this cytokine peak. Whether short-course ART impacted on IL-6 and D-dimer levels at week 60 is currently unknown. Although all individuals have stopped treatment by week 60, due to the blinded nature of the SPARTAC trial, week 60 results were analysed together regardless of randomisation arm and represent a heterogeneous population in terms of treatment history.

In the SMART study, baseline D-dimer levels were significantly higher in untreated individuals compared to those with HIV RNA <400 copies/ml on ART ($p<0.001$), but baseline IL-6 levels did not vary according HIV RNA (Kuller, et al. 2008). In untreated SMART participants at baseline there was no correlation between HIV RNA levels and IL-6 or D-dimer (Baker, et al. 2010). In contrast, both IL-6 and D-dimer were significantly associated with HIV RNA levels in PHI. When divided by HIV RNA strata, there was a linear relationship between VL strata and D-dimer levels. Interestingly, for IL-6, higher than expected levels were seen in the 13 individuals with VL <400 copies/ml at baseline. These individuals may represent natural elite controllers and when the trial is unblinded it will be

possible to examine the specific characteristics of these participants in more detail. A recent study of individuals with HIV-2 infection showed that in those with detectable HIV-2 RNA, VL correlates directly with level of immune activation, yet in those with natural elite control, a wide range of levels of immune activation is seen demonstrating that control of HIV RNA may not necessarily equate to low levels of immune activation and subsequent inflammation (Leligidowicz, et al. 2010).

Both IL-6 and D-dimer in PHI were also associated with age, with increased age being associated with higher biomarker levels. These data are consistent with observations in chronic infection (Kuller, et al. 2008). It is well-established that older subjects experience faster rates of CD4 cell decline (Touloumi, et al. 1998) and high levels of inflammation and immune activation, independent of HIV RNA levels, may in part explain this enhanced disease progression. The predictive value of baseline IL-6 and D-dimer levels in PHI on disease outcome measures (rate of CD4 cell decline, HIV-1 related events and AIDS diagnoses, and HIV-1 unrelated events such as CVD) is currently unknown but will comprise an interesting analysis once the trial is unblinded.

Participants in SPARTAC were randomised 1:1:1 to one of the three arms. For the assessment of the effects of ART on IL-6 and D-dimer, the two treatment arms (12 and 48 weeks ART) were combined and analysed together. No comparison was made between the two treatment arms as the SPARTAC study is still ongoing. It is possible that the length of treatment could impact on immune inflammatory processes although there are no current data comparing relative lengths of treatment on biomarkers levels. It is also unknown how the changes in biomarkers observed in patients receiving ART compare with individuals with PHI who did not receive treatment. Once the SPARTAC study is unblinded in 2011, the randomized nature of the study will allow a direct comparison of biomarker levels in treated compared to untreated individuals, allowing us to assess the natural history of IL-6 and D-dimer changes in patients with PHI.

As no comparison can be made with the control arm of SPARTAC until the study is unblinded, we cannot yet confirm whether the observed reductions were definitely due to the effect of ART or the natural history of biomarker changes following PHI. Recent analyses of SMART participants who were either naive to ART or not on ART at the time of randomisation showed significant reductions in D-dimer in individuals with chronic HIV-1 infection starting ART compared to those deferring ART (Baker, et al. 2010), consistent with other studies in chronic HIV-1 (Wolf, et al. 2002) and with our findings in PHI. However, no reductions were observed in IL-6 and hs-CRP in ART treated individuals compared to untreated individuals who deferred ART ($p=0.12$ and $p=0.17$ for IL-6 and hs-CRP, respectively, at six months). It should be noted that only 62% of individuals commencing ART in the analysis by Baker et al. suppressed HIV RNA to <400 copies/ml at six months, which may explain

these findings, and significant reductions were seen in IL-6, hs-CRP and D-dimer at six months compared to baseline in those achieving viral suppression (Baker, et al. 2010).

In this analysis, IL-6 and D-dimer levels fell significantly within 12 weeks of starting ART in PHI, but rebounded to baseline levels 4 weeks after stopping therapy. The clinical significance of these findings, in the setting of PHI, is not yet known. Factors associated with larger decrease on starting ART were also associated with a larger increase on stopping ART and there was no difference between baseline levels and those 4 weeks after stopping treatment. A high biomarker level pre-ART was associated with a larger fall of biomarker on commencing ART, and a low value on ART associated with a higher rise on stopping ART. Likewise, high BMI was associated with a smaller fall in D-dimer on starting and also a smaller rise on stopping, indicating that those with high BMI have less response to ART in terms of biomarker changes. Interestingly, in the SMART study, higher BMI was associated with higher levels of both IL-6 and D-dimer at baseline (Kuller, et al. 2008) and in the recent analysis by Baker et al, lower BMI was associated with greater reductions in hs-CRP, consistent with our finding for D-dimer (Baker, et al. 2010). In our analysis, high baseline CD4 cell count was associated with a larger fall in IL-6 on starting ART, suggesting a possible benefit of starting early ART, however there was no association between CD4 cell count at time of stopping ART and biomarker rebound. Baseline biomarker levels were associated with higher HIV RNA, however change in VL on stopping ART was not associated with change in either biomarker. This is in contrast to the SMART study where rise in VL on stopping ART was significantly associated with change in IL-6 and D-dimer levels. The study is limited by small numbers of available plasma at later time points of stopping and starting ART, and the lack of association may be due to small numbers in each VL stratum.

We performed a comparison of the laboratory techniques used between SPARTAC and the SMART trial, with samples from the same patient and time points analysed in the two laboratories. Although highly correlated, IL-6 results were consistently lower when measured in the SPARTAC study than in the SMART laboratory, indicating poor agreement between samples. For D-dimer, SPARTAC results were consistently higher. In a series of studies at the University of California, Los Angeles, which measured cytokines and soluble markers of activation in HIV-1 infected patients, substantial differences were found when ELISA kits from different manufacturers were used and analytic results from different lots of ELISA kits supplied by a single manufacturer also differed by up to 50% (Aziz, et al. 1999a). These studies highlight the need for quality control procedures when samples are analysed in more than one laboratory. Our study confirms a marked variation in performance characteristics between laboratories and demonstrates the need for caution when comparing results between different published studies.

The assays used in the SPARTAC trial were chosen after careful consideration on the basis of kit sensitivity and feasibility with available laboratory facilities, and expert opinion was that the assays used would be comparable between SPARTAC and SMART (personal communication Elaine Cornell, University of Vermont, USA). In SPARTAC, standard operating procedures mean that all samples have been treated in the same way, although the effect of shipping from different sites is not known. Several quality control procedures were undertaken as described in methods chapter 2.4. Interestingly both IL-6 assays were from the same manufacturer. As D-dimer is a routinely used clinical test, SPARTAC D-dimer samples were measured in an accredited NHS laboratory. The discrepancy between results comparing the techniques used by the two studies may indicate differences in the assays used or possibly differences in laboratory techniques or storage conditions. The effect of shipping samples is unclear. However this comparison of techniques is limited by the small number of samples included and a larger sample would give a better indication of the discrepancy between laboratories.

Although it was disappointing not to be able to undertake a direct comparison between SPARTAC RCT and SMART using individual patient data, the crude comparison with published data may give an indication of level of biomarker rebound after treatment interruption of ART initiated in PHI versus chronic HIV-1 infection. Although we found no significant differences in biomarker rebound when compared to the SMART study, there was a trend towards lower levels of IL-6 and D-dimer rebound compared to that observed in chronic infection. For IL-6 this comparison must be interpreted with caution as IL-6 results were consistently lower in SPARTAC than those in SMART in the comparison of techniques used in the two studies. However as the comparison of techniques showed consistently higher D-dimer results for SPARTAC, our results indicate that D-dimer rebound is likely to be lower on discontinuing ART commenced in PHI (SPARTAC) compared to that commenced in chronic infection (SMART). PHI represents a very different immunological stage of HIV-1 mediated disease to chronic infection, and biomarker changes may therefore differ. It is also possible that the clinical consequences of biomarker rebound may differ between chronic and PHI due to the short duration of HIV-1 infection at ART initiation and short exposure to ART.

In conclusion we observed raised levels of IL-6 and D-dimer in PHI and at week 60 compared to healthy controls. Both biomarkers were related to age and HIV RNA levels. A significant reduction in IL-6 and D-dimer occurred on commencing ART in PHI. On discontinuation of therapy, both biomarkers rebounded to baseline levels but did not rebound beyond baseline levels at week 4. Once the SPARTAC trial is unblinded in 2011, the clinical significance of these findings will be investigated.

Chapter 5: HIV-1 VL dynamics following cessation of ART started in PHI or during chronic infection

Introduction

The level of HIV-1 VL is a strong predictor of disease progression when measured in both early (Lyles, et al. 2000) and chronic HIV-1 infection (Mellors, et al. 1997; Mellors, et al. 1996; Phillips and Pezzotti 2004). In addition, VL is also a critical determinant of risk of onward HIV-1 transmission (Quinn, et al. 2000). The level of viral rebound following cessation of ART therefore has important consequences.

Interruption of ART started in chronic infection is no longer a recommended management strategy (El-Sadr, et al. 2006) and viral rebound following treatment cessation reaches levels comparable to pre-treatment values (El-Sadr, et al. 2006; Oxenius, et al. 2002; Wit, et al. 2005). In spite of effective therapies, the early establishment of a stable, inaccessible reservoir of latently infected resting memory CD4 T-cells is hypothesised to be the major source contributing to rebound viraemia after cessation of ART (Siliciano and Siliciano 2006; Trono, et al. 2010).

Early treatment with ART during PHI may reduce the number of infected cells and prevent the establishment of a larger viral reservoir, conferring enhanced control of viral replication when therapy is stopped. Small studies quantifying the viral reservoir in treated PHI participants confirm that although a reduction in reservoir size is observed after even short-course ART initiated in PHI, complete abolition of viral replication is not achieved and the viral reservoir may be re-expanded even after short-term rebound of viraemia (Lillo, et al. 2002). Initial control of plasma viraemia after stopping ART in PHI had been reported in some studies (Hecht, et al. 2006; Rosenberg, et al. 2000; Steingrover, et al. 2008), but durable long-term effects were not observed in others (Desquilbet, et al. 2004; Fidler, et al. 2002; Hecht, et al. 2006; Kaufmann, et al. 2004; Koegl, et al. 2009; Streeck, et al. 2006). An observational study comparing treatment interruption in 24 patients with PHI with 46 patients initiating ART in chronic infection, showed delayed or absent viral rebound following cessation of ART commenced in PHI and significantly lower median time to viral rebound, suggesting different viral dynamics between PHI and chronic infection (Steingrover, et al. 2008).

In addition to considering the influence of short-course ART during PHI on viral rebound post-ART interruption, it is important to address the clinical safety of this strategy. In the SMART study, the level of plasma HIV RNA rebound following cessation of ART was highly correlated with increases in inflammatory and pro-coagulatory biomarkers, and biomarker levels were predictive of mortality (Kuller, et al. 2008). Based on these observations, the consequences of discontinuing treatment commenced in early infection on levels of plasma viraemia are important.

It is possible that ART started in PHI may confer enhanced virological control once ART is stopped compared with stopping ART initiated in chronic HIV-1 disease. Moreover, sustained reductions in VL set-point resulting from ART given in PHI could dampen immune activation and inflammatory consequences of HIV-1 infection, leading to a long-term improvement in clinical outcome. Possible mechanisms resulting in reduced viral rebound after treatment in PHI compared with chronic infection could include a smaller size viral reservoir in PHI, preserved immunological function conferring enhanced natural viral control, or lower levels of inflammation and immune activation, thereby limiting viral replication which preferentially occurs in activated cells.

The aims of this chapter were to:

- i. describe viral dynamics associated with stopping and starting ART in PHI
- ii. compare the level of plasma VL when ART is discontinued in patients starting treatment in PHI (SPARTAC) to those in whom ART was commenced in chronic HIV-1 Infection (SMART).

Results

5.1 Patient characteristics

Viral dynamics associated with starting and stopping ART were examined in SPARTAC participants who were randomised to either one of the two treatment arms. Data from the two treatment arms from all SPARTAC centres were combined and analysed together. Individual patient data from SMART study participants were obtained from the International Network for Strategic Initiatives in Global HIV Trials (INSIGHT) group. Viral dynamics following treatment interruption were then compared between SPARTAC and SMART trial participants.

In the SMART study, participants with a CD4 cell count >350 cells/ μ l were randomised to either a Drug Conservation (DC) or Viral Suppression (VS) arm. SPARTAC and SMART study participants were included in this analysis if they underwent protocol-indicated ART cessation, i.e. if they were receiving ART at time of randomisation to the DC arm in SMART, or randomised to one of the two treatment arms in SPARTAC, and had a CD4 cell count and plasma VL available when ART was stopped. The closest CD4 cell count and VL to the time of ART cessation were used, up to 24 weeks and 12 weeks before stopping for CD4 cell count and VL respectively and no more than 2 weeks after stopping treatment. Viral load measurements were scheduled at 4, 12, 24, 36, 48 and 4, 8, 16, 32, 40, 48 weeks after stopping ART in the SPARTAC and SMART studies, respectively, and were defined by those closest to the scheduled visits (allowing a ± 2 week window around the week 4 and 6 visits and a ± 4 week window around subsequent visits). Enrolment into SMART was stopped on 11th January 2006 due to the results of an interim analysis which indicated clear superiority of the VS arm, and it was recommended that participants in the DC arm re-initiate ART at this point (El-Sadr, et al. 2006).

In SPARTAC, 243 participants were randomised to one of the two treatment arms, and 238 of these initiated therapy. Of these, 11 were excluded because they did not stop ART ($n=6$), were on ART for <15 days ($n=4$) or did not have a VL at ART stop ($n=1$). Of 2290 SMART participants receiving ART at the time of randomisation to the DC arm, 256 were excluded from this analysis because ART was not stopped ($n=42$, 25 of whom were randomised in the month prior to 11th January 2006), stopped ART after 11th January 2006 ($n=20$), did not have a VL at ART stop ($n=1$) or did not have any subsequent treatment-naïve VLs at scheduled visits ($n=193$, 153 of whom had only stopped in the month prior to 11th January 2006). Analyses also were restricted to participants who had suppressed VL to <400 copies/ml at the time of ART stop, as this was the limit of the least sensitive assay used across both trials. Forty-three SPARTAC and 384 SMART participants had VL ≥ 400 copies/ml at ART stop. VL data were $\log_{10}$ -transformed and values <400 copies/ml were treated as equal to 400 for all participants to avoid confounding by trial as a greater proportion of VLs were measured using this detection limit

in the PHI compared to chronically infected participants. In addition analyses were restricted to those in whom HIV-1 had been sexually transmitted, as few were from other risk groups in SPARTAC compared to SMART. In SPARTAC no participants were known to have acquired HIV-1 through injecting drug use (IDU) and 1% had unknown route of transmission, compared to 8% and 5% for IDU and unknown in SMART. Therefore a further 125 SMART participants who reported IDU as their transmission risk, and 2 SPARTAC and 75 SMART participants with other or unknown route of HIV-1 transmission, were excluded. Overall, 182 PHI and 1450 chronically infected participants were included in this analysis.

SPARTAC participants recruited from the following sites were included: UK and Ireland n=86 (47.3%), South Africa n=54 (29.7%), Australia n=19 (10.4%), Italy n=12 (6.6%), Brazil n=9 (4.9%), and Spain n=2 (1.1%). Participant demographics, ART exposure and CD4 cell count at time of treatment discontinuation for included SPARTAC and SMART participants are shown in Table 5.1. Compared to participants with chronic infection, SPARTAC participants were younger (median 34 versus 44 years), more likely to be female (33% versus 24%), had considerably less ART exposure (6% versus 44% ever exposed to ≥ 3 classes) and were more likely to be on a protease inhibitor regimen at the time of ART stop (94% versus 36%). At ART stop, CD4 cell count was slightly higher among SPARTAC compared to SMART participants (707 versus 646 cells/ μ l). Among SMART participants, 77% had a nadir CD4 cell count of <350 cells/ μ l. Two SMART participants had CD4 cell count <350 cells/ μ l at ART stop (contrary to SMART inclusion criteria), but both were measured on the day of ART stop and both participants had previous CD4 cell count >350 cells/ μ l within the previous 6 weeks. The estimated time since first initiating ART was 3 (3, 11) versus 72 (48, 96) months for SPARTAC compared to SMART participants, respectively. As SMART participants were enrolled into the trial with prevalent HIV-1 infection, duration of HIV-1 infection and time since first initiation of ART were often not known. The intervals available for the chronically infected participants can, therefore, be regarded as the minimum values.

Follow-up data for this analysis was included from the date of first stopping ART and was censored at the last available VL measurement, the 48 week visit, when ART was re-initiated, or 11th January 2006 for SMART participants, whichever was earliest. Due to the premature stop of the SMART trial, 559 (39%) of chronically infected participants were censored on 11th January 2006. A further 17 (9%) and 463 (32%) SPARTAC and SMART participants respectively were censored before their 48 week visit due to ART re-initiation. The median follow-up was 48 (IQR 45, 49) and 27 (IQR 12, 43) weeks for the PHI and chronically infected participants, respectively, and the median number of RNA measurements included per individual was 6 (IQR 5, 6) and 4 (IQR 3, 6) for SPARTAC and SMART respectively.

Table 5.1. Characteristics of participants at time of stopping ART in the SPARTAC and SMART trials.

	SMART Chronic HIV infection N=1450	SPARTAC PHI N=182
Sex, female	350 (24%)	60 (33%)
Age, years	44 (38, 51)	34 (28, 42)
HIV-1 exposure		
MSM	890 (61%)	114 (63%)
Sex between men & women (male)	222 (15%)	8 (4%)
Sex between men & women (female)	338 (23%)	60 (33%)
Time since first diagnosed HIV-positive, months	96 (60, 144)	6 (4, 13)
Number of ART drugs, ever	5 (4, 7)	3 (3, 3)
Number of ART classes, ever		
1	48 (3%)	0 (0%)
2	763 (53%)	171 (94%)
≥3	639 (44%)	11 (6%)
ART type at stop		
NNRTI based	674 (46%)	8 (4%)
PI based	521 (36%)	171 (94%)
3 NRTI	132 (9%)	2 (1%)
3 class	84 (6%)	0 (0%)
NRTI sparing	3 (<1%)	0 (0%)
Suboptimal ART	36 (2%)	1 (1%)
Nadir CD4 cell count up to ART stop, cells/μl	230 (132, 340)	-
<200	592 (41%)	-
200-349	515 (36%)	-
350-499	226 (16%)	-
≥500	117 (8%)	-
CD4 cell count at ART stop, cells/μl	646 (495, 848)	707 (586, 919)
CD4 nadir <200 cells/μl	568 (456, 724)	-
CD4 nadir 200-349 cells/μl	618 (494, 784)	-
CD4 nadir 350-499 cells/μl	806 (646, 993)	-
CD4 nadir ≥500 cells/μl	948 (784, 1176)	-

Participant characteristics for those randomised to discontinue ART, at the time of ART cessation, are shown for the SPARTAC and SMART trials. Values are median (IQR) for continuous variables and n (%) for categorical variables. CD4 values are shown for closest up to 24 weeks before ART was stopped.

5.2 Time to VL reaching pre-ART levels in SPARTAC participants

Among SPARTAC participants, the time to reach pre-ART VL levels following treatment interruption was estimated using survival methods. Pre-ART VLs in PHI participants were estimated as the mean of all available VLs before ART initiation (9%, 85%, 5%, 1% participants had 1, 2, 3 and 4 pre-ART VLs available, respectively). The median (IQR) pre-ART VL was 4.5 (IQR 3.9, 5.1) $\log_{10}$ copies/ml. Eleven participants had pre-ART VL <400 copies/ml and were, therefore, not included in this analysis. Nine participants were censored before reaching pre-ART levels due to ART re-initiation (one at 5 week and the remainder at least 25 weeks after ART stop; the median pre-ART VL was somewhat higher in these participants at 5.5 $\log_{10}$ copies/ml). A total of 71 (42% of 171) participants were observed to rebound to pre-ART VL levels, at a median 50 (95% CI 48, 51) weeks after treatment interruption. A quarter of participants had rebounded to pre-ART levels by 15 (95% CI 12, 26) weeks.

5.3 VL rebound at 4 weeks after ART stop by PHI versus chronically infected participants

Viral load at 4 weeks after stopping ART was compared between SPARTAC (PHI) and SMART (chronic infection). Using linear and ordered logistic regression, respectively, differences in absolute levels and in the proportions with VL <400, 400-3499, 3500-9999, 10,000-49,000 or $\geq 50,000$ copies/ml were examined at 4 weeks after stopping ART. Table 5.2 shows factors associated with HIV RNA level at four weeks after stopping ART on linear regression. Four weeks after stopping therapy, VL levels were 0.45 (95% CI 0.26, 0.64) $\log_{10}$ copies/ml lower among PHI compared to chronically infected participants ($p < 0.001$; median 3.7 [IQR 2.7, 4.3] versus 4.4 [IQR 3.0, 5.0] $\log_{10}$ copies/ml respectively). There was a trend towards an association between lower CD4 cell count at time of stopping ART and higher week 4 VL, $p = 0.08$). Persons infected through heterosexual sex had significantly lower week 4 VL than MSM ($p < 0.001$), but with no evidence that this effect differed between chronic infection and PHI ($p = 0.4$). There was no association between age at time of treatment discontinuation and VL at week 4 after stopping ART ($p = 0.6$). The proportions of participants with VL <400 copies/ml four weeks after stopping therapy were similar between the PHI and chronic infection populations (22% versus 21%, respectively; $p = 0.6$). Table 5.3 shows the number of participants within each VL stratum for SPARTAC and SMART participants. The proportions of participants with VL rebound to 400-3499, 3500-9999, 10,000-49,999 and $\geq 50,000$ copies/ml were 22, 23, 16 and 17% among SPARTAC participants compared to 12, 8, 18 and 40% among the SMART participants, indicating higher levels of VL rebound in chronically infected individuals (adjusted $p < 0.001$).

Table 5.2. Factors associated with viral load four weeks after stopping ART

	Coefficient (95% CI)	p value
PHI, versus chronic HIV-1 infection	-0.45 (-0.64, -0.26)	<0.001
Age at ART stop, per 10 years	0.01 (-0.04, 0.07)	0.6
Sex/risk group, versus men infected through sex with men		<0.001
Male, infected through sex with women	-0.14 (-0.30, 0.03)	
Female, infected through sex with men	-0.33 (-0.47, -0.20)	
CD4 cell count at ART stop, per 100 cells/ μ l	-0.02 (-0.04, 0.002)	0.08

Linear regression model of factors associated with HIV RNA levels ($\log_{10}$ copies/ml) 4 weeks after stopping ART in SPARTAC vs. SMART participants respectively. The regression coefficient (95% CI) and p-value are given for each variable

Table 5.3. Level of viral load 4 weeks after stopping ART in PHI and chronic infection.

VL (copies/ml)	Chronic HIV-1 infection N=1327*	PHI N=156*
<400	284 (21%)	35 (22%)
400-3499	161 (12%)	34 (22%)
3500-9999	110 (8%)	36 (23%)
10,000-49,999	235 (18%)	25 (16%)
$\geq 50,000$	537 (40%)	26 (17%)

Number of SMART (chronic HIV-1 infection) and SPARTAC (PHI) participants with VL within each VL stratum are shown 4 weeks after stopping ART. Values are n (%). *participants with a week 4 VL available.

5.4 VL rebound up to 48 weeks after stopping ART in PHI and chronic infection

Differences in VL levels and slopes over 4-48 weeks after stopping ART were examined using linear mixed models. In addition the time averaged viral burden, reflecting circulating virus at a given time point, was estimated by calculating the area under the VL curve (AUC), over the whole period after stopping ART, and dividing by time. Models were adjusted for the effect of sex/risk group (MSM, heterosexual men (MSW), and heterosexual women), age and CD4 cell count at ART stop. No adjustments were made for time on ART, as this is confounded by duration of infection and, therefore, by trial, nor for ART class which was also confounded by trial. Differences in the effects of participant characteristics at ART stop, and nadir CD4 cell count up to ART stop, were investigated for PHI compared to chronically infected participants, where appropriate, using interactions.

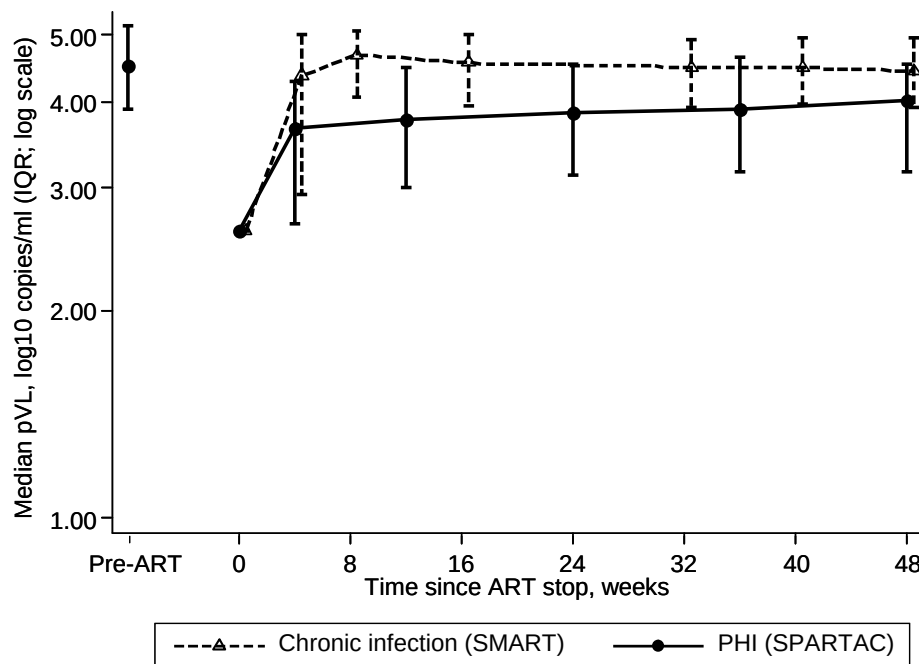
Over 48 weeks after stopping ART, the median VL remained lower in the PHI group compared to chronically infected group, demonstrated in Figure 5.1a. Modelling VL 4-48 weeks after stopping ART, there was evidence to suggest that the chronic infection participants rebounded more rapidly than the PHI participants ($p < 0.0001$). Results of this model are demonstrated in Figure 5.1b. The predicted VL for a representative participant with PHI (male infected through sex between men, aged 40 years and with CD4 cell count 600 cells/ μ l at ART stop) at weeks 4, 12 and 36 after stopping ART were 3.36 (3.27, 3.45), 3.53 (3.43, 3.63) and 4.04 (3.88, 4.20) $\log_{10}$ copies/ml, respectively, compared to 3.75 (95% CI 3.71, 3.79), 4.10 (4.05, 4.14) and 5.13 (5.05, 5.21) $\log_{10}$ copies/ml, respectively for chronic infection.

The median (IQR) time-averaged viral burden was 1.12 (0.56, 1.69) and 1.55 (1.03, 1.99) $\log_{10}$ copies/ml amongst participants with PHI and chronic infection, respectively. After adjustment, total viral burden was, on average, 0.28 (95% CI 0.17, 0.39) $\log_{10}$ copies/ml lower for PHI compared to chronically infected participants ($p < 0.001$).

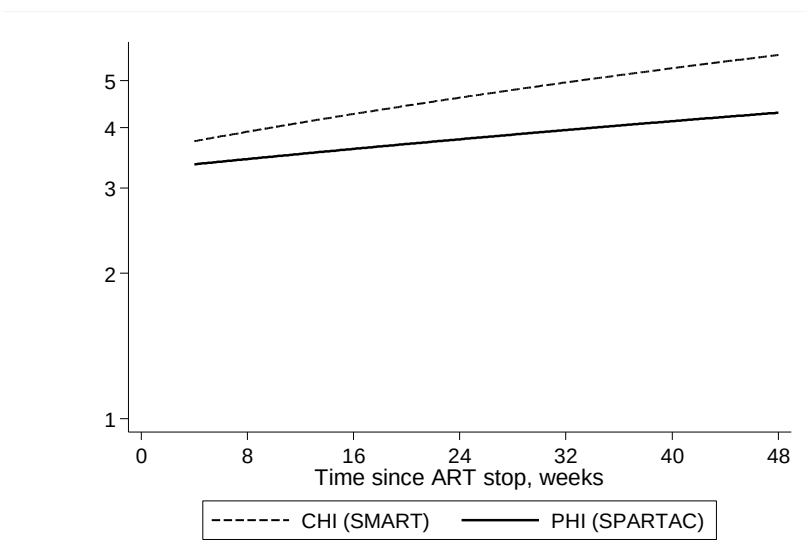
The associations between longer-term VL rebound and sex/risk group, CD4 cell count and age at ART stop were qualitatively similar to those for the week 4 VL rebound. There was evidence of higher VL over 48 weeks among MSM compared to MSW and with lower CD4 cell count (0.03 [-0.04, -0.01] per 100 cells, $p < 0.001$), but no difference in these effects by PHI/chronic infection status ($p = 0.2$ and 0.3 respectively). Older age at time of stopping ART was associated with higher VL rebound, although the magnitude of this effect was small (0.04 [0.01, 0.08] $\log_{10}$ copies/ml per 10 years, $p = 0.02$) and there was no difference by PHI/chronic infection status ($p = 0.4$).

Figure 5.1

a) VL up to 48 weeks after stopping ART in PHI versus chronic infection



b) Predicted VL over 4-48 weeks after stopping ART in PHI versus chronic infection on mixed effect model



a) Plasma VL in PHI vs. Chronic HIV-1 infection (CHI) from 0 to 48 weeks after treatment cessation is shown, alongside pre-treatment VL for PHI

b) Predicted VL over 4-48 weeks after ART stop by PHI versus chronic infection (CHI), based on a representative participant (male infected through sex with men, aged 40 years and with CD4 cell count 600 cells/ μ l at time of ART stop)

5.5 VL rebound up to 48 weeks after stopping ART, categorising the chronically infected participants by nadir CD4 cell count

At time of stopping ART, SMART participants were categorised according to their nadir CD4 cell count, in order to assess whether any differences over 48 weeks between the populations could be explained solely by CD4 T-cell levels. Compared to PHI participants, the VL rebound after 4 weeks, up to 48 weeks, was significantly higher in the chronically infected participants with CD4 T-cell nadir <500 cells/μl, and lower CD4 T-cell nadir was associated with faster rebound (Figures 5.2 a and b). For every 8 weeks, on average the VL in PHI participants increased by 0.17 (95% CI 0.14, 0.20) log₁₀ copies/ml compared to 0.71 (0.60, 0.81), 0.46 (0.36, 0.57), 0.33 (0.21, 0.44) and 0.15 (0.01, 0.29) log₁₀ copies/ml in chronically infected participants with nadir CD4 cell counts of <200, 200-349, 350-499 and ≥500 cells/μl, respectively. Predicted VL at week 4 and 36 after stopping ART are demonstrated in Table 5.4. and were lower in PHI and for higher nadir CD4 cell count.

We observed a similar relationship for the time-averaged AUC: chronically infected participants with a lower nadir CD4 cell count had higher viral burden, compared with PHI participants (0.39 [95% CI 0.27, 0.51], 0.29 [0.18, 0.41] and 0.20 [0.06, 0.33] log₁₀ copies/ml higher viral burden for nadir CD4 cell counts <200, 200-349 and 350-499 cells/μl respectively). There was no difference in viral burden between chronically infected participants with nadir CD4 cell count ≥500 cells/μl and PHI participants (0.04 [-0.12 to 0.20] log₁₀ copies/ml).

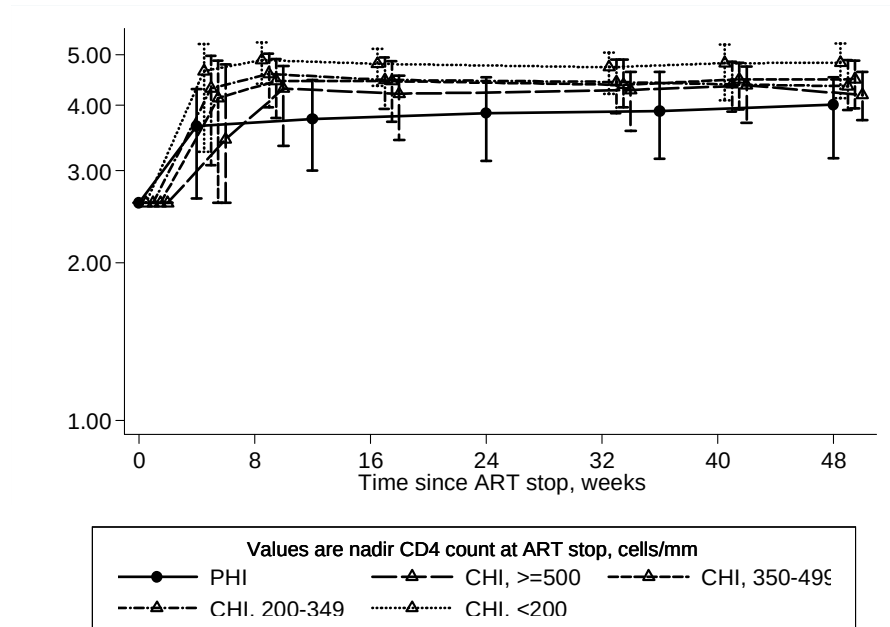
Table 5.4. Predicted VL in PHI and chronic infection, stratified by nadir CD4 cell count

	Week 4 VL log ₁₀ copies/ml (95% CI)	Week 36 VL log ₁₀ copies/ml (95% CI)
SPARTAC	3.33 (3.24, 3.42)	4.01 (3.85, 4.18)
SMART, nadir CD4 ≥500	3.46 (3.34, 3.57)	4.34 (4.11, 4.56)
SMART, nadir CD4 350-499	3.61 (3.52, 3.69)	4.71 (4.53, 4.88)
SMART, nadir CD4 200-349	3.72 (3.66, 3.77)	5.01 (4.89, 5.14)
SMART, nadir CD4 <200	3.88 (3.83, 3.94)	5.82 (5.68, 5.96)

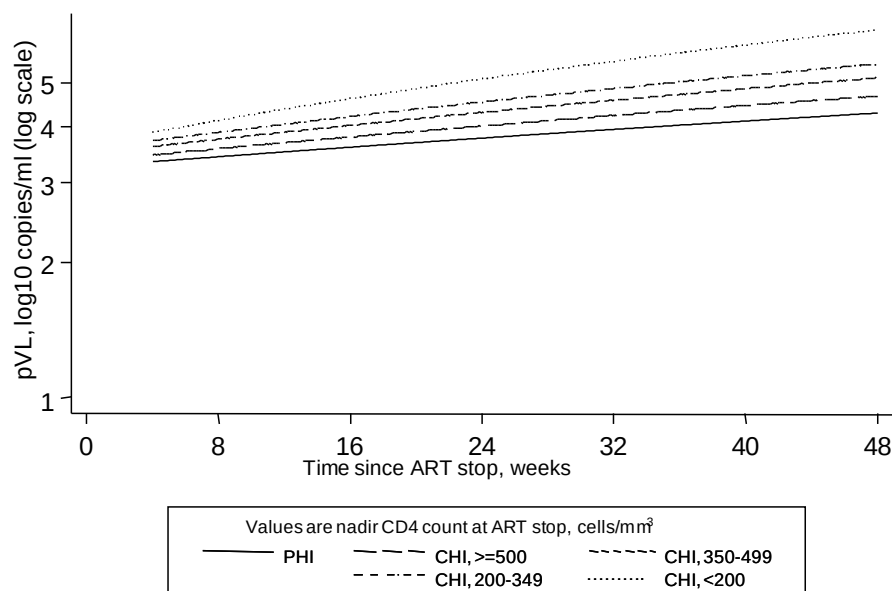
Predicted VL at week 4 and week 36 from mixed effect model for PHI (SPARTAC) vs. Chronic infection (SMART), with participants with chronic infection categorised by nadir CD4 cell count (cells/μl), based on a representative participant (MSM, aged 40 years, CD4 cell count 600 cells/μl at time of stopping ART).

Figure 5.2

a) VL up to 48 weeks after stopping ART, with participants with chronic HIV-1 infection categorised by nadir CD4 cell count



b) Predicted VL on mixed effect model over 4-48 weeks after stopping ART



a) Plasma VL in PHI (SPARTAC) versus chronic infection (CHI; SMART) is shown. CHI is categorised by nadir CD4 cell count.
b) Results from mixed effect model for VL over 4-48 weeks after stopping ART with CHI participants categorised by nadir CD4 cell count (cells/ μ l), based on a representative participant (MSM, aged 40 years, CD4 cell count 600 cells/ μ l at time of stopping ART).

5.6 Effect of CD4 cell count at ART stop on VL rebound

There was evidence of an interaction between nadir CD4 cell count in SMART participants and CD4 cell count at time of stopping ART, in relation to VL rebound over 48 weeks ($p=0.04$ and 0.0006 in the linear mixed model for VL up to 48 weeks and the linear regression for AUC, respectively). Among chronically infected participants with a nadir CD4 cell count <200 cells/ μl , lower CD4 cell nadir in association with higher CD4 cell count at stop resulted in higher viral load AUC after stopping ART, with a trend for such an association in participants with CD4 cell count <350 cells/ μl . For participants with nadir CD4 cell counts of <200 and $200\text{--}349$ cells/ μl , a CD4 cell count of 100 cells/ μl higher at ART stop was associated with an AUC 0.05 (95% CI $0.03, 0.08$) and 0.02 ($-0.002, 0.05$) $\log_{10}$ copies/ml higher, respectively. In contrast, among chronically infected participants with nadir CD4 cell count of ≥ 350 cells/ μl , there was no evidence of an association between CD4 cell count at stop and subsequent AUC (0.002 [$-0.03, 0.04$] and -0.01 [$-0.05, 0.03$] $\log_{10}$ copies/ml per 100 cells/ μl higher CD4 cell count at stop, for participants with nadir CD4 cell counts of $350\text{--}499$ and ≥ 500 cells/ μl , respectively). Among PHI participants, there was evidence to suggest that a higher CD4 cell count at time of stopping ART was associated with lower AUC (0.04 [95% CI $0.003, 0.08$] $\log_{10}$ copies/ml lower per 100 cells/ μl higher CD4 cell count at stop). The effect on AUC of PHI versus chronic infection/nadir CD4 cell count remained robust with or without adjustment for the CD4 cell count at time of stopping ART.

5.7 Summary of results in this chapter

In the SPARTAC trial, 42% of participants rebounded to pre-ART VL levels, at a median of 50 weeks after stopping therapy. Four weeks after stopping ART, although the proportion of participants with detectable VL ≥ 400 copies/ml was similar, VL levels were significantly lower in SPARTAC compared to those in SMART participants, and remained lower up to 48 weeks. Lower CD4 cell nadir in the SMART study was associated with higher VL after stopping ART, and VL rebound for SMART participants with CD4 cell nadir >500 cells/ μl was comparable to that seen in SPARTAC.

Discussion

This is the first study to compare the viral dynamics between PHI and chronic infection in individuals undergoing a randomised protocol-indicated interruption of ART. We observed that viral rebound after stopping ART initiated in PHI was lower than that observed in chronic infection, 4 weeks after treatment interruption. This difference was sustained over 48 weeks of follow-up. In addition, the time averaged viral burden was significantly lower in PHI patients versus the chronically infected population. These data are consistent with those from Steingrover et al. (2008) who reported differences in VL rebound when comparing treatment interruption in PHI to a non-randomised cohort of individuals who started ART in chronic infection. However, whereas Steingrover observed delayed or absent viral rebound in PHI, a similar proportion of PHI and chronically infected subjects in our study had HIV RNA levels >400 copies/ml at 4 weeks, and stopping ART either during PHI or chronic infection was associated with a rebound in VL in most individuals, although to lower levels in PHI. This discrepancy may be due to the small numbers investigated and the non-randomised nature of the treatment interruption in the Steingrover cohort, or due to inherent differences between the study populations.

The stage of infection prior to commencing therapy was predictive of viral dynamics after withdrawing treatment, indicating that the ability to control viral replication after discontinuation of ART is related to preservation of the immune response. When chronic infection participants were stratified by CD4 cell count at time of stop ART, nadir CD4 cell count prior to starting ART predicted the capacity to control viral replication, with lower nadir CD4 cell count associated with higher VL after ART stop. Viral rebound was higher across all CD4 cell strata when compared with rebound in PHI. Interestingly, in chronic infection patients with CD4 cell nadir <350, there was an association between CD4 cell count at stop and degree of viral rebound as measured by area under VL time curve, with higher CD4 cell count at stop being associated with higher AUC. Thus, the degree of viral rebound could be related to degree of immune reconstitution occurring on ART, or to the number of CD4 target cells available for viral infection upon stopping ART (Douek, et al. 2002) in those with severely depleted CD4 T-cell resources prior to commencing ART. In contrast, this effect was not apparent in PHI and higher CD4 cell count at stop was associated with lower VL rebound, indicating different VL dynamics between chronic and PHI.

These data may reflect differences in the size of the viral reservoir which is likely to be considerably smaller in those commencing ART early. Strain et al (2005) showed that in patients treated in PHI, within 6 months of infection, a more pronounced reduction in the size of the latent reservoir was seen compared with those treated in chronic infection (Strain, et al. 2005). Another study of NNRTI based ART during recent HIV-1 infection found that ART in PHI reduces HIV-1 DNA to levels

comparable to those seen in long-term non-progressors, and these effects were not apparent if therapy was started during chronic infection (Pires, et al. 2004). The size of the latent viral reservoir in patients with chronic HIV-1 infection has been shown to predict viral rebound after structured treatment interruption (Yerly, et al. 2004). Together, these findings suggest an association between timing of initiation of ART and decay of the HIV-1 reservoir, and highlight the potential benefits of early ART.

We observed higher viral rebound in MSM compared with heterosexual study participants. This difference remains unexplained. In SPARTAC, MSM were more likely to have subtype B virus than heterosexual participants. Some VL assays have been reported to underestimate HIV RNA in individuals with non-B subtypes (Holguin, et al. 2008), although this is not thought to affect more recent VL assays. However, subtype data were not available for SMART so are not included in this analysis. Another explanation could be gender differences in VL, as VL has previously been noted to be lower in women than in men in individuals with higher CD4 cell counts of >350 cells/ μ l, and most participants this analysis would fall into this CD4 cell category (Donnelly, et al. 2005). Our data are in agreement with findings from the CASCADE cohort which showed that among men, those infected heterosexually and by injecting drug use had on average 56% lower HIV RNA levels than homosexual men (Touloumi, et al. 2004). However, as no significant differences in HIV-1 progression are known to occur according to risk group (CASCADE 2000), the clinical relevance of this finding is not clear.

As the majority of studies examining short-course ART in PHI are observational in nature, potential bias associated with reason for stopping or starting therapy is likely to confound the results. In our analysis, participants underwent protocol-indicated cessation of ART, and the randomised reason for treatment interruption in both chronic infection and PHI populations will reduce bias in this analysis. However this study does have some limitations. No adjustment for ART duration was made, although length of ART was significantly longer for the chronic infection (SMART) participants compared to the PHI (SPARTAC) participants. This would have had the effect, in any case, of exaggerating the difference in VL rebound between the two populations. In addition, we were unable to compare the two ART arms of the SPARTAC trial, as follow-up is currently blinded. Of note, the SPARTAC participants randomised to the 12 weeks of ART may contribute disproportionately to those SPARTAC PHI participants with detectable VL at ART stop, who were then excluded from the analysis. Longitudinal analysis of both SMART and SPARTAC populations is confounded by exclusion of data due to informative censoring, for example, when individuals initiated long-term ART. This potential source of bias was accounted for to some extent in the mixed effect models. However, the result of failing to account for this completely would result in an underestimate of the observed difference between the two populations, due to the higher number of SMART patients reinitiating

therapy compared to SPARTAC. Finally, we did not examine CD4 cell count evolution in this analysis, or the relationship between VL rebound and clinical disease progression, because the SPARTAC trial is ongoing.

In both SMART and SPARTAC, VL was not assayed until 4 weeks post-treatment interruption. More frequent testing would give a true indication of immediate viral dynamics following ART cessation. It is possible that initial viral rebound will peak immediately following treatment interruption, prior to VL quantification in this study. However, models comparing rate of viral replication after withdrawal of treatment to initial acute infection show the rate in initial acute infection to be nearly five times faster (Little, et al. 1999). Although treatment discontinuation in PHI was associated with viral rebound, this was not observed to rebound to pre-ART levels until a median of 50 weeks, and it is unlikely that potentially greater increases would have been observed if VL had been measured sooner after treatment cessation.

Data from African heterosexual transmission studies have shown that for individuals in HIV serodiscordant relationships, where the HIV-infected partner's VL is less than 1500 copies RNA/ml, HIV-1 transmission is rare (Quinn, et al. 2000; Wawer, et al. 2005). In our study, the majority of participants in both SMARTAC and SMART had viral rebound at one month to levels sufficient to pose a risk of onward transmission. An additional goal of treating individuals with PHI may be to limit the enhanced risk of onward viral transmission and the role of ART as a public health tool to limit HIV-1 transmission is receiving heightened attention. The high VL in infected blood during acute HIV-1 infection is reflected in genital tract secretions, and seminal and cervical VLs strongly correlate with plasma VLs for the first 6 months of infection, and are significantly higher than levels in chronic infection (Miller, et al. 2010; Morrison, et al. 2010). Specific characteristics of recently transmitted viral variants make PHI particularly infectious (Keele and Derdeyn 2009; Ma, et al. 2009). It is unknown how long after transmission these characteristics remain or whether they are preserved after intermittent ART. Targeting individuals with ART during PHI could have a marked impact on HIV-1 transmission, but it is crucial that strategies investigating the use of short-term ART as a prevention measure examine the consequences of ART discontinuation and viral rebound on onward transmission. In the SMART study, individuals did not reduce high risk sexual behaviour, despite treatment interruption and detectable VL (Burman, et al. 2008). In a recent study looking at onward HIV-1 transmission amongst individuals treated in PHI who stopped ART, there were at least five new primary infections originating from these patients within 16-61 weeks after stopping early ART (Rieder, et al. 2010). The level of viral rebound following cessation of ART in PHI should therefore be anticipated when designing a strategy aimed at limiting secondary transmission, and it is likely that long-term ART would be preferable with this goal in mind.

Treatment interruption in the SMART study was associated with adverse clinical outcome and this strategy is no longer recommended in chronic infection (El-Sadr, et al. 2006). The levels of plasma HIV RNA in PHI correlates with biomarkers of inflammation, coagulation and immune activation (Chapter 4 and 6). Levels of viral rebound may therefore affect risk, not only of HIV-1 disease progression and development of opportunistic infection, but also of conditions not traditionally related to HIV such as CVD. The clinical effects of short-course treatment in PHI currently remain unknown; however the differences in VL dynamics between PHI and chronic infection demonstrated in this analysis indicate that the consequences of treatment interruption may differ. Such differences may reflect immunological status, immune activation and reservoir size. This observed difference in plasma VL rebound between PHI and chronic infection may translate into differences in levels of immune activation and inflammation on discontinuation of ART commenced in PHI versus chronic infection, and supports the hypothesis that treatment interruption following ART in PHI may not be associated with same adverse clinical effects as seen in the SMART trial.

Chapter 6: T-cell Immune Activation in PHI

HIV-1 infection induces a state of chronic immune hyper-activation which is thought to contribute towards the immunological destruction that precedes clinical disease (Cadogan and Dalgleish 2008). In untreated HIV-1 infected patients, the level of T-cell immune activation is an independent predictor of disease progression (Bofill, et al. 1996; Deeks, et al. 2004; Hazenberg, et al. 2003b; Liu, et al. 1997) and in some studies has been shown to be a stronger predictor of disease progression than CD4 cell count or VL (Giorgi, et al. 2002; Liu, et al. 1997).

Following HIV-1 acquisition, massive depletion of CCR5 positive CD4 memory T-lymphocytes occurs, focused primarily in the GI mucosa through cytopathic and apoptotic mechanisms, resulting in depletion of T-cell reservoirs (Cadogan and Dalgleish 2008; Douek 2007; Mattapallil, et al. 2005). It has been hypothesized that this irreversible GALT damage creates a permanently imbalanced immunological environment instigating chronic homeostatic events as the immune system struggles to replace its losses, leading to a high turnover of T-cells, continuous T-cell activation, and a gradual loss of peripheral CD4 cells (Cadogan and Dalgleish 2008). In addition, damage to gut mucosal surfaces allows translocation of microbial products across the gut epithelium and into the systemic circulation, resulting in persistent antigenic stimulation of the immune system and hypothesized to be a major cause of HIV-associated immune activation (Brenchley, et al. 2006).

Deeks et al. (2004) showed that the level of T-cell activation is established early in the course of HIV-1 infection and varies widely between individuals, but is generally stable within a given person. It is possible that short-course ART given during PHI will reduce long-term immune activation even after discontinuation of ART, by rapidly limiting VL and thereby altering the immunological events described above. This hypothesis will be tested after the SPARTAC study has been unblinded. To gain some understanding of the processes occurring during PHI, we undertook an in-depth analysis of different markers of CD8 and CD4 T-cell activation, and assessed both viral and host factors associated with these parameters.

CD38 and HLA-DR are cell surface molecules which are known to be upregulated upon antigenic activation *in vivo* and are commonly used as markers of T-cell activation. In addition, the relationship between expression of PD-1, a marker of T-cell exhaustion which is also upregulated on activated T-cells, with other markers of activation was investigated. PD-1 expression in chronic infection has been associated with impaired functional capacity of CTL such as reduced cytokine production and loss of viral control (Day, et al. 2006). PD-1 positive cells undergo high rates of apoptosis and have limited proliferative capacity (Petrovas, et al. 2006) and the interactions between PD-1 and its

ligands are thought to be involved in negative regulation of immune activation (Cai, et al. 2004; Freeman, et al. 2000; Latchman, et al. 2001). The role of PD-1 in PHI has not been fully explored.

The aims of this chapter were to:

- i. compare immune activation in PHI to those at SPARTAC week 60 and to healthy controls (methods chapter 2.3).
- ii. explore the relationship between different markers of immune activation, PD-1 expression, inflammation and coagulation in individuals with PHI.
- iii. assess host and viral factors associated with immune activation and PD-1 expression in PHI

Results

6.1 Immune activation in PHI, at week 60 and in healthy controls

PBMC samples for T-cell activation assays were available from the UK recruited SPARTAC participants at week 0 and week 60. In order to investigate the effect of duration of infection on markers of T-cell activation, a comparison was undertaken between week 0 and week 60 of the study. Due to the blinded nature of the SPARTAC trial, week 60 results were analysed together regardless of randomisation arm, although ultimately a comparison between the 3 arms of SPARTAC will be undertaken at week 60 to assess the effect of short-course ART on immune activation after discontinuing treatment. All individuals had discontinued treatment given as a trial intervention by week 60. PBMC samples from 10 healthy individuals were analysed to assess whether T-cell activation was higher in HIV-1 infected individuals compared to healthy controls.

One-hundred-and-forty-nine SPARTAC participants were recruited in the UK and baseline characteristics of these individuals are shown in Table 6.1. The majority of participants were MSM with subtype B virus. The median age was 34 years (range 19-60). The median estimated time from seroconversion at the baseline study visit was 73 days (range 8 to 156 days).

The following T-cell activation markers were assessed on both CD8 and CD4 T-cells: CD38 expression (both proportion of cells expressing CD38 (CD38%) and Median Fluorescent Intensity (MFI), HLA-DR expression (both HLA-DR% and HLA-DR MFI), proportion of cells with dual expression of CD38 and HLA-DR (CD38 HLA-DR%), and proportion of cells expressing PD-1 (PD-1%).

Table 6.1. Baseline characteristics of UK SPARTAC participants

Baseline variable		Number or Median	(%) or [IQR]
Sex:	Male	143	(96)
	Female	6	(4)
Risk group:	MSM	139	(93)
	Heterosexual	9	(6)
	Other / unknown	1	(1)
Age (years)		34	[28,41]
Estimated time from seroconversion (days)		73	[47,93]
BMI		23.3	[21.6, 25.4]
Systolic blood pressure		127	[116, 135]
Diastolic blood pressure		76	[68, 82]
Clade	B	134	(90)
	non-B	15	(10)
Viral tropism *	R5	135/146	(92)
	X4	11/146	(8)
CD4 (cells/ μ l) overall		540	[406, 669]
<350		18	(12)
350-499		42	(28)
>500		89	(60)
HIV RNA (copies/ml) overall		57050	[11774, 195,517]
<400		7	(5)
400-9999		26	(17)
10000-99999		55	(37)
>100000		61	(41)
HLA type*	B*57 +ve	10/120	(7)
	B*27 +ve	14/120	(10)

Baseline characteristics of UK recruited SPARTAC participants (n=149) are shown. *Tropism available in 146 participants; HLA typing was available for 120 participants only

CD8 T-cell activation

Baseline PBMC samples at week 0 were available from 122 individuals and from 124 individuals at week 60. Results for 122 and 124 participants at week 0 and 60, respectively, are shown except for PD-1 where results for 120 and 114 participants were available at week 0 and week 60, respectively (results for PD-1 were disregarded due technical problems with the APC laser during analysis of one batch of 12 samples). Median values and IQR are shown in Table 6.2 and data are further demonstrated on box-plot graphs in Figure 6.1.

In PHI (week 0) the median percentage of CD8 cells expressing CD38 (CD8 CD38%) was 41.5%, median percentage of CD8 cells expressing HLA-DR (CD8 HLA-DR%) was 32% and median dual expression of CD38 and HLA-DR activation markers (CD8 CD38 HLA-DR%) was 17.3%. Median PD-1% expression on CD8 cells was 4.5%. Differences in immune activation at week 60 were compared to week 0 using the Wilcoxon signed rank test. At week 60 there was a significant reduction in the percentage of CD8 cells expressing CD38 ($p<0.0001$), in CD38 MFI ($p<0.0001$), and in the percentage of CD8 cells co-expressing CD38 and HLA-DR ($p<0.0001$). There was a trend toward reduced CD8 HLA-DR% expression at week 60 ($p=0.08$) although no change was observed in HLA-DR MFI ($p=0.2$).

Figure 6.2 shows flow cytometry plots in a single representative individual and demonstrates a clear population of activated CD8 cells at week 0 which has reduced in size considerably by week 60. There was no change in CD8 PD-1 expression between week 0 and week 60.

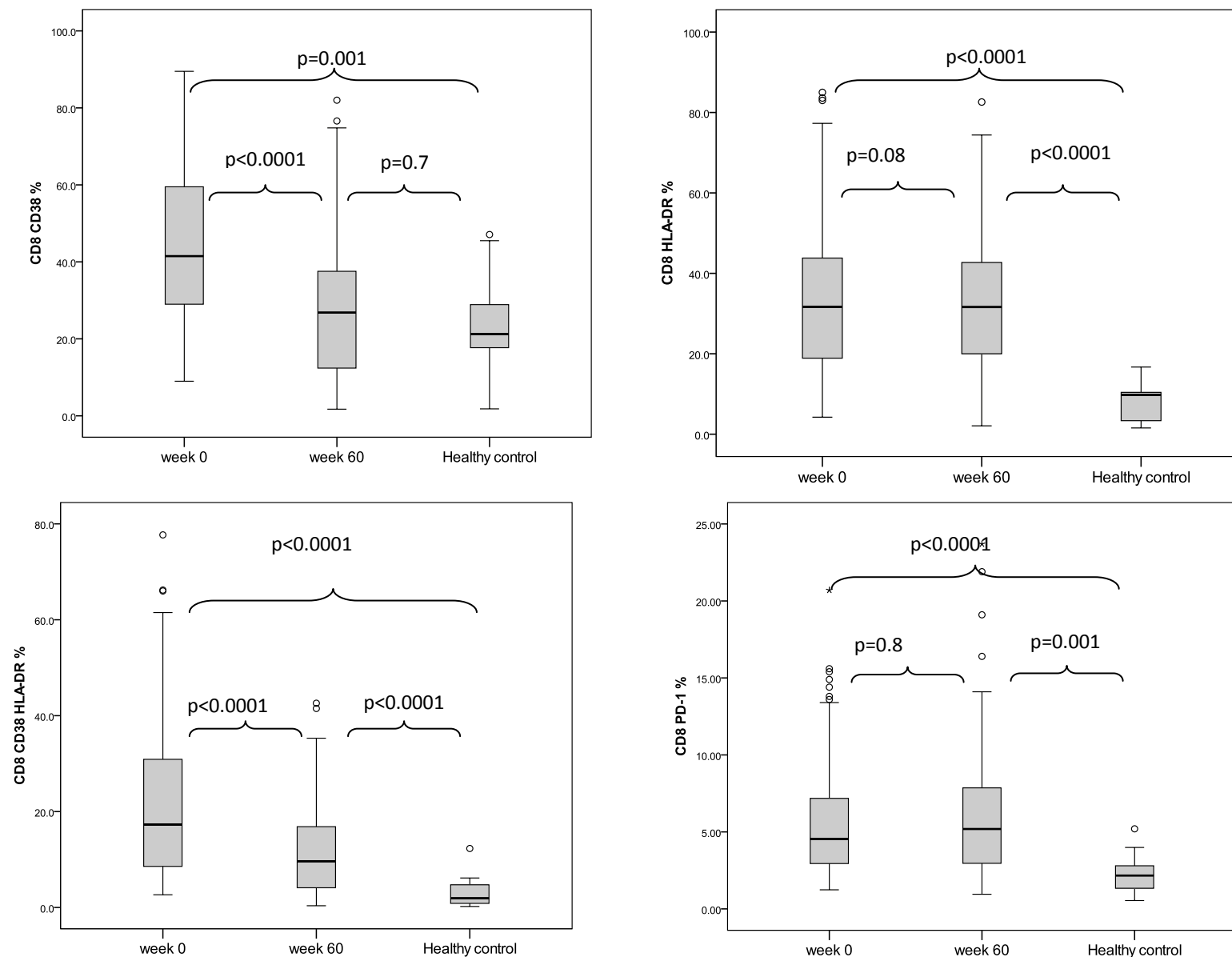
Levels of immune activation in HIV-1 infected individuals were compared with healthy controls using Mann-Whitney tests. All markers of CD8 T-cell activation were significantly higher at week 0 when compared to healthy controls ($p\leq 0.001$ for all). At week 60, CD38 activation (both percentage expression and MFI) was not significantly higher than in healthy controls, although HLA-DR% expression, HLA-DR MFI, dual HLA-DR CD38 % expression, and PD-1 expression remained significantly elevated ($p\leq 0.001$ for all).

Table 6.2. T-cell activation and PD-1 expression in SPARTAC participants and in healthy controls

Parameter		Week 0			Week 60			Healthy control		
		N	Median	[IQR]	N	Median	[IQR]	N	Median	[IQR]
CD8 cells	CD38%	122	41.5	29, 59.5	124	26.8	12.4, 37.5	10	21.2	17.7, 28.9
	CD38 MFI	122	402	269, 630	124	276.5	153.5, 437.5	10	219	140, 323
	HLA-DR %	122	32.0	18.9, 43.8	124	31.5	20.0, 42.7	10	9.8	3.4, 10.4
	HLA-DR MFI	122	178.5	109, 303	124	187	113.5, 303	10	63.5	36.1, 75.5
	CD38 HLA-DR %	122	17.3	8.5, 30.9	124	9.6	4.1, 16.8	10	1.9	0.8, 4.7
	PD-1%	120	4.5	2.9, 7.2	114	5.2	2.3, 7.9	10	2.2	1.3, 2.8
CD4 cells	CD38%	122	52.3	38, 65.3	124	45.1	29.7, 57.1	10	44.1	29.1, 57.7
	CD38 MFI	122	463	310, 594	124	362	229, 560.5	10	263	177, 587
	HLA-DR %	122	10.8	6.7, 15.4	124	11.2	6.8, 18.3	10	5.7	1.7, 8.2
	HLA-DR MFI	122	51.5	35.9, 64.4	124	56.0	35.9, 81.7	10	43.8	21.1, 50.4
	CD38 HLA-DR %	122	5.3	3.3, 9.0	124	5.0	2.2, 8.8	10	3.4	0.9, 4.5
	PD-1%	120	8.4	5.9, 10.9	114	7.5	4.4, 10.7	10	1.8	1.0, 2.3

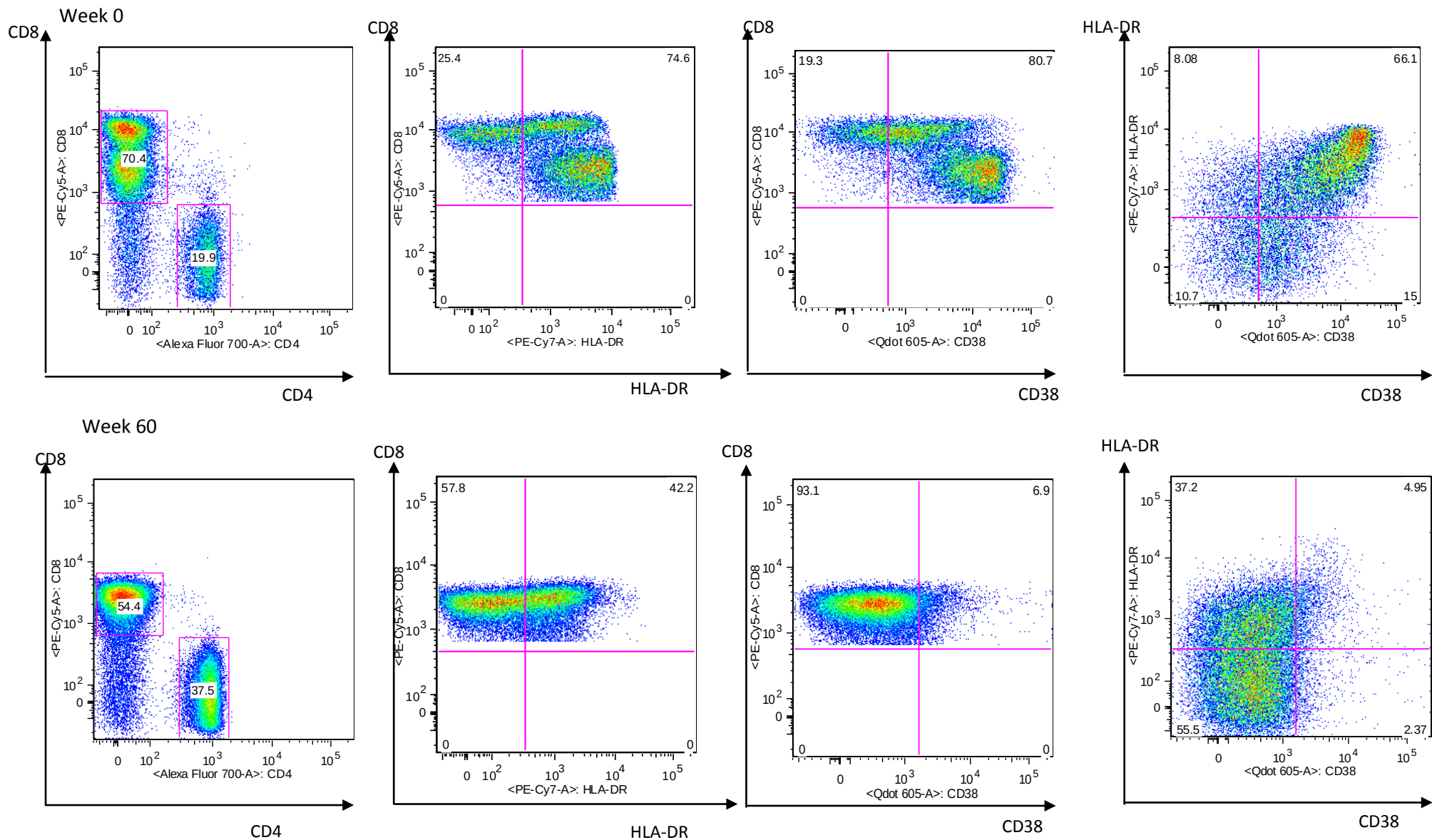
Median values and IQR for markers of CD8 and CD4 T-cell activation are shown at SPARTAC baseline visit (PHI), SPARTAC week 60 and in healthy controls

Figure 6.1. CD8 T-cell activation and PD-1 expression in SPARTAC participants and in healthy controls



Boxplots showing median, IQR and upper and lower adjacent values for CD8 T-cell activation markers in SPARTAC participants at week 0 (n=122) and week 60 (n=124), and in healthy controls (n=10). For PD-1 n=120 at week 0 and n=114 at week 60. Week 0 was compared to week 60 using Wilcoxon signed ranks test for paired samples. Mann-Whitney U tests were used to compare HIV-negative controls to SPARTAC participants

Figure 6.2. Flow cytometry plot demonstrating CD8 T-cell activation in a single representative individual at week 0 and week 60



Flow cytometry dot-plots of single and dual expression of HLA-DR and CD38 on CD8 cells at Week 0 (HIV-1 seroconversion) and Week 60 are shown in the same individual. 128 Cell are gated on CD8. There is a clear population of activated CD8 cells, expressing both CD38 and HLA-DR, which has reduced considerably by week 60

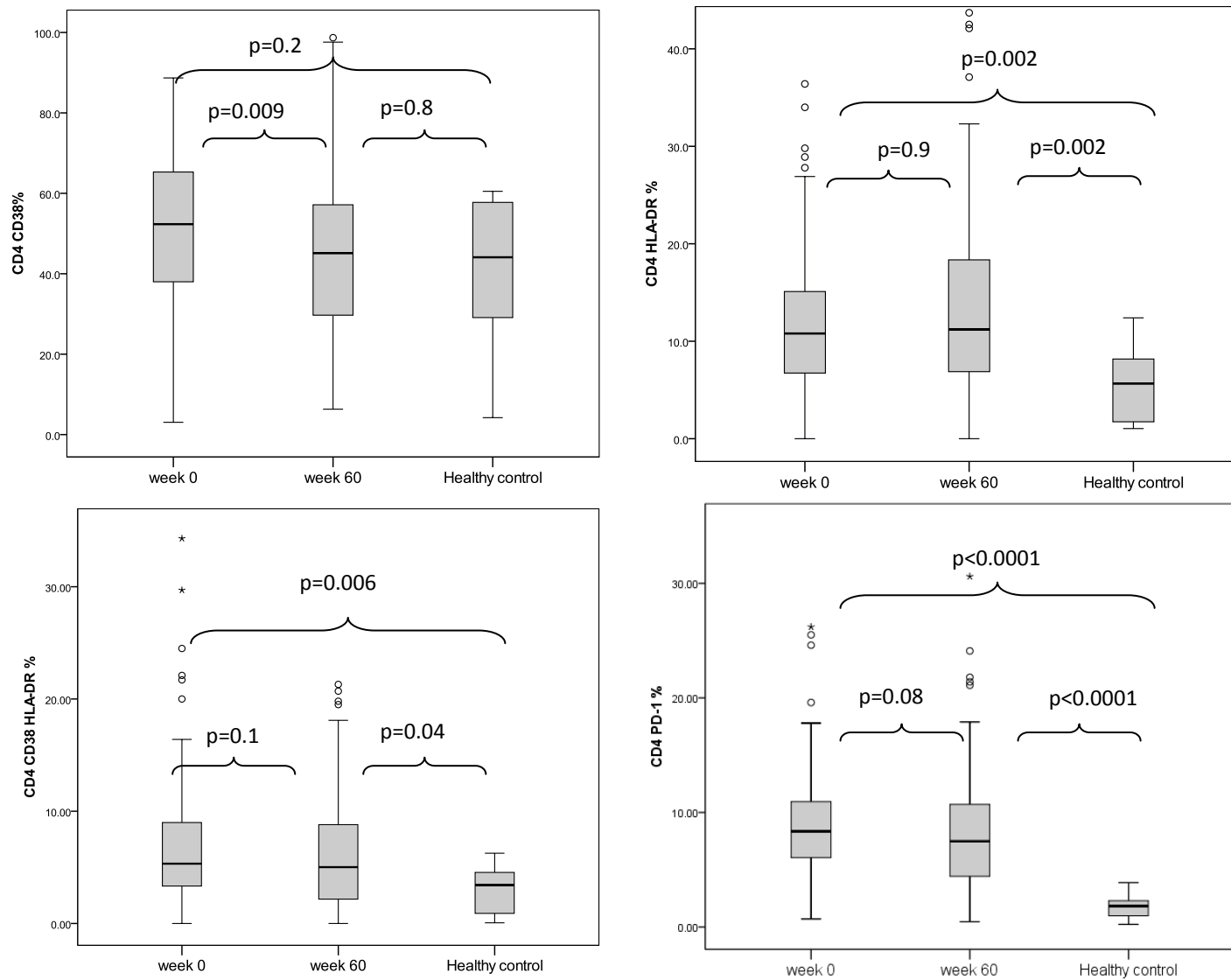
CD4 T-cell activation

In PHI, the median percentage of CD4 cells expressing CD38 (CD4 CD38%) was 52.3%, median percentage of cells expressing HLA-DR (CD4 HLA-DR%) was 10.8% and median dual expression of CD38 and HLA-DR% (CD4 CD38 HLA-DR) was 5.3%. Median CD4 PD-1% expression was 8.4%. Results at week 0, week 60 and in healthy controls are shown in Table 6.2 and data are further demonstrated on box-plot graphs in Figure 6.3.

Compared to week 0, at week 60 there was a significant reduction in CD38% expression on CD4 cells ($p=0.009$) and a significant reduction in CD4 CD38 MFI ($p=0.03$). There was a trend towards reduced PD-1 % expression ($p=0.08$). There was no significant change in HLA-DR % expression ($p=0.9$), HLA-DR MFI ($p=0.2$) or dual expression of HLA-DR and CD38 ($p=0.1$) between weeks 0 and 60.

HLA-DR expression, dual CD38 HLA-DR% expression and PD-1% expression were significantly elevated when compared to healthy controls at both week 0 and week 60 (Figure 6.3). HLA-DR MFI was also significantly elevated at week 0 and 60 compared to healthy controls ($p=0.03$ and $p=0.01$ for week 0 and week 60, respectively). There was no significant difference between CD4 CD38% expression or CD38 MFI in HIV-1 infected individuals compared to healthy controls in this cohort.

Figure 6.3. CD4 T-cell activation levels in SPARTAC participants and in healthy controls



Box-plots showing median, IQR and upper and lower adjacent values for CD4 T-cell activation markers in SPARTAC participants at week 0 (n=122) and week 60 (n=124), and in healthy controls (n=10). For PD-1 n=120 at week 0 and n=114 at week 60. Week 0 was compared to week 60 using Wilcoxon signed ranks test for paired samples. Mann-Whitney U tests were used to compare healthy controls to SPARTAC participants

6.2 Relationship between markers of T-cell immune activation, PD-1 expression, and inflammatory biomarkers in PHI

The relationship between markers of T-cell immune activation, PD-1 expression, and inflammatory biomarkers in individuals with PHI were examined. With the exception of CD4 and CD8 cell CD38% expression which were normally distributed, activation marker results were skewed. Therefore non-parametric testing was used to assess the associations between variables, although results from parametric Pearson correlations of both the absolute and log-transformed variables gave similar results. Table 6.3 shows Spearman Rho correlations between different markers of immune activation. Different measures of CD8 T-cell activation were correlated strongly with each other ($p < 0.0001$ for all). CD8 PD-1 expression correlated with all markers of CD8 activation ($p < 0.0001$ for all).

Different measures of CD4 T-cell activation also correlated with each other ($p < 0.0001$ for all). CD4 PD-1 expression was not associated with CD4 CD38 activation, but did correlate with dual CD4 CD38 HLA-DR expression ($p = 0.003$).

CD8 T-cell activation markers (CD38, HLA-DR and dual expression) were correlated with all markers of CD4 T-cell activation ($p < 0.0001$ for all). CD8 PD-1% expression correlated with CD4 PD-1% expression, and with CD4 CD38% expression and dual CD4 CD38 HLA-DR% expression. CD4 PD-1% expression was also associated with CD8 CD38% expression and dual CD8 CD38 HLA-DR% expression.

The relationship between markers of immune activation and IL-6 and D-dimer were also examined (Table 6.3). Biomarker results for IL-6 and D-dimer were available in 129 and 120 UK participants, respectively, of whom 111 and 105 also had cells available for T-cell activation studies. There was a significant correlation between IL-6 and CD8 CD38, CD8 CD38 MFI and dual CD8 expression of CD38 and HLA-DR ($p = 0.008$, $p = 0.004$ and $p = 0.01$, respectively). D-dimer correlated with CD8 CD38% expression, dual CD8 CD38 HLA-DR% expression and CD8 PD-1 expression ($p = 0.003$, $p = 0.03$, $p = 0.02$, respectively). There was no significant correlation between CD4 T-cell activation markers and IL-6 or D-dimer.

Table 6.3. Relationship between different markers of CD8 and CD4 T-cell activation, IL-6 and D-dimer

		CD8 cells						CD4 cells						Plasma Biomarkers	
	Parameter	CD38 %	CD38 MFI	HLA-DR %	HLA-DR MFI	CD38 HLA-DR %	PD-1 %	CD38 %	CD38 MFI	HLA-DR %	HLA-DR MFI	CD38 HLA-DR %	PD-1%	IL-6	D-dimer
CD8 cells	CD38 % coefficient <i>p-value</i>		0.78 0.000	0.39 0.000	0.39 0.000	0.76 0.000	0.54 0.000	0.63 0.000	0.50 0.000	0.17 0.06	0.18 0.048	0.51 0.000	0.33 0.000	0.25 0.008	0.30 0.003
	CD38 MFI coefficient <i>p-value</i>	0.79 0.000		0.56 0.000	0.56 0.000	0.73 0.000	0.40 0.000	0.67 0.000	0.71 0.000	0.33 0.000	0.25 0.006	0.62 0.000	0.07 0.5	0.27 0.004	0.16 0.1
	HLA-DR % coefficient <i>p-value</i>	0.39 0.000	0.56 0.000		0.96 0.000	0.81 0.000	0.31 0.000	0.38 0.000	0.40 0.000	0.74 0.000	0.64 0.000	0.73 0.000	0.09 0.3	0.12 0.2	0.03 0.75
	HLA-DR MFI coefficient <i>p-value</i>	0.39 0.000	0.56 0.000	0.96 0.000		0.78 0.000	0.31 0.000	0.40 0.000	0.42 0.000	0.72 0.000	0.63 0.000	0.71 0.000	0.05 0.59	0.14 0.13	0.03 0.72
	dual CD38 HLA-DR % coefficient <i>p-value</i>	0.76 0.000	0.73 0.000	0.0.81 0.000	0.78 0.000		0.45 0.000	0.50 0.000	0.45 0.000	0.55 0.000	0.50 0.000	0.70 0.000	0.24 0.009	0.23 0.01	0.21 0.03
	PD-1% coefficient <i>p-value</i>	0.54 0.000	0.40 0.000	0.31 0.000	0.31 0.000	0.45 0.000		0.26 0.000	0.14 0.12	0.16 0.07	0.18 0.048	0.28 0.002	0.45 0.000	0.07 0.5	0.23 0.02
CD4 cells	CD38 % coefficient <i>p-value</i>	0.63 0.000	0.68 0.000	0.38 0.000	0.40 0.000	0.5 0.000	0.26 0.004		0.90 0.000	0.36 0.000	0.41 0.000	0.65 0.000	0.14 0.13	0.06 0.50	-0.01 0.9
	CD38 MFI coefficient <i>p-value</i>	0.50 0.000	0.71 0.000	0.40 0.000	0.42 0.000	0.45 0.000	0.14 0.12	0.90 0.000		0.39 0.000	0.41 0.000	0.63 0.000	-0.05 0.6	0.1 0.3	-0.09 0.4
	HLA-DR % coefficient <i>p-value</i>	0.17 0.06	0.33 0.000	0.74 0.000	0.72 0.000	0.55 0.000	0.16 0.07	0.36 0.000	0.39 0.000		0.89 0.000	0.87 0.000	0.16 0.08	0.08 0.4	0.11 0.28
	HLA-DR MFI coefficient <i>p-value</i>	0.18 0.048	0.25 0.006	0.64 0.000	0.63 0.000	0.50 0.000	0.18 0.048	0.41 0.000	0.41 0.000	0.89 0.000		0.81 0.000	0.19 0.04	0.05 0.64	0.07 0.46
	dual CD38HLA-DR % coefficient <i>p-value</i>	0.51 0.000	0.62 0.000	0.73 0.000	0.71 0.000	0.70 0.000	0.28 0.000	0.65 0.000	0.64 0.000	0.87 0.000	0.81 0.000		0.27 0.003	0.16 0.09	0.15 0.13
	PD-1% coefficient <i>p-value</i>	0.33 0.000	0.07 0.5	0.09 0.3	0.05 0.06	0.24 0.009	0.45 0.000	0.14 0.13	-0.05 0.6	0.16 0.08	0.19 0.04	0.27 0.003		-0.002 0.98	0.13 0.18
Plasma Biomarkers	IL-6 coefficient <i>p-value</i>	0.25 0.008	0.27 0.004	0.12 0.2	0.14 0.13	0.24 0.01	0.07 0.5	0.06 0.5	0.1 0.3	0.08 0.4	0.05 0.6	0.16 0.09	-0.002 0.98		0.28 0.002
	D-dimer coefficient <i>p-value</i>	0.29 0.003	0.16 0.11	0.03 0.7	0.03 0.7	0.21 0.03	0.23 0.02	-0.01 0.9	-0.09 0.4	0.11 0.29	0.07 0.46	0.15 0.13	0.13 0.18	0.28 0.002	

The association between different CD8 and CD4 cell markers of T-cell activation, and plasma biomarkers IL-6 and D-dimer, are shown. Non parametric Spearman rho correlation coefficients are given, although parametric Pearson correlations were similar. Significant p-values are shown in bold. N=122 for T-cell activation markers, N=120 for PD-1, N=111 for IL-6 and N=105 for D-dimer. Percentage (%) expression on CD8 and CD4 T-cells and median fluorescent intensity (MFI) are given for CD38 and HLA-DR.

6.3 Viral and Host factors associated with T-cell activation in PHI

Viral factors and immune activation

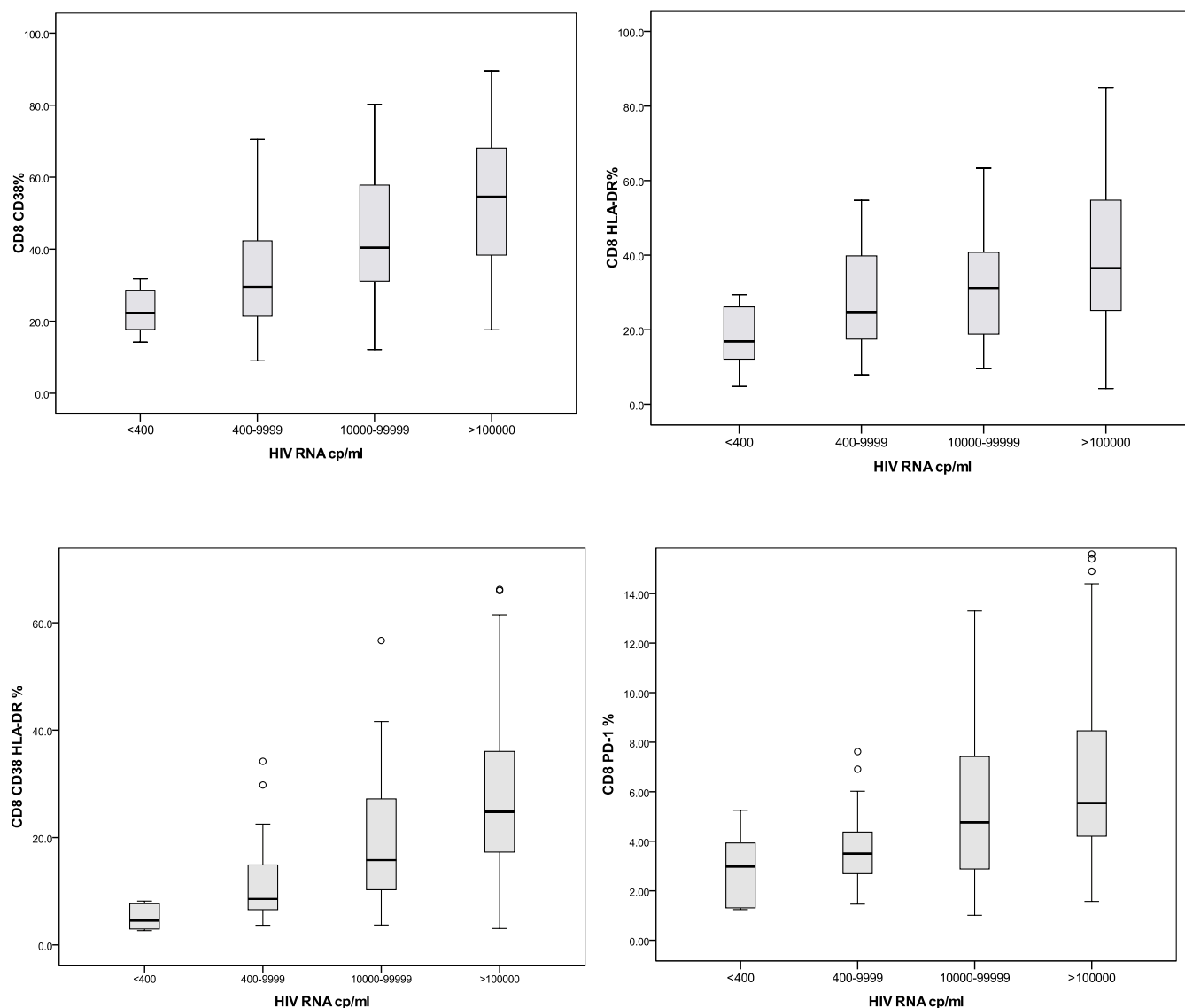
Viral load

The relationship between VL and markers of CD8 and CD4 cell activation was investigated. There was a highly significant association between HIV RNA levels and all markers of CD8 T-cell activation (CD38% and HIV RNA: Spearman $\rho=0.51$, $p<0.0001$; CD38 MFI and HIV RNA: $\rho=0.52$ $p<0.0001$; HLA-DR% and HIV RNA: $\rho=0.29$, $p=0.001$; HLA-DR MFI and HIV RNA: $\rho=0.31$, $p=0.001$; dual CD38 HLA-DR% and HIV RNA: $\rho=0.54$ $p<0.0001$).

There was a highly significant association between PD-1 expression and HIV RNA levels (Spearman $\rho=0.4$, $p<0.0001$). Due to the known relationship between PD-1 expression and T-cell activation, it is possible that this observed correlation between PD-1 expression and HIV RNA may be related to the correlation between CD38 expression and HIV RNA, i.e. the effect of VL on PD-1 expression is mediated through the effect of VL on CD38 expression. In order to control for this, unadjusted Pearson correlations between $\log_{10}$ -transformed PD-1 and $\log_{10}$ VL were undertaken, and compared to partial correlations, controlling for CD38 expression. Unadjusted Pearson correlation showed a highly significant relationship between $\log_{10}$ PD-1 and $\log_{10}$ VL (coefficient 0.4, $p<0.0001$). On controlling for CD38%, the strength of this correlation reduced substantially (coefficient 0.18, $p=0.044$) indicating that the relationship between PD-1 and VL is at least in part mediated through upregulation of PD-1 in response to immune activation. However controlling for HLA-DR expression did not significantly alter the strength of the association between $\log_{10}$ VL and $\log_{10}$ PD-1 (coefficient 0.3, $p<0.0001$).

The relationship between immune activation and HIV RNA was further examined according to VL strata. There was a rise in CD8 T-cell activation and CD8 PD-1 expression with each VL stratum (Figure 6.4).

Figure 6.4. Effect of VL on level of CD8 T-cell activation in UK SPARTAC patients at baseline

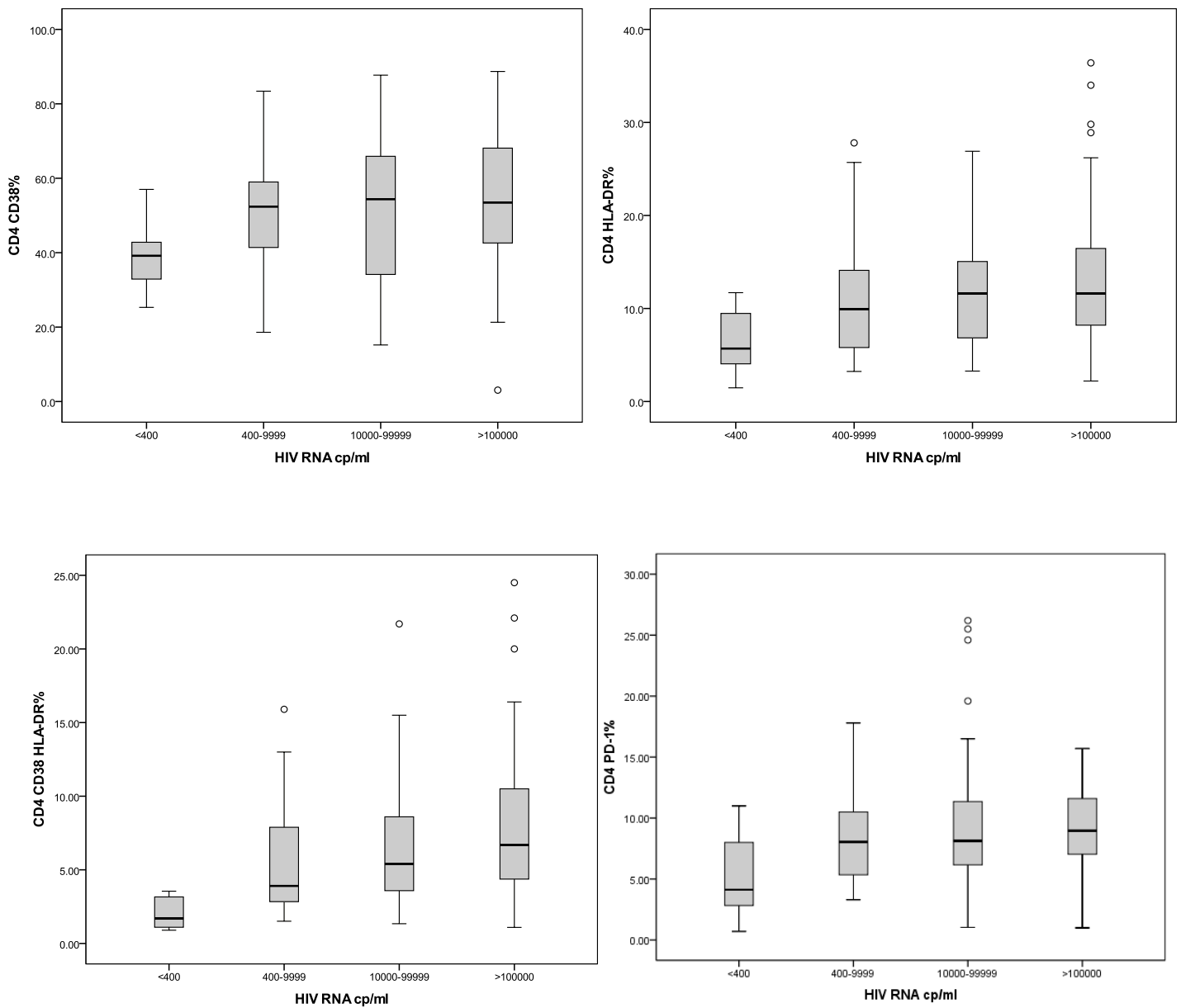


Box-plots showing CD8 T-cell activation markers CD38%, HLA-DR% and dual CD38 HLA-DR%, and PD-1% expression (median, IQR and upper and lower adjacent values) for individuals as divided by VL strata (VL<400 n=7; VL 400-9999 n=26; VL 10,000-99999 n=55, VL>100,000 n=60)

The association between CD4 cell activation and HIV RNA levels was less robust. There was a trend towards a correlation between CD4 CD38 expression and HIV RNA levels (CD38% and HIV RNA: Spearman rho 0.17, $p=0.06$; CD38 MFI and HIV RNA: rho=0.17, $p=0.06$) and similar results for HLA-DR expression (HLA-DR% and HIV RNA: rho=0.17, $p=0.07$; HLA-DR MFI: rho=0.16 $p=0.07$). Dual expression of CD38 and HLA-DR on CD4 cells was significantly associated with HIV RNA (rho=0.33, $p<0.0001$). There was a trend towards an association between CD4 PD-1 expression and HIV RNA levels (rho=0.17, $p=0.07$).

For CD4 CD38%, CD4 HLA-DR-% and PD-1%, lower levels of immune activation were seen in the lower 2 viral strata, but the relationship is less clear for the upper VL strata (Figure 6.5). There is a clear rise in dual CD38 HLA-DR% for every VL stratum.

Figure 6.5. Effect of VL on level of CD4 T-cell activation in UK SPARTAC patients at baseline



Box-plots showing CD4 T-cell activation markers CD38%, HLA-DR% and dual CD38 HLA-DR%, and PD-1% expression (median, IQR and upper and lower adjacent values) for individuals as divided by VL strata (VL<400 n=7; VL 400-9999 n=26; VL 10,000-99999 n=55, VL>100,000 n=60)

Viral tropism

Primary HIV infection is characterized by the predominance of CCR5 tropic virus. CXCR4 tropic virus is associated with faster disease progression (Koot, et al. 1993; Tersmette, et al. 1989). We hypothesised that increased immune activation associated with CXCR4 tropic variants may be a potential mechanism explaining this observed enhanced rate of immunological decline. Viral tropism was assessed genotypically from samples taken at baseline and was available in 146 patients of whom 135 (92.5 %) had R5 and 11 (7.5 %) had X4 tropic virus. PBMCs for T-cell activation were available in 120 of these individuals, of whom 110 (93.7%) had R5 virus and 10 (8.3%) had X4 virus. HIV-1 parameters (CD4 and VL), markers of T-cell activation, PD-1 expression, and biomarkers IL-6 and D-dimer were compared in individuals with R5 versus X4 using virus using Mann-Whitney U tests. Results are shown in Table 6.4.

Overall, for the 146 individuals in whom tropism results were available, median VL was 64,046 (IQR 17602, 193758) copies/ml in individuals with R5 virus and 28,800 (IQR 3682, 40937) copies/ml in those with X4 virus ($p=0.08$). Median CD4 cell count was 540 (IQR 401, 664) cells/ μ l in those with R5 virus and 550 (IQR 441, 563) cells/ μ l for those with X4 viruses ($p=0.9$). In the 120 individuals with available PBMCs for T-cell activation included in this analysis, median CD4 cell count was 550 (IQR 412, 668) vs. 520 (460, 563) cells/ μ l in those with R5 and X4 variants respectively ($p=0.7$), and median VL was 59623 (IQR 14965, 184103) vs. 28462 (IQR 4855, 44713) copies/ml in those with R5 and X4 variants respectively ($p=0.2$).

X4 variants were associated with higher levels of CD4 T-cell activation as measured by percentage of CD4 cells expressing HLA-DR (19.6% vs. 10.6% $p=0.01$), HLA-DR MFI (76.9 vs. 49.5, $p=0.005$), and dual CD38 HLA-DR% expression (9.3% vs. 4.9% $p=0.01$). There was a trend toward higher CD4 CD38 MFI in those with X4 using variants (591.5 vs. 451.5, $p=0.06$). The difference in CD4 T-cell activation between individuals with R5 vs. X4 variants is further demonstrated on box-plot graphs shown in Figure 6.6.

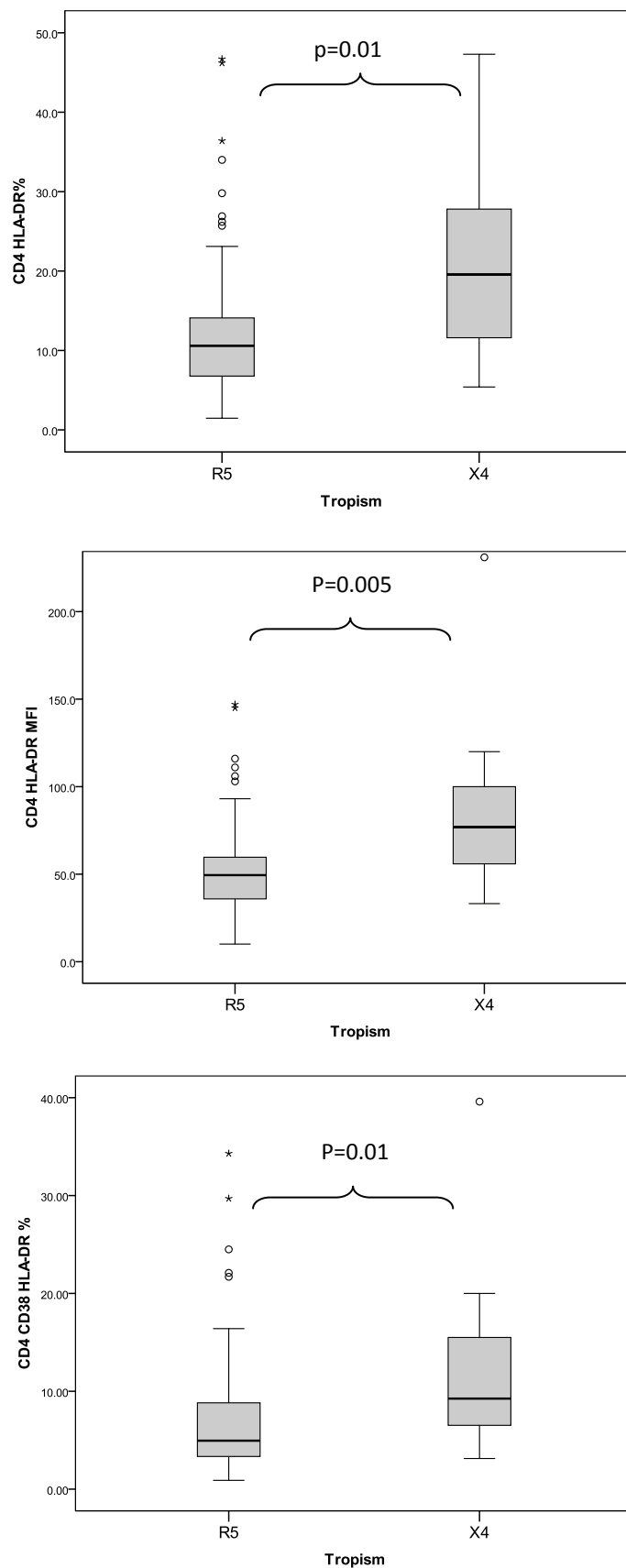
There was no significant association between CD8 T-cell activation and viral tropism. However there was a trend towards higher CD8 HLA-DR expression in those with X4 variants (HLA-DR%: 41.2% vs. 30.9%, $p=0.06$; HLA-DR MFI: 262.5 vs. 178.5, $p=0.07$). PD-1 expression was not related to viral tropism (Table 6.4).

Table 6.4. Effect of viral tropism on HIV-1 parameters, markers of immune activation and inflammation

	Parameter	R5 virus		X4 virus		p-value
		N	median (IQR)	N	median (IQR)	
HIV-1	CD4 cell count	135	540 (401, 664)	11	550 (441, 563)	0.9
parameters	HIV RNA	135	64,046 (17602, 193758)	11	28,800 (3682, 40937)	0.08
CD8 cells	CD38	110	41.5 (29.6, 59.5)	10	42.7 (26.5, 54.9)	0.9
	CD38 MFI	110	401.5 (262, 630)	10	439 (296, 1563)	0.4
	HLA-DR	110	30.9 (18.9, 43.0)	10	41.2 (26.5, 73.4)	0.06
	HLA-DR MFI	110	178.5 (108, 303)	10	262.5 (153, 885)	0.07
	CD38 HLA-DR	110	17.3 (8.3, 30.1)	10	23.5 (13.3, 40.1)	0.2
	PD-1	110	4.7 (2.3, 7.4)	10	4.4 (2.1, 5.3)	0.4
CD4 cells	CD38	110	50.1 (37.0, 65.1)	10	57.2 (51.2, 73.1)	0.1
	CD38 MFI	110	451.5 (310, 579)	10	591.5 (474, 919)	0.06
	HLA-DR	110	10.6 (6.7, 14.1)	10	19.6 (11.6, 27.8)	0.01
	HLA-DR MFI	110	49.5 (35.9, 59.7)	10	76.9 (55.9, 100)	0.005
	CD38 HLA-DR	110	4.9 (3.3, 8.8)	10	9.3 (6.5, 15.5)	0.01
	PD-1	110	8.4 (6.0, 10.1)	10	8.4 (4.3, 11.8)	0.9
Biomarkers	IL-6	116	1.5 (0.9, 2.8)	11	1.4 (0.9, 2.4)	0.8
	D-dimer	108	0.4 (0.2, 0.7)	10	0.4 (0.3, 0.5)	0.7

Median (IQR) HIV RNA, CD4 cell count and immune activation and inflammation parameters are shown in individuals with CCR5 versus CXCR4 tropic virus. Patients with R5 virus were compared to those with X4 virus using Mann-Whitney U tests. Significant results are shown in bold type.

Figure 6.6. CD4 T-cell activation in individuals with R5 vs. X4 virus



Levels of CD4 activation are compared between individual with R5 (n=110) and X4 tropic virus (n=10) using Mann-Whitney tests. Median, IQR & upper and lower adjacent values for HLA-DR%, HLA-DR MFI and dual 139 CD38 HLA-DR% expression are shown.

Viral subtype

Levels of immune activation were compared between B (n=107) and non-B (n=15) subtypes using Mann-Whitney U tests. There was no relationship between non-B subtypes and levels of immune activation.

Transmitted drug resistance

Of 149 UK SPARTAC participants, at baseline, 12 (8%) had transmitted ART drug resistance classified at Stanford level 3 to 5 (low, intermediate or high level resistance to commonly used ART drugs), 7 (4.7%) had resistance at Stanford level 4 to 5 (intermediate or high-level resistance), and 4 (2.7%) had Stanford level 5 high level ART drug resistance. In 122 individuals with available PBMCs for immune activation studies, there was no association between transmitted genotypic drug resistance and T cell activation.

Host factors and immune activation

CD4 cell count

Lower levels of CD4 cell count were associated with higher levels of immune activation. There was an inverse correlation between CD8 CD38 expression and CD4 cell count (CD38% and CD4 cell count: Spearman Rho -0.22, p=0.01; CD38 MFI and CD4 cell count: Rho -0.24, p=0.008) and CD8 CD38 HLA-DR dual expression (Rho -0.2, p=0.02) but not CD8 HLA-DR expression alone (HLA-DR% and CD4 cell count: Rho = -0.1, p=0.28; HLA-DR MFI and CD4: Rho = -0.15, p=0.1). CD8 PD% was inversely correlated with CD4 cell count (Spearman Rho = -0.2, p=0.02)

There was a trend toward an inverse correlation between CD4 CD38 expression and CD4 cell count (CD38% and CD4 cell count: Rho = -0.17, p=0.06; CD38 MFI and CD4 cell count: Rho = -0.17, p=0.06). HLA-DR expression was not related to CD4 cell count (HLA-DR% and CD4 cell count: Rho = 0.03, p=0.7; HLA-DR MFI and CD4 cell count: Rho = -0.03, p=0.7). There was a trend towards inverse correlation between CD4 PD-1% and CD4 cell count (rho = -0.16, p=0.09)

Age

HLA-DR expression on CD8 T-cells increased with age (HLA-DR% and age: Spearman Rho = 0.23, p=0.01; HLA-DR MFI: Rho = 0.27, p=0.003). However there was no relationship between CD4 HLA-DR expression and age (CD4 HLA-DR% and age: Rho = 0.14, p=0.1; CD4 HLA-DR MFI and age: Rho = 0.12, p=0.2).

There was no relationship between CD8 CD38 expression and age, however, consistent with previous studies (McCloskey, et al. 1997) there was an inverse correlation between CD4 CD38 expression and age (CD38% and age: $Rho=-0.25$, $p=0.005$; CD38 MFI and age: $Rho=-0.21$, $p=0.02$). There was no relationship between age and dual CD38 HLA-DR expression on CD8 or CD4 T-cells (CD8 CD38 HLA-DR% and age: $Rho=0.13$, $p=0.15$; CD4 CD38 HLA-DR% $Rho=0.02$, $p=0.9$).

There was a trend towards higher CD8 PD-1 expression with higher age (CD8 PD-1% and age: $Rho=0.15$, $p=0.08$). CD4 PD-1 expression was not significantly correlated with age (CD4 PD-1% and age: $Rho=0.09$, $p=0.3$).

Gender

Levels of T-cell activation were compared between male ($n=117$) and female participants ($n=5$) using Mann-Whitney U tests. Female gender was associated with significantly lower levels of HLA-DR expression and dual CD38 HLA-DR expression on both CD8 and CD4 T-cells (CD8 HLA-DR% $p=0.03$; CD8 CD38 HLA-DR% $p=0.04$; CD4 HLA-DR $p=0.03$; CD4 CD38 HLA-DR $p=0.02$).

Blood pressure and BMI

There was no significant correlation between CD8 or CD4 cell immune activation and systolic or diastolic blood pressure or BMI.

HLA type

Carriers of class 1 HLA alleles B*27 and B*57 have delayed HIV-1 disease progression. We hypothesized that reduced levels of immune activation could be a contributing factor to reduced disease progression in patients with protective HLA types. To assess the effect of protective HLA types on immune activation, IL-6 and D-dimer, patients were categorised as being HLA B*57 positive, HLA B*27 positive or having another HLA type. Markers of T-cell activation, PD-1 expression, IL-6 and D-dimer were compared between HLA positive and negative individuals using Mann-Whitney U tests. The effect of HLA type adjusting for other factors was further examined using linear regression.

HLA type was available in 145/149 UK participants. 10 (7%) and 14 (10%) individuals were heterozygous carriers of HLA B*57 and HLA B*27 respectively. The prevalence of these alleles in the general UK population is similar (estimated 6.8-8.7% for HLA B*57 and 8.3-9.3% for HLA B*27 (Middleton D 2003)). There were no homozygous carriers of either HLA type. All HLA B*57 positive patients were HLA B*5701 and B*27 positive patients were B27*05 on 4 digit typing. Of those with

available HLA typing, 120 individuals had PBMCs available for T-cell activation studies and of these 10 were HLA B*27 positive and 13 were HLA B*27 positive.

Table 6.5 shows VL, CD4 cell count, biomarker and immune activation results in HLA B*57 positive and negative individuals respectively. HLA B*57 positive individuals had significantly lower VL ($p=0.001$) and higher CD4 cell counts ($p=0.002$).

Positivity for HLA B*57 was associated with lower levels of both CD8 and CD4 T-cell activation as measured by CD38% expression and CD38 MFI on CD8 and CD4 T-cells (CD8 CD38%: 43.5% vs. 14.6% for HLA B57 negative vs. positive, respectively, $p<0.0001$; CD8 CD38 MFI: 414 vs. 193, $p<0.0001$; CD4 CD38%: 53.4% vs. 36.7%, $p=0.005$; CD4 CD38 MFI 470 vs. 307 $p=0.02$). In addition CD8 CD38 HLA-DR dual expression was significantly lower in HLA B*57 positive individuals (CD8 CD38 HLA-DR %: 18.5% vs. 6.8% for HLA B57 negative vs. positive, respectively, $p<0.0001$) and there was a trend toward lower dual expression on CD4 T-cells (CD4 CD38 HLA=DR%: 5.6 vs. 3.7 $p=0.07$). PD-1 expression on CD8 T-cells did not differ between HLA B*57 negative and positive individuals although there was a trend towards lower CD4 PD-1% expression (8.5% vs. 6.4% $p=0.08$). There was no association between HLA B*57 positivity and level of IL-6 or D-dimer.

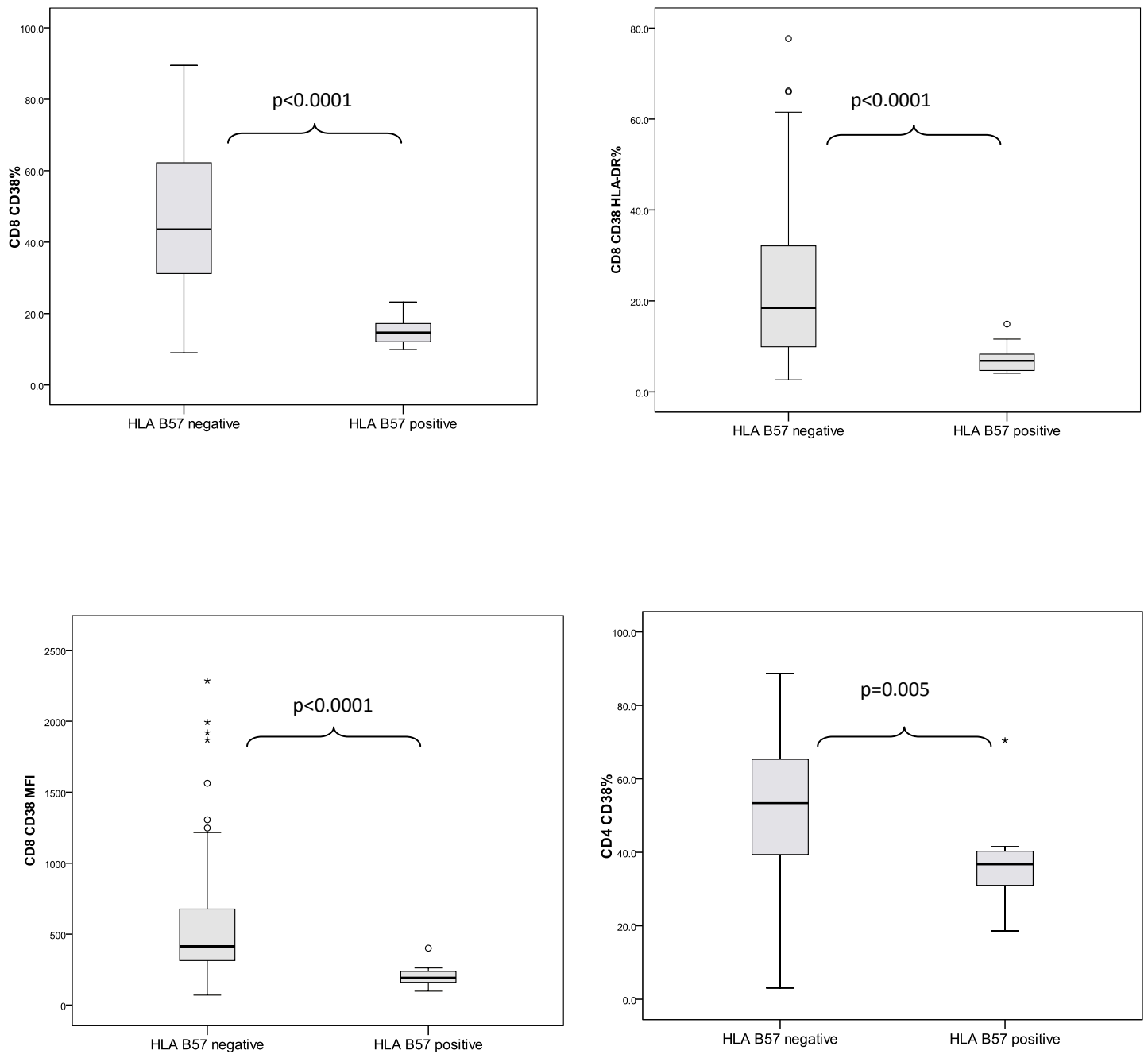
Differences in immune activation between individuals who were HLA B*57 positive versus negative is further demonstrated in Figure 6.7. On linear regression analysis, the differences between HLA B*57 positive and negative individuals remained robust on adjustment for VL and other variables, described in section 6.3.3.

Table 6.5. HIV-1 and immune activation / inflammation parameters in individuals with protective HLA type B*57

	Parameter	HLA B*57 –ve		HLA B*57 +ve		p-value
		N	Median (IQR)	N	Median (IQR)	
HIV-1	HIV RNA	135	64046 (17603, 94859)	10	7245 (411, 26851)	0.001
parameters	CD4	135	530 (401, 658)	10	712 (635, 830)	0.002
CD8 cells	CD38%	110	43.5 (31.2, 61.1)	10	14.6 (12.1, 17.2)	<0.0001
	CD38 MFI	110	414 (314, 677)	10	193 (161, 238)	<0.0001
	HLA-DR%	110	32.3 (18.8, 45.1)	10	25.5 (20.2, 31.2)	0.3
	HLA-DR MFI	110	181 (110, 311)	10	155 (91.4, 175)	0.15
	CD38 HLA-DR %	110	18.5 (9.9, 32.1)	10	6.8 (4.7, 8.3)	<0.0001
	PD-1	110	4.9 (2.3, 7.4)	10	3.6 (2.9, 4.4)	0.1
CD4 cells	CD38%	110	53.4 (39.4, 65.3)	10	36.7 (31.0, 40.3)	0.005
	CD38 MFI	110	470 (319, 604)	10	307 (257, 365)	0.02
	HLA-DR%	110	10.6 (6.7, 15.9)	10	11.7 (10.7, 12.0)	0.8
	HLA-DR MFI	110	51.5 (35.1, 66.8)	10	51.2 (43.2, 55.0)	0.8
	CD38 HLA-DR%	110	5.6 (3.3, 9.3)	10	3.7 (2.7, 4.8)	0.07
	PD-1	110	8.5 (6.2, 11.8)	10	6.4 (5.1, 8.5)	0.08
Biomarkers	IL-6	116	1.54 (0.9, 2.7)	10	1.16 (0.8, 1.8)	0.4
	D-dimer	109	0.38 (0.26, 0.61)	8	0.32 (0.18, 0.58)	0.4

HLA B*57 positive and negative populations were compared using the Mann-Whitney U test.

Figure 6.7. Levels of CD4 and CD8 T-cell activation in HLA B*57 positive and negative individuals.



Immune activation in HLA B*57 negative (n=110) and positive individuals (n=10) were compared using Mann-Whitney U tests. Median, IQR & upper and lower adjacent values for CD8 CD38%, CD8 CD38 MFI, CD8 dual CD38 HLA-DR expression and CD4 CD38% expression are shown on box-plot graphs.

Table 6.6 shows VL, CD4 cell count, biomarker and immune activation results in HLA B*27 positive and negative individuals respectively. HLA B*27 positive individuals had significantly lower VL than HLA B*27 negative individuals ($p=0.01$) although there was no significant difference in CD4 cell count.

There was no significant association between CD8 T-cell activation (CD38 or HLA-DR expression) and HLA B*27 positivity, although there was a trend towards lower CD8 CD38% in HLA B*27 positive individuals (44.5% vs. 13%, $p=0.08$). CD8 PD-1 expression was significantly lower in HLA B*27 positive individuals (4.9% vs. 2.9%, $p=0.008$, figure 6.8) and this difference remained significant on linear regression analysis adjusting for VL (see next section). There were no significant differences in CD4 T-cell activation or PD-1 expression between those who were HLA*B27 positive and HLA B*27 negative.

D-dimer was significantly lower in HLA B*27 positive individuals (0.24 mg/L vs. 0.42 mg/L, $p=0.002$, figure 6.8). To adjust for other variables associated with D-dimer, linear regression analysis was carried out using $\log_{10}$ transformed D-dimer as the dependant variable. On adjusting for log HIV RNA and age, D-dimer was 0.69 (95% CI 0.51, 0.93) mg/L lower, equating to a difference of 31%, (95% CI 7%, 49%) between HLA B*27 positive and negative individuals ($p=0.016$). There was no association between IL-6 and HLA B*27 positivity.

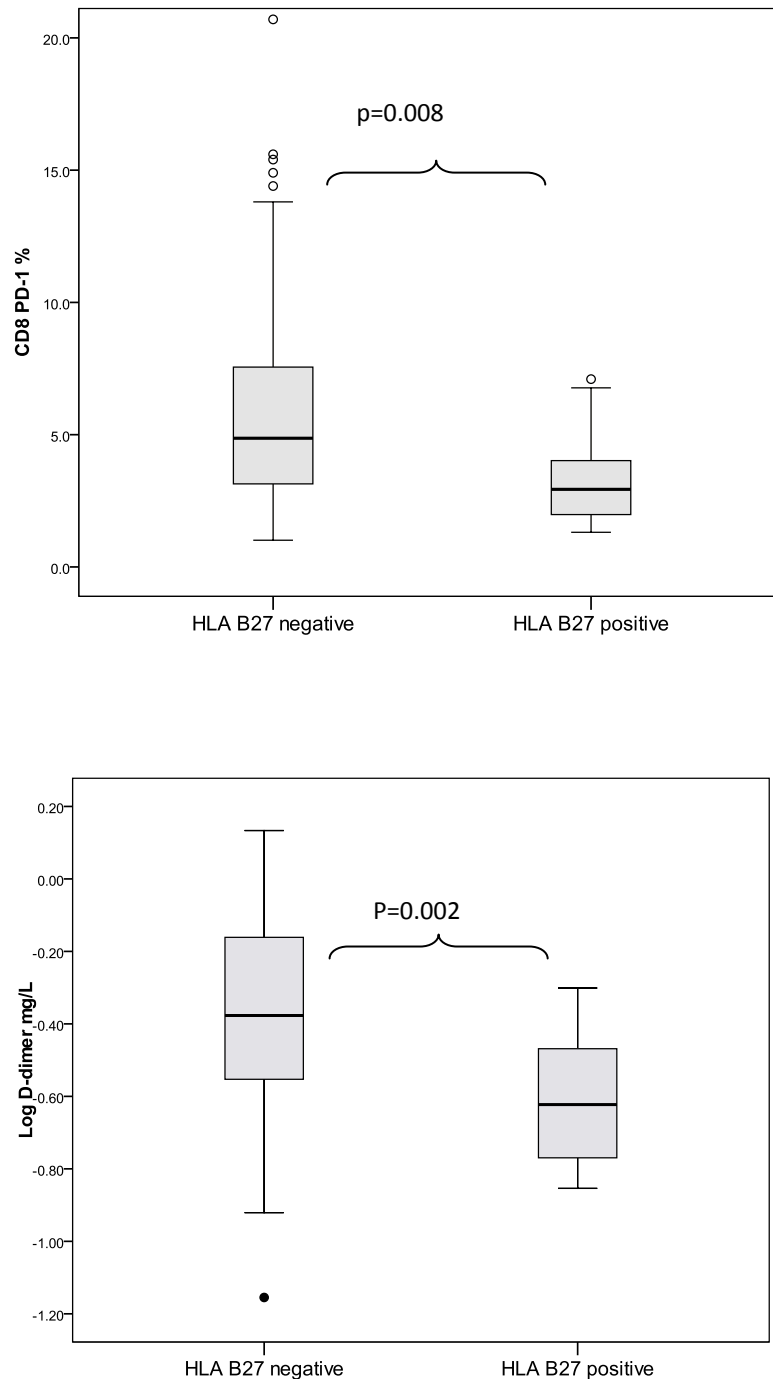
The prevalence of protective HLA alleles was examined in patients displaying HIV-1 control in PHI, defined in this analysis as patients with HIV RNA <400 copies/ml at baseline. As seen in figures 6.4 and 6.5, CD8 and CD4 immune activation was lower in these individuals. Seven of 149 UK patients (4.7%) had VL<400copies/ml. Of 120 patients with available HLA typing 6/120 (5%) had HIV RNA <400 copies/ml. Of these, HLA B*27 was present in one (16%) individual and HLA B*57 present in one individual (16%).

Table 6.6. HIV-1 and immune activation / inflammation parameters in individuals with protective HLA type B*27

Parameter		HLA B*27 –ve		HLA B*27 +ve		p-value
		N	Median (IQR)	N	Median (IQR)	
HIV-1 parameters	HIV RNA	131	62386 (17121,196182)	14	22921 (1248, 40200)	0.01
	CD4	131	535 (400, 670)	14	571 (502, 691)	0.2
CD8 cells	CD38%	107	44.5 (28.8, 61.5)	13	37.0 (31.8, 39.2)	0.08
	CD38 MFI	107	402 (258, 680)	13	373 (393, 418)	0.5
	HLA-DR%	107	31.2 (18.8, 45.1)	13	26.5 (23.0, 37.0)	0.5
	HLA-DR MFI	107	179 (103, 307)	13	173 (133, 280)	0.9
	CD38 HLA-DR %	107	18.2 (8.7, 32.4)	13	14.2 (8.6, 17.5)	0.2
	PD-1	107	4.9 (3.1, 7.6)	13	2.9 (2.0, 4.0)	0.008
CD4 cells	CD38%	107	50.1 (36.3, 66.3)	13	54.4 (48.6, 57.7)	0.8
	CD38 MFI	107	447 (290, 584)	13	546 (458, 590)	0.2
	HLA-DR%	107	10.7 (6.7, 15.1)	13	11.7 (7.8, 16.4)	0.7
	HLA-DR MFI	107	49.9 (35.1, 61.5)	13	55.9 (47.7, 70.2)	0.2
	CD38 HLA-DR%	107	4.9 (3.2, 9.5)	13	6.5 (3.9, 7.9)	0.9
	CD4 PD-1	107	8.2 (6.2, 11.)	13	8.8 (3.7, 11.0)	0.9
Biomarkers	IL-6	112	1.55 (0.94, 2.68)	14	1.22 (0.80, 1.42)	0.1
	D-dimer	103	0.42 (0.28, 0.69)	14	0.24 (0.17, 0.34)	0.002

HLA B*27 positive and negative populations were compared using Mann-Whitney U tests.

Figure 6.8. PD-1 and D-dimer levels in HLA B*27 positive and negative individuals



Box-plots displaying PD-1 and D-dimer (median, IQR & upper and lower adjacent values) in HLA B*27 negative and positive individuals. Mann-Whitney U tests were used to compare populations. For PD-1: HLA B*27 –ve n=107; HLA B*27 +ve n=13; for D-dimer HLA B*27 –ve n=103, HLA B*27 +ve n=14.

Linear regression model of factors associated with immune activation in PHI

To further explore the relationship between immune activation, plasma VL and other significant baseline characteristics (age, sex, CD4 cell count, HLA type, viral tropism), a linear regression analysis was undertaken. Time since estimated seroconversion was also included in the model in order to assess whether immune activation was related to duration of HIV-1 infection. Multivariate regression models were constructed using backwards selection and variables were included if the *p*-value was <0.1 on univariate analysis. VL and T-cell activation markers (all except CD38% which was already normally distributed) were log₁₀ transformed to achieve normality for analysis purposes.

Factors associated with CD8 T-cell activation

Overall linear regression results for factors associated with CD8 T-cell activation are shown in Table 6.7. On univariate analysis, higher CD8 CD38% expression was significantly associated with increasing VL, and inversely associated with HLA B*57 positivity, CD4 cell count and duration of infection. There was a trend toward lower CD38% in HLA B*27 positive individuals. On multivariate analysis, VL, duration of infection and HLA B*57 positivity remained significant. Adjusting for baseline variables, CD38% remained 21% lower in HLA B*57% positive individuals (95% CI 9.9%, 32%). For every log increase in VL there was an 8.6% rise in CD38% expression (95% CI 5.4%, 11.8%) and for every estimated day since seroconversion, CD38% was 0.1% lower (95% CI 0.01%, 0.2%).

Higher CD8 HLA-DR% expression was associated with increasing age, male sex and higher VL on univariate analysis. There was a trend towards higher HLA-DR levels associated with X4 tropism. On multivariate analysis, adjusting for age, gender and HIV RNA, X4 virus was significantly related to CD8 HLA-DR expression. X4 tropism was associated with a 51.3% relative increase in HLA-DR expression compared to R5 tropism (95% CI 4.7%, 99.5%). VL was associated with a 20.2% relative increase in HLA-DR expression for every log increase in HIV RNA (95% CI 7.1%, 25.8%) and there was 12.2% relative increase in HLA-DR% for every 10 year increase in age (95% CI 1.0%, 25.8%).

Higher dual expression of CD38 HLA-DR on CD8 T-cells was significantly associated with higher HIV RNA and male sex, and inversely associated with CD4 cells count and HLA B*57 positivity on univariate analysis. On multivariate analysis, HIV RNA and HLA B*57 positivity remained significant. After accounting for HIV RNA, HLA B*57 positivity was associated with a 47.0% relative reduction in dual CD38 HLA-DR expression (95% CI 10.9%, 60.2%). A log increase in HIV RNA was associated a relative increase in HLA-DR expression of 47.9% (95% CI 31.8%, 69.8%).

PD-1% expression on CD8 cells was associated with increased age and higher HIV RNA levels and was lower in HLA B*27 positive individuals on both univariate and multivariate analysis. HLA-B*27

positivity was associated with a relative reduction in PD-1 expression of 30.9% (95% CI 0.2%, 49.9%). A log increase in HIV RNA was associated with a 25.8% relative increase in PD-1 expression (95% CI 12.2%, 38.0%) and an 10 year increase in age was associated with a 12.2% relative increase in PD-1 expression on CD8 T-cells (1%, 25.8%)

Factors associated with CD4 T-cell activation

Overall results for factors associated with CD4 T-cell activation are shown in Table 6.8. CD38% expression on CD4 cells was inversely associated with age, CD4 cell count and with HLA B*57 positivity on univariate analysis. On multivariate analysis, after adjusting for age and CD4 cell count, CD4 CD38% activation remained 12% lower ($p=0.04$, 95% CI 0.8%, 23%) in HLA B*57 positive individuals. CD4 cell count was no longer related to CD38 expression on adjusting for other factors. Age remained inversely related to CD38 activation; for every 10 year increase in age there was a 4.2% reduction in CD8 CD38% (0.08%, 7.5%).

Higher HLA-DR% expression on CD4 cells was associated with HLA RNA, X4 tropism and male sex, with a borderline association with increasing age. On multivariate analysis both X4 tropism and HIV RNA remained significant. X4 tropism was associated with a relative increase in CD4 HLA-DR expression of 77.8% (95% CI 20.2%, 150%). For a log increase in HIV RNA, there was a relative increase in HLA-DR expression of 14.8% (95% CI 14.8, 25.8%).

Dual expression of CD38 and HLA-DR on CD4 T-cells was significantly associated with X4 tropism, male sex and HIV RNA levels on univariate analysis. X4 tropism and HIV RNA remained significant in the multivariate model. X4 tropism was associated with a relative increase of 113.7% (95% CI 34.8%, 246.7%). A log increase in HIV RNA was associated with a 34.5% relative increase in dual expression (17.5%, 47.9%)

CD4 PD-1% expression was not associated with any baseline covariates except for HIV RNA. A log increase in HIV RNA was associated with a relative increase in PD-1 expression of 12.2% (95% CI 1%, 25.8%).

Table 6.7. Factors associated with CD8 T-cell activation and PD-1 expression in PHI

	Parameter	Univariate			Multivariate		
		Coefficient	95% CI	p-value	Coefficient	95% CI	p-value
CD8 CD38 %	Age(+10 years)	-0.5	-4.3 , 3.4	0.8			
	Sex (female)	-12.5	-30.5, 5.5	0.2			
	CD4 (+100 cells)	-2.5	-4.2, -0.8	0.004			
	HIV RNA (+1log)	10.6	7.3, 13.9	<0.0001	8.6	5.4, 11.8	<0.0001
	HLA B*57 +ve	-31.6	-43.8 , -19.8	<0.0001	-21.0	-32.1, -9.9	<0.0001
	HLA B*27 +ve	-10.6	-22.1, 0.8	0.07			
	X4 tropism	0.04	-13, 13	0.9			
	Duration (+1day)	-0.1	-0.2, -0.01	0.03	-0.1	-0.2, -0.01	0.02
Log ₁₀ CD8 HLA-DR %	Age(+10 years)	0.07	0.02, 0.2	0.005	0.05	0.003, 0.1	0.04
	Sex (female)	-0.26	-0.49, -0.03	0.03			
	CD4 (+100 cells)	-0.01	-0.03, 0.01	0.6			
	HIV RNA (+1log)	0.08	0.04, 0.1	0.001	0.08	0.03, 0.1	0.002
	HLA B*57 +ve	-0.07	-0.2, 0.1	0.4			
	HLA B*27 +ve	-0.06	-0.2, 0.1	0.4			
	X4 tropism	0.2	-0.004, 0.3	0.06	0.18	0.02, 0.3	0.03
	Duration (+1day)	0.000	-0.002,	0.5			
Log ₁₀ CD8 CD38 HLA-DR%	Age(+10 years)	0.06	-0.009, 0.1	0.09			
	Sex (female)	-0.33	-0.63, -0.03	0.03			
	CD4 (+100 cells)	-0.03	-0.06, -0.003	0.03			
	HIV RNA (+1log)	0.2	0.1, 0.3	<0.0001	0.17	0.12, 0.23	<0.0001
	HLA B*57 +ve	-0.4	-0.6, -0.2	<0.0001	-0.2	-0.4, -0.05	0.015
	HLA B*27 +ve	-0.1	-0.3, 0.07	0.2			
	X4 tropism	0.2	-0.07, 0.4	0.2			
	Duration (+1day)	-0.002	-0.003,	0.1			
Log ₁₀ CD8 PD-1 %	Age(+10 years)	0.07	0.02, 0.1	0.01	0.05	0.000, 0.1	0.05
	Sex (female)	-0.1	-0.34, 0.15	0.43			
	CD4 (+100 cells)	-0.02	-0.04, 0.007	0.2			
	HIV RNA (+1log)	0.1	0.07, 0.2	<0.0001	0.1	0.05, 0.14	<0.0001
	HLA B*57 +ve	-0.1	-0.2, 0.07	0.2			
	HLA B*27 +ve	-0.2	-0.4, 0.06	0.008	-0.16	-0.3, -0.001	0.01
	Tropism X4	-0.08	-0.3, 0.1	0.4			
	Duration (+1day)	-0.001	-0.003,	0.2			

Linear regression analysis of factors associated with CD8 T-cell activation and PD-1 expression in PHI (n=120). HIV RNA, HLA-DR%, dual CD38 HLA-DR% and PD-1% were log transformed to normalize the distribution. Multivariate regression models were constructed using backwards selection and variables were included if the p-value was <0.1 on univariate analysis. The regression coefficient (95% CI) and p-value are given for each variable.

Table 6.8. Factors associated with CD4 T-cell activation and PD-1 expression

	Parameter	Univariate			Multivariate		
		Coefficient	95% CI	p-value	Coefficient	95% CI	p-value
CD4 CD38 %	Age (+10 years)	-4.1	-7.5, -0.7	0.02	-4.2	-7.5, -0.08	0.02
	Sex (female)	-2.8	-19.1, 13.3	0.72			
	CD4(+100 cells)	-1.6	-3.2, -0.08	0.04			
	HIV RNA (+1log)	2.6	-0.7, 5.9	0.12			
	HLA B*57 +ve	-16.1	-27.6, -4.7	0.006	-12.3	-24.0, -0.8	0.04
	HLA B*27 +ve	1.1	-9.4, 11.5	0.8			
	X4 tropism	9.7	-1.8, 21.4	0.1			
	Duration (+1day)	-0.03	-0.1, 0.07	0.5			
Log ₁₀ CD4 HLA-DR%	Age (+10 years)	0.05	-0.005, 0.1	0.08			
	Sex (female)	-0.27	-0.51, -0.02	0.03			
	CD4(+100 cells)	0.002	-0.02, 0.03	0.9			
	HIV RNA +1log)	0.06	0.01, 0.1	0.015	0.06	0.01, 0.1	0.02
	HLA B*57 +ve	0.02	-0.2, 0.2	0.8			
	HLA B*27 +ve	-0.02	-0.2, 0.1	0.8			
	X4 tropism	0.2	-0.06, 0.4	0.009	0.26	0.08, 0.4	0.004
	Duration (+1day)	0.000	-0.001, 0.002	0.7			
Log ₁₀ CD4 CD38 HLA-DR%	Age (+10 years)	0.01	-0.05, 0.08	0.7			
	Sex (female)	-0.35	-0.65, -0.05	0.02			
	CD4(+100 cells)	-0.02	-0.05, 0.01	0.3			
	HIV RNA (+1log)	0.1	0.06, 0.2	<0.0001	0.13	0.07, 0.17	<0.0001
	HLA B*57 +ve	-0.2	-0.4, 0.04	0.1			
	HLA B*27 +ve	-0.01	-0.2, 0.1	0.9			
	X4 tropism	0.3	-0.08, 0.5	0.007	0.33	0.13, 0.54	0.001
	Duration (+1day)	0.000	-0.002, 0.002	0.7			
Log ₁₀ CD4 PD-1%	Age (+10 years)	0.02	-0.03, 0.07	0.4			
	Sex (female)	0.05	-0.19, 0.3	0.7			
	CD4(+100 cells)	-0.02	-0.04, 0.004	0.1			
	HIV RNA (+1log)	0.05	0.000, 0.1	0.05	0.05	0.000, 0.1	0.05
	HLA B*57 +ve	-0.08	-0.2, 0.08	0.3			
	HLA B*27 +ve	-0.1	-0.3, 0.03	0.1			
	Tropism X4	-0.002	-0.2, 0.2	0.9			
	Duration (+1day)	0.000	-0.001, 0.002	0.6			

Linear regression analysis of factors associated with CD4 T-cell activation and PD-1 expression in PHI (n=120). HIV RNA, HLA-DR%, dual CD38 HLA-DR% and PD-1% were log transformed to normalize the distribution. Multivariate regression models were constructed using backwards selection and variables were included if the p-value was <0.1 on univariate analysis. The regression coefficient (95% CI) and p-value are given for each variable

6.4. Summary of results in this chapter

Levels of T-cell activation were raised in PHI and at week 60 compared to healthy controls and highest closest to seroconversion. Markers of T-cell activation were correlated with each other, with HIV RNA and with IL-6 and D-dimer. Host factors, such as age and HLA type, and viral factors such as viral tropism, affect levels of T-cell activation in PHI. PD-1 expression in PHI correlates with T-cell activation and HIV RNA, and HLA B*27 positive individuals have lower CD8 PD-1 expression.

Discussion

The level of immune activation is an important predictor of HIV-1 disease progression. In this chapter, we describe levels of T-cell activation in a UK cohort of 122 individuals with PHI, and assess both viral and host factors associated with immune activation at this time.

Consistent with previous studies we demonstrated raised levels of T-cell activation in HIV-1 infection compared to healthy controls. Higher levels of T-cell activation were seen in PHI compared with the same individuals at week 60. In addition, in individuals with PHI, a significant inverse association was seen between estimated time since seroconversion and level of immune activation as measured by CD38 expression. The baseline study visit in this group of individuals represents a wide range of estimated time since seroconversion (8 to 156 days) and in some, the acute events associated with seroconversion may have resolved by the time of the baseline visit. Our data indicate that the high VL associated with PHI is also associated with high levels of immune activation which reduces naturally over time, but remains higher than seen in healthy controls, even in the chronic phase of disease. It should be noted, however, that our healthy controls were not age or gender matched, nor were they formally tested for HIV infection, thus their comparability may be limited. It should be also be noted that the week 60 results in this cohort represent a heterogeneous group of individuals, two thirds of whom have received either 12 or 48 weeks of ART as per the SPARTAC study protocol. Once the SPARTAC study is unblinded it will be possible to perform a comparison of levels of immune activation across the 3 arms of the study to assess whether short-course ART, by dampening the immunological and virological events associated with PHI, impacts on long-term levels of immune activation, even after treatment is discontinued.

In this study, T-cell activation was evaluated by assessing expression of CD38 and HLA-DR on both CD4 and CD8 T-cells. We assessed both the percentage of cells expressing these activation markers, and the median fluorescent intensity, which represents the median density of activation marker per cell. The MFI method has some advantages as it is not dependant on the placement of negative gates during flow cytometry analysis and therefore could be seen as a more objective measure of T-

cell activation than percentage. Liu et al (1996) compared relative fluorescent intensity for CD38 to percentage expression, and found that elevated mean fluorescence intensity of CD38 expression on CD8 cells had prognostic value for development of AIDS that was almost identical to the prognostic value of the percentage of CD8 cells expressing CD38 (Liu, et al. 1996b), however other studies provide conflicting reports as to the best predictive activation marker for HIV-1 disease progression (Hazenbergh, et al. 2003b). In our study, all measures of immune activation on both CD4 and CD8 T-cells were highly correlated with each other, and when assessing factors associated with immune activation in PHI, results were similar when measured by percentage or MFI. Once SPARTAC is unblinded it will be possible to compare the different measures of immune activation used in this study with HIV-1 outcomes to determine the best predictive markers for disease progression.

In this study we measured PD-1 expression, a marker associated with T-cell exhaustion, alongside T-cell activation. We demonstrated that in PHI there was a close relationship between CD8 T-cell expression of PD-1, CD38 and HLA-DR. This supports data reported by Sauce et al., which showed that the proportion of total CD8+ PD-1+ T-cell population positively correlate with the proportion of total CD8+ CD38+ T-cell population in chronic infection. This has not been previously demonstrated in PHI. In addition we demonstrate a correlation between CD4 PD-1 expression and CD4 cell activation (HLA-DR and dual expression) indicating a relationship between CD4 cell activation and PD-1 expression in this population.

We also demonstrate a significant correlation between both CD8 and CD4 PD-1% expression and HIV RNA levels. However, as described here and by others (Sauce, et al. 2007) it is not possible to determine if PD-1 expression is a cause of increased plasma VL, as a result of T-cell exhaustion and impaired viral control, or a consequence of high VL leading to T-cell activation and subsequent PD-1 upregulation. On controlling for CD38 expression, the magnitude of the correlation between PD-1 and HIV RNA, whilst remaining statistically significant, was reduced, indicating that the relationship between PD-1 and HIV RNA may, in part, be due to T-cell activation. Adjusting for HLA-DR expression had little effect on the strength of the relationship between PD-1 and HIV RNA indicating a direct relationship between PD-1 and HIV-1 viraemia independent of activation status of the total CD8 population. However, in a related study, using specimens obtained from SPARTAC participants at baseline, Hickling et al. (2010) showed that, on HIV-1 specific cells, PD-1 expression correlated with CD38 expression but not plasma HIV RNA, supporting the hypothesis that PD-1 expression is due to T-cell activation in this setting (personal communication Stephen Hickling, Oxford University).

Although high PD-1 expression has been associated with worse disease outcome in studies of chronic infection (Holm, et al. 2008; Zhang, et al. 2007) there are few data on the clinical

consequence of PD-1 expression when measured in PHI. Once the SPARTAC study is unblinded, it will be possible to establish the prognostic value of measuring PD-1 expression in the total CD8 and CD4 T-cell population by evaluating the relationship between baseline PD-1 expression in PHI and clinical disease outcomes in the untreated arm of SPARTAC.

Differences in clinical outcome in HIV-1 infected individuals may represent differences in genetic variants amongst HIV-1 infecting strain, host genetics differences, or differences in the virus specific immune response (Altfeld, et al. 2003). This is the first study to assess the impact of host factors such as HLA type and viral factors such as tropism on markers of T activation in PHI. It has been previously established that the level of immune activation in recent infection correlates with HIV-1 viraemia (Deeks, et al. 2004) and this is confirmed in our study. CD8 T-cell activation was highly and significantly correlated with HIV-1 viraemia for all activation markers. Unlike Deeks, however, we did not see a significant correlation between CD4 CD38 expression and HIV RNA, although there was a trend, and this may be due a difference in laboratory techniques or cohort differences. However HIV RNA was correlated with HLA-DR expression and dual CD38 HLA-DR expression on CD4 cells confirming that CD4 T-cell activation is associated with HIV RNA levels in PHI.

The majority of transmitted HIV-1 variants are CCR5 using viruses (Keele, et al. 2008). Recent publications have reported faster disease progression for individuals newly infected with CXCR4 tropic HIV-1 variants. In a cohort of individuals with PHI, CXCR4-using viruses were associated with an accelerated rate of disease progression, estimated by a faster decline of CD4 T-cell count to less than 350 cells/ μ l and by reduced time to initiating ART (Raymond, et al. 2010). We demonstrated that increased levels of immune activation were associated with more pathogenic CXCR4-tropic variants and this may be a potential mechanism explaining this observed enhanced rate of immunological decline in previous studies. Our study supports findings by Hazenberg et al. which showed that in individuals followed up since PHI over a 5 year period, increased proportions of CD4 T-cells positive for Ki67, a marker of T-cell division, were seen in individuals in whom emergence of X4 variants occurred, compared to those maintaining R5 virus (Hazenberg, et al. 2003a). Hazenberg previously showed that as the number of proliferating Ki-67 positive cells decreases on ART, its expression is likely to be driven by generalised immune activation associated with HIV-1 viraemia rather than being a T-cell homeostatic response to compensate for T-cell depletion (Hazenberg, et al. 2000). In our study, the significantly higher levels of T-cell activation in patients infected with CXCR4-tropic viruses were seen in the absence of any significant differences in VL or CD4 cell count, indicating that immune activation may be an early step reflecting underlying pathogenic processes at this initial stage of HIV-1 infection.

Previously reported prevalence of X4 viruses in PHI varies according to subtype and methodology of determining tropism, with a low frequency of CXCR4-using viruses described in PHI patients with non-B subtype infection (Frange, et al. 2010), compared to frequencies of up to 15.9% of X4 or mixed-tropic viruses at the time of PHI in individuals with subtype B infection (Frange, et al. 2009). In SPARTAC, the prevalence of X4 variants was 8.3%, measured using the geno2pheno algorithm with the false positive rate (FPR) set at 5.75%. This is comparable to findings by Raymond, et al. who described a prevalence of 6.4% in PHI when using an ultrasensitive phenotypic assay and a prevalence of 5.6% when using the geno2pheno algorithm with the FPR set at 5.75% (Raymond, et al. 2010).

This is the first study correlating HLA type with markers of immune activation, inflammatory and coagulation biomarkers in PHI. We found that HLA type B*57 was associated with lower levels of CD4 and CD8 T-cell activation and HLA B*27 was associated with lower levels of PD-1 expression and fibrinolysis. These protective HLA types have been associated with slower rates of HIV-1 progression in previous studies (O'Brien, et al. 2001). These findings were independent of levels of plasma HIV RNA. It is thought that the interaction between the genetic HLA background in an individual and the viral epitopes recognised by that HLA type direct both the magnitude of HIV-1 specific T-cell responses and the ability for viral escape. Our findings support the hypothesis that reduced levels of immune activation associated with these protective HLA alleles contribute towards reduced levels of HIV-1 disease progression. In addition, the reduced PD-1 expression seen in HLA B*27 positive individuals could reflect a mechanism for enhanced viral control due to a less exhausted T-cell population. Our findings are consistent with studies in elite controllers, who are known to have higher prevalence of protective HLA alleles. Hunt et al. showed that elite controllers have lower levels of immune activation, compared with individuals with detectable HIV RNA levels, however the prevalence of protective HLA types in this cohort was not directly examined (Hunt, et al. 2008). A further study in elite controllers demonstrated a peculiar activation phenotype where viral control was associated with low CD38 expression and high HLA-DR expression on HIV-1 specific cells (Saez-Cirion, et al. 2007). Although we did not specifically look at HIV-1 specific cells, our study demonstrated significantly lower CD38 activation in those with protective HLA types but no difference in HLA-DR activation, in keeping with these findings.

It is not understood whether high levels of viral replication in PHI initially lead to higher levels of immune activation, or whether the initial level of immune activation dictates subsequent VL. We demonstrate that host genetic differences contribute to level of immune activation supporting the latter hypothesis. This is consistent with data from Hazenburg et al (2003) which shows that the

level of CD70 expression, a further marker of immune activation, dictates HIV-1 progression even prior to HIV-1 acquisition, and other studies showing that immune activation can predict disease progression in the absence of detectable virus (Hunt, et al. 2008; Hunt, et al. 2003).

We see a similar prevalence of protective HLA alleles to that seen in the general UK population. This is in contrast to previous data reporting lower prevalence of these alleles in PHI. Altfeld et al reported 3.4% prevalence of HLA B*57 in individuals with symptomatic PHI in contrast to 9.6% prevalence in chronically infected individuals, and concluded that, individuals with protective HLA alleles are less likely to present with symptoms and therefore may be underrepresented in acute infection cohorts (Altfeld, et al. 2003). Participants entered into the SPARTAC study reflect a more representative cohort of individuals due to the broader entry criteria which are not restricted to symptomatic disease.

CD38 is an early T-cell differentiation marker and expression is known to be increased in children and decline with age (McCloskey, et al. 1997). Consistent with previous studies, we saw an inverse relationship between the proportion of CD4 CD38 expression, and age. However, we did not see a relationship between CD8 CD38 expression and age, possibly reflecting differences in our cohort which is restricted to adults aged 18 to 65, in contrast to other studies which include children. HLA-DR expression, a known marker of immune senescence, was positively correlated with age and HIV RNA, and this may reflect one mechanism by which the accelerated aging that has been reported in HIV-1 infected individuals occurs. In addition the increased immune activation seen in older participants, alongside higher levels of IL-6 and D-dimer (chapter 4) may explain why older individuals experience faster disease progression.

In conclusion, we demonstrate high levels of immune activation associated with PHI, which reduce over time but remain higher than in healthy controls. Immune activation was correlated with PD-1 expression, IL-6 and D-dimer, and determined by both host factors, such as age and HLA type, and viral factors such as levels of HIV RNA and viral tropism. Whether initial immune activation drives viraemia, or whether the reverse is true, remains unclear, yet this is likely to be a self-perpetuating cycle.

Chapter 7: Assessment of markers of microbial translocation, endothelial activation and tissue factor in PHI

Introduction

Irrespective of the route of HIV-1 acquisition there is early trafficking of HIV-1 to the GALT during PHI, resulting in high level HIV-1 replication and massive depletion of CCR5 positive memory T-lymphocytes (McMichael, et al. 2010). It has been hypothesized that HIV-1 related immunological and structural deficits in the GI tract allow an increase in gut permeability, permitting translocation of gut-colonising bacteria and their metabolites across the gut epithelium and into the systemic circulation. This process, known as microbial translocation, has been postulated to be a major factor contributing towards the persistent immune activation characteristic of chronic HIV-1 infection (Douek 2007).

Microbial translocation has been quantified by measuring circulating plasma levels of LPS, a cell wall component of gram negative bacteria. LPS binds soluble LPS binding protein (LBP) in plasma which transfers it onto a peripheral membrane protein, CD14, expressed on the cell surface of monocytes and macrophages. The LPS-CD14 complex triggers recognition by Toll-like Receptor 4 (TLR 4) on the monocyte cell surface, activating the cell to produce proinflammatory cytokines and chemokines, thus inducing further immune activation. LPS recognition through the innate immune system therefore results in macrophage activation and subsequent initiation of adaptive immune responses (Janeway and Medzhitov 2002). Brenchley et al. (2006) demonstrated raised levels of LPS in individuals with chronic HIV-1 infection, compared to HIV-negative controls, and showed that LPS directly correlated with activated CD38+HLA-DR+ CD8 T-cells, but not with CD4 cell count or VL. This was supported by a further study showing an association between plasma levels of bacterial rDNA and T-cell activation (Jiang, et al. 2009).

Soluble CD14 (sCD14) is secreted by CD14 positive monocytes in response to LPS stimulation and directly correlates with LPS levels in HIV-1 infected individuals (Brenchley, et al. 2006; Papasavvas, et al. 2009). Baseline plasma levels of sCD14 were independently predictive of mortality in the SMART trial (Sandler, et al. 2010). LPS-Binding Protein (LBP) is produced by GI and hepatic epithelial cells and correlates with both sCD14 (Brenchley, et al. 2006) and LPS (Ancuta, et al. 2008). Naturally occurring antibodies to LPS core oligosaccharide are produced in response to LPS to neutralise LPS activity (Strutz, et al. 1999). These endotoxin core antibodies (EndoCAb) are increased in conditions of chronic microbial translocation such as inflammatory bowel disease (Barclay 1995), but have been shown to inversely correlate with LPS in chronic HIV-1 infection (Ancuta, et al. 2008; Brenchley, et al.

2006; Papasavvas, et al. 2009), suggesting that they are insufficient to neutralise LPS in this setting (Brenchley, et al. 2006).

Exposure to LPS is a key inducer of procoagulant tissue factor expression both *in vivo* and *in vitro* (Breitenstein, et al. 2010; Derhaschnig, et al. 2004; Funderburg, et al. 2010b). Kuller et al. hypothesised that activation of tissue factor pathways could be a key event explaining the adverse rise in all cause mortality seen in those stopping ART in the SMART study (Kuller, et al. 2008). Tissue factor is expressed on monocytes and the sub-endothelium of the blood vessel wall, and becomes activated when vascular endothelium is damaged to initiate the extrinsic coagulation pathway. Funderburg et al. showed that in chronic HIV-1 infection, monocyte tissue factor expression correlated with soluble CD14 levels, T-cell activation, and D-dimer levels, as well as with HIV-1 viraemia and inversely with CD4 cell count, and concluded that drivers of immune activation such as HIV-1 viraemia and microbial translocation activate clotting cascades through this pathway. In chronic HIV-1 infection raised soluble tissue factor levels have been linked to cardiac events (Ford, et al. 2010). There are no data examining tissue factor in PHI.

HIV-1 infected individuals have increased endothelial dysfunction as measured by brachial artery flow mediated vasodilation (Blum, et al. 2005; Solages, et al. 2006), and raised levels of endothelial adhesion molecules such as ICAM-1 and VCAM-1 (Wolf, et al. 2002). ICAM-1 and VCAM-1 levels decrease with ART (Wolf, et al. 2002), and increase on ART treatment interruption (Papasavvas, et al. 2008). Animal models have shown that enteric microflora directly contribute to constitutive ICAM-1 expression on vascular endothelial cells (Komatsu, et al. 2000), and studies on human coronary artery endothelial cells show that LPS directly results in TLR 4 mediated inflammatory activation of the endothelium and induction of adhesion molecules such as ICAM-1 (Zeuke, et al. 2002). There are no current data on examining endothelial dysfunction in PHI.

Several studies have now confirmed raised levels of microbial translocation in HIV-1 infected compared to HIV-negative cohorts (Brenchley, et al. 2006; Cassol, et al. 2010; Nowroozalizadeh, et al. 2010; Troseid, et al. 2010; Wallet, et al. 2010), and this process provides a potential mechanism for T-cell activation, endothelial activation, and initiation of pro-coagulatory pathways in HIV-1 infection. No longitudinal studies have assessed LPS levels in relation to HIV-1 disease progression in untreated individuals in Western cohorts, and thus the clinical significance of microbial translocation remains uncertain. A longitudinal African study found no relationship between LPS and sCD14 levels and HIV-1 disease progression (Redd, et al. 2009). In contrast to data from Brenchley et al. (2006), several studies have failed to demonstrate a direct association between T-cell activation and LPS levels (Cassol, et al. 2010; Papasavvas, et al. 2009; Wallet, et al. 2010). In addition there are few data

on the relationship between microbial translocation, immune activation, endothelial activation and HIV-1 disease parameters in individuals with PHI.

The aims of this chapter were to:

- i. estimate levels of microbial translocation in PHI and how these change over time.
- ii. assess the relationship between surrogate markers of microbial translocation and other bio-markers of inflammation/coagulation (IL-6, D-dimer) and cellular markers of immune activation (CD38+ and HLA-DR+ expression on CD4 and CD8 T-cells) in PHI and at week 60.
- iii. assess levels of soluble tissue factor (sTF) and soluble ICAM-1 in PHI and compare these to markers of immune activation, microbial translocation, and coagulation.

Results

7.1 Baseline characteristics

The overall assays employed for this analysis are described in detail in the materials and methods (chapter 2.4). As SPARTAC samples were not collected specifically for LPS analysis, the following markers of LPS activity were measured: sCD14, LBP and EndoCAb. Samples from 90 UK and Australian SPARTAC participants with paired available samples at week 0 and week 60, and from 30 healthy controls (chapter 2.3), were analysed. Subjects were chosen on the basis of available plasma at the specific SPARTAC time points, and on the availability of results on inflammatory and coagulatory biomarkers and markers of immune activation from SPARTAC analyses (chapter 4 and 6). Baseline characteristics of the 90 SPARTAC participants included in this analysis are shown in Table 7.1. Participants were predominantly MSM with subtype B virus. The median age was 34 years. Estimated time since seroconversion was 73.5 [IQR 52, 93.5] days.

Table 7.1. Baseline characteristics of SPARTAC participants included in microbial translocation studies

Baseline variable		Number or Median	(%) or [IQR]
Sex:	Male	87	(97)
	Female	3	(3)
Risk factor	MSM	84	(93)
	Heterosexual sex	5	(6)
	Not reported	1	(1)
Age (years)		34.6	[30, 41.4]
Estimated time from seroconversion (days)		73.5	[52, 93.5]
Clade B		82	(91)
CD4 (cells/ μ l)		550	[412, 682]
HIV RNA (copies/ml) overall		50783	[9010, 190000]
	<1000	8	(9)
	1000-9999	15	(17)
	10000-99999	31	(34)
	>100000	36	(40)
Site of recruitment	UK	85	(94)
	Australia	5	(6)

Baseline characteristics of 90 SPARTAC participants included in microbial translocation analysis. Subjects were chosen on the basis of available plasma.

7.2 Markers of LPS activity (sCD14, EndoCAb and LBP) in SPARTAC participants and healthy controls

Absolute results for median values and interquartile (IQR) range are given below in Table 7.2. All markers of LPS activity fell within similar ranges to those reported by Brenchley et al. (2006), except EndoCAb levels for healthy controls which tended to be lower in our analysis (median 174 MMU/ml vs. >500 MMU/ml).

Results at week 0 and 60 were compared to healthy controls using Mann-Whitney U tests, and differences in biomarkers at week 60 compared to week 0 were assessed using the Wilcoxon signed rank test. Figure 7.1 shows box-plot graphs demonstrating changes between week 0 and week 60 in SPARTAC participants and comparison with healthy controls.

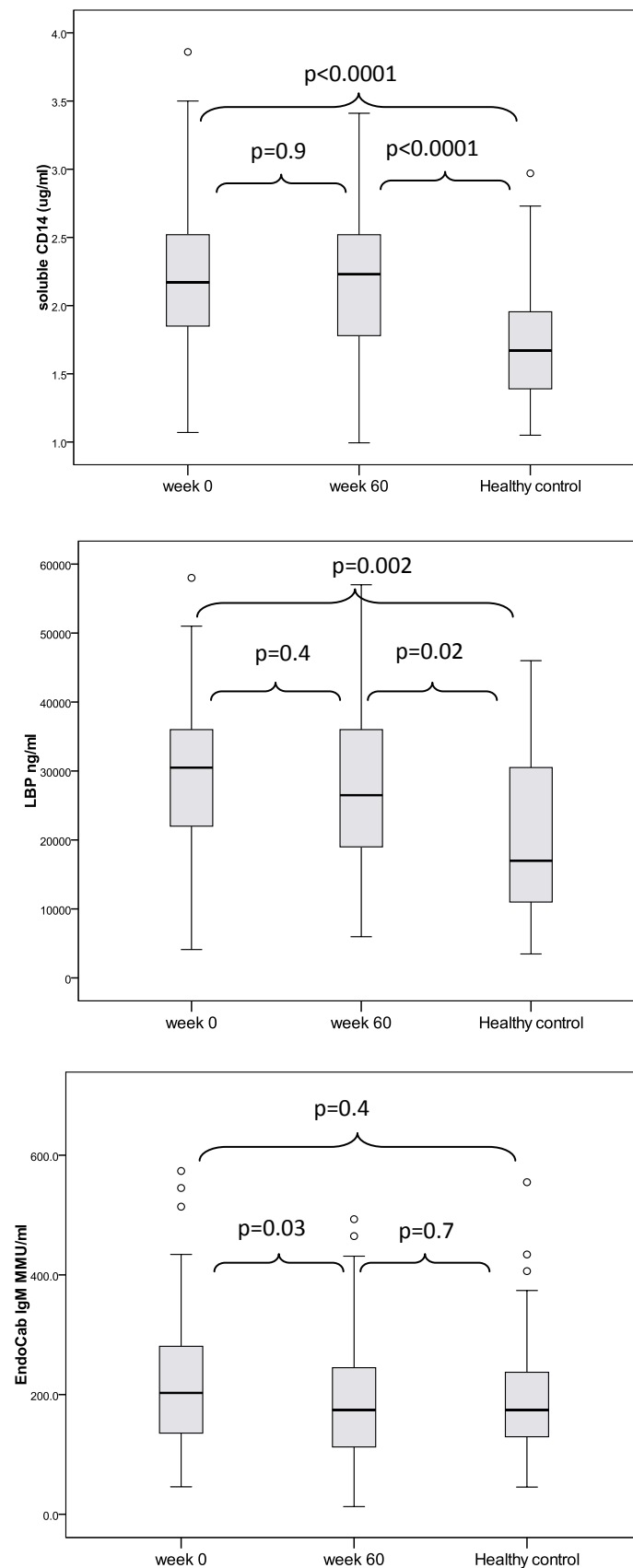
sCD14 and LBP were significantly higher in HIV-1 infected individuals at both week 0 and week 60 compared to healthy controls, suggesting that levels of microbial translocation are higher in HIV-1 infected individuals in both PHI and early chronic infection in patients in this cohort. There was no significant change between the 2 study time points in either biomarker. There was no difference in EndoCAb levels compared to healthy controls at week 0 or week 60. However, a significant decline in EndoCAb was seen between week 0 and week 60 in SPARTAC participants.

Table 7.2. LPS activity at week 0, week 60 and healthy controls

Biomarker	Week 0 (n=90)		Week 60 (n=90)		Healthy control (n=30)	
	Median	[IQR]	Median	[IQR]	Median	[IQR]
sCD14 x 10 ⁶ pg/ml	2.17	[1.85, 2.52]	2.23	[1.78, 2.52]	1.66	[1.39, 1.94]
LBP x 10 ⁴ ng/ml	3.05	[2.2, 3.6]	2.65	[1.90, 3.6]	1.85	[1.1, 3.1]
EndoCAb MMU/ml	202.7	[135.6, 280.7]	174.3	[112.5, 241.3]	174.1	[129.5, 237.4]

Markers of LPS activity (median [IQR]) are shown in 90 SPARTAC participants and in 30 healthy controls.

Figure 7.1. Markers of LPS activity in SPARTAC participants at week 0 and week 60 and in healthy controls



Box-plots showing median, IQR and upper and lower adjacent values for sCD14, LBP and EndoCAB in SPARTAC participants at week 0 and week 60, and in healthy controls. Week 0 was compared to week 60 using Wilcoxon signed ranks test for paired samples. Mann-Whitney U tests were used to compare healthy controls to SPARTAC participants

7.3 Factors associated with microbial translocation in PHI

Relationship between markers of LPS activity and each other

All biomarkers measured in this chapter were normally distributed except sTF and EndoCAb which had skewed distributions. On $\log_{10}$ transformation sTF and EndoCAb became normally distributed. To assess the relationship between different surrogate markers of microbial translocation, Pearson correlations were undertaken. In keeping with findings by Brenchley et al (2006), there was a significant correlation between sCD14 and LBP (Pearson correlation coefficient 0.24, $p=0.02$). There was no significant relationship between levels of EndoCAb and either LBP or sCD14 ($\log_{10}$ EndoCAb and sCD14: Pearson correlation coefficient 0.08, $p=0.5$; $\log_{10}$ EndoCAb and LBP: 0.01, $p=0.9$). Non parametric Spearman correlations gave similar results.

Baseline patient characteristics and markers of LPS activity

The relationship between baseline characteristics and markers of microbial translocation was examined using linear regression. There was no association between surrogate markers of microbial translocation and age, BMI, blood pressure or estimated time since seroconversion.

Relationship between HIV-1 parameters and markers of LPS activity in PHI

On linear regression analysis, there was a significant correlation between HIV RNA levels at week 0 and both sCD14 and LBP (Table 7.3). A one log rise in HIV RNA was associated with a rise in sCD14 of 0.16×10^6 pg/ml ($p=0.005$) and a rise in LBP of 3007 ng/ml ($p=0.006$). This is in contrast to findings by Brenchley et al. (2006) in chronic infection, which did not show an association with HIV RNA levels and sCD14 or LBP. No significant relationship was seen between $\log_{10}$ EndoCAb and $\log_{10}$ HIV RNA.

Participants were divided according to HIV RNA strata (HIV RNA <10,000 $n=23$; 10,000-99999 $n=31$, >100,000 $n=36$). The relationship between HIV RNA strata and biomarkers of microbial translocation is demonstrated in Figure 7.2. LBP and sCD14 increased with increasing VL strata, although the relationship was less clear for sCD14 at high VL strata.

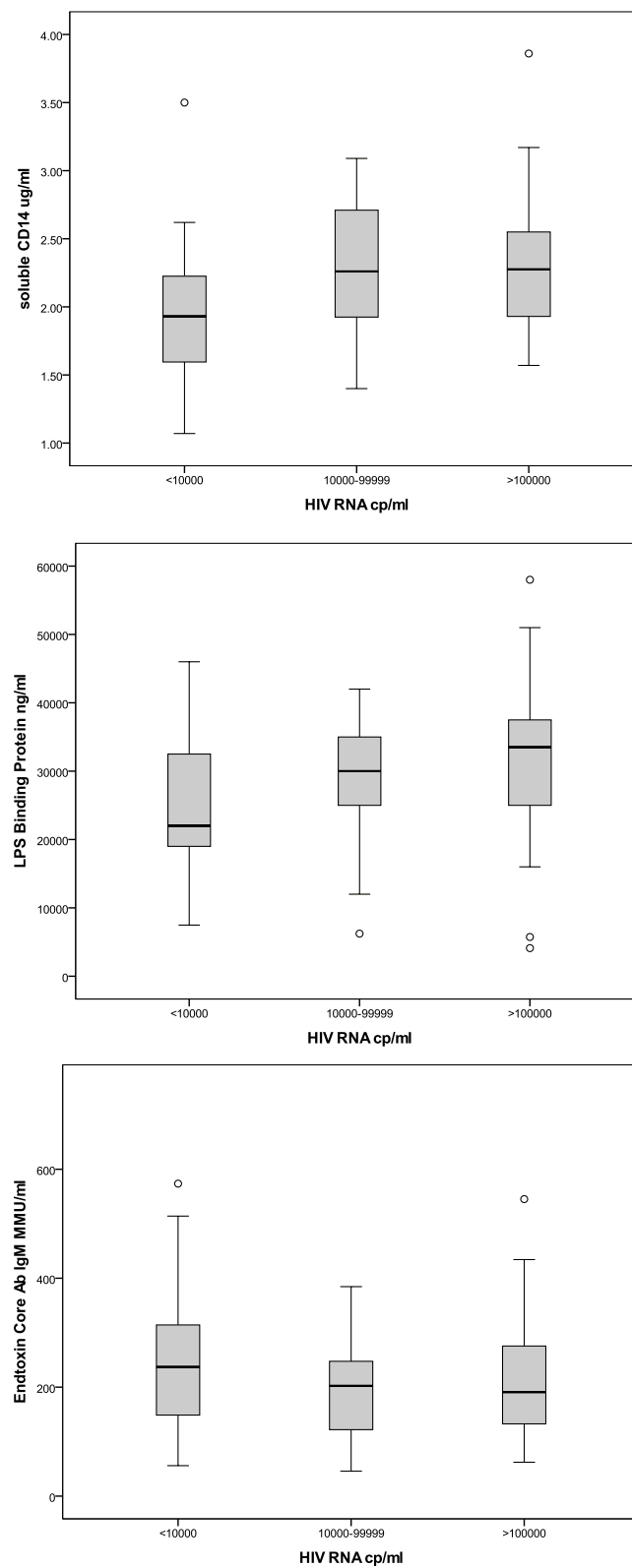
sCD14 was inversely associated with CD4 cell count. A rise in CD4 cell count of 100 cells/ μ l was associated with 0.05×10^6 pg/ml lower levels of sCD14 (95% CI 0.001×10^6 , 0.1×10^6 , $p=0.046$). There was no relationship between EndoCAb or LBP and CD4 cell count.

Table 7.3. Linear regression analysis of factors related to LPS activity in PHI

	Parameter	Coefficient	95% CI	P-value
sCD14	HIV RNA (+1 log)	0.16	0.05, 0.26	0.005
x10 ⁶ pg/ml	CD8 CD38% (+1%)	-0.004	-0.006, 0.005	0.9
	CD8 HLA-DR (+1 log)	0.19	-0.22, 0.61	0.4
	CD8 CD38 HLA-DR (+1 log)	0.21	-0.10, 0.22	0.2
	CD4 CD38 (+ 1%)	-0.001	-0.007, 0.005	0.7
	CD4 HLA-DR (+1log)	0.29	-0.10, 0.68	0.1
	CD4 CD38 HLA-DR (+1 log)	0.24	-0.07, 0.56	0.1
	IL-6 (+1 log)	0.20	-0.08, 0.49	0.2
	D-dimer (+1 log)	0.56	0.15, 0.98	0.007
LBP	HIV RNA (+1 log)	3007	876.8, 5138	0.006
ng/ml	CD8 CD38% (+1%)	36.2	-75.6, 148.2	0.5
	CD8 HLA-DR (+1 log)	2444.4	-5363, 10254.6	0.5
	CD8 CD38 HLA-DR (+1 log)	2879.7	-3006.8, 8766.3	0.3
	CD4 CD38 (+ 1%)	-30.6	-151.0, 89.6	0.6
	CD4 HLA-DR (+1log)	2738.4	-4708.7, 10185.5	0.5
	CD4 CD38 HLA-DR (+1 log)	3085.4	-2945, 9116.6	0.3
	IL-6 (+1 log)	9159.8	3812.6, 14505.9	0.001
	D-dimer (+1 log)	8482.3	328.6, 16636.1	0.04
Log ₁₀	HIV RNA (+1 log)	-0.004	-0.06, 0.05	0.9
EndoCAb	CD8 CD38% (+1%)	0.000	-0.003, 0.003	0.8
MMU/ml	CD8 HLA-DR (+1 log)	-0.06	-0.3, 0.2	0.6
	CD8 CD38 HLA-DR (+1 log)	-0.009	-0.2, 0.1	0.9
	CD4 CD38 (+ 1log)	0.002	-0.001, 0.005	0.3
	CD4 HLA-DR (+1log)	0.003	-0.2, 0.2	0.9
	CD4 CD38 HLA-DR (+1 log)	0.04	-0.1, 0.2	0.6
	IL-6 (+1log)	0.02	-0.1, 0.2	0.8
	D-dimer (+1 log)	0.005	-0.2, 0.2	0.9

Linear regression analysis of factors associated with surrogate markers of microbial translocation (sCD14, LBP and EndoCAb) in 90 SPARTAC participants with PHI (week 0). EndoCAb, IL-6, D-dimer, and HLA-DR% and CD38 HLA-DR% expression on CD4 and CD8 T-cells were log₁₀ transformed to achieve normality for analysis purposes. The regression coefficient (95% CI) and p-value are given for each variable.

Figure 7.2. Relationship between VL strata and markers of LPS activity



Relationship between surrogate markers of LPS activity and different VL strata are shown. In previous studies LBP and sCD14 have been shown to correlate with LPS levels and EndoCAB inverse correlates with LPS. Individuals are divided according to VL (HIV RNA<10,000 n=23, 10,000-99,999 n=31 and>100,000 n=36). Box-plot graphs demonstrate median values, IQR and upper and lower adjacent values.

Relationship between markers of LPS activity and immune activation in PHI (SPARTAC week 0).

Microbial translocation has been hypothesized to be a key cause of immune activation in chronic HIV-1 infection (Brenchley, et al. 2006). We assessed the relationship between markers of LPS activity and markers of T-cell immune activation, IL-6 and D-dimer in PHI, using samples from SPARTAC patients at baseline (week 0). Of the 90 individuals included in this analysis, 80 had results available from T-cell activation studies, and 89 and 87 individuals had IL-6 and D-dimer results available, respectively.

Results from linear regression analysis of the relationship between markers of immune activation and microbial translocation are shown in Table 7.3. Results for CD38% and HLA-DR% expression are given although results examining CD38 MFI and HLA-DR MFI were similar.

In this cohort of individuals with PHI, there was no significant relationship between markers of LPS activity and T-cell activation. However, higher sCD14 was significantly associated with a rise in D-dimer and higher LBP was significantly associated with increases in both IL-6 and D-dimer. There was no association between EndoCAb levels and T-cell activation, IL-6 or D-dimer.

7.4 Factors associated with microbial translocation at week 60

The relationship between markers of LPS activity measured at SPARTAC week 60, and markers of immune activation at this time point, was examined using linear regression. At week 60, T-cell activation results were available in 77 individuals and IL-6 and D-dimer available in 89 individuals. Due to the blinded nature of the SPARTAC trial, week 60 results were analysed together regardless of randomisation arm, and it should be noted that two-thirds of participants had received either 12 weeks or 48 weeks treatment by this time point, whilst one third had not been treated at all. All individuals had discontinued the ART given as a trial intervention by week 60.

Results from linear regression are given in Table 7.4. Results for CD38 and HLA-DR percentage expression are given although MFI gave similar values. In contrast to our observations in PHI, there was a significant association between these surrogate markers of microbial translocation and T-cell activation at week 60. sCD14 was associated with rises in CD4 T-cell activation as measured by HLA-DR% expression and dual CD38 HLA-DR% expression, as well as increases in IL-6 and D-dimer. sCD14 was not associated with HIV RNA levels at this time point. LBP was associated with CD8 activation (CD8 CD38% and CD8 CD38 HLA-DR% dual expression) with a trend towards an association with raised CD4 HLA-DR% expression ($p=0.08$). LBP was also significantly associated with rises in IL-6 and D-dimer, and with higher HIV RNA. EndoCAb was significantly associated with CD4 T-cell activation as measured by CD38% expression and dual CD38 HLA-DR% expression. There was no significant relationship between EndoCAb levels and HIV RNA.

There was a trend towards an inverse association between sCD14 and total CD4 cell count at week 60 (0.04×10^6 reduction in sCD14 for every 100 rise in CD4 cell count 95% CI -0.004×10^6 , 0.1×10^6 , $p=0.07$). CD4 cell count was not related to LBP or EndoCAb levels.

Table 7.4. Linear regression analysis of factors related to LPS activity at week 60

	Week 60 Parameter	Coefficient	95% CI	P-value
sCD14	HIV RNA (+1 log)	-0.02	-0.12, 0.08	0.7
x10 ⁶ pg/ml	CD8 CD38% (+1%)	0.03	-0.005, 0.01	0.5
Week 60	CD8 HLA-DR% (+1 log)	0.15	-0.31, 0.61	0.5
	CD8 CD38 HLA-DR% (+1 log)	0.09	-0.22, 0.40	0.6
	CD4 CD38% (+ 1%)	-0.002	-0.009, 0.004	0.5
	CD4 HLA-DR % (+1 log)	0.70	0.28, 1.12	0.001
	CD4 CD38 HLA-DR % (+1 log)	0.29	0.00, 0.59	0.05
	IL-6 (+1 log)	0.47	0.18, 0.76	0.002
	D-dimer (+1 log)	0.52	0.10, 0.96	0.02
LBP	HIV RNA (+1 log)	2759.7	729.5, 4789.9	0.01
ng/ml	CD8 CD38% (+1%)	188.6	39.9, 337.3	0.01
Week 60	CD8 HLA-DR% (+1 log)	2494.1	-7516.1, 12504.5	0.6
	CD8 CD38 HLA-DR% (+1 log)	7001.0	470.4, 13531.5	0.04
	CD4 CD38% (+ 1%)	12.3	-137.7, 162.2	0.8
	CD4 HLA-DR% (+1log)	8519.8	-1034, 18073.5	0.08
	CD4 CD38 HLA-DR% (+1 log)	5287	-1241.5, 11815.6	1.1
	IL-6 (+1 log)	8489.7	2248.8, 14730.7	0.01
	D-dimer (+1 log)	13874.7	5184.3, 22565.2	0.002
Log ₁₀	HIV RNA (+1 log)	0.002	-0.01, 0.02	0.7
EndoCAb	CD8 CD38% (+1%)	0.0001	-0.001, 0.001	0.7
MMU/ml	CD8 HLA-DR% (+1 log)	0.03	-0.03, 0.08	0.4
Week 60	CD8 CD38 HLA-DR% (+1 log)	0.005	-0.03, 0.05	0.8
	CD4 CD38% (1%)	0.001	0.0004, 0.002	0.02
	CD4 HLA-DR% (+1log)	0.04	-0.01, 0.1	0.1
	CD4 CD38 HLA-DR% (+1 log)	0.04	0.007, 0.08	0.02
	IL-6 (+1log)	-0.001	-0.04, 0.04	0.9
	D-dimer (+1 log)	0.004	-0.05, 0.06	0.9

Linear regression analysis of factors associated with surrogate markers of microbial translocation (sCD14, LBP and EndoCAb) in 90 SPARTAC participants with at week 60. EndoCAb, IL-6, D-dimer, and HLA-DR% and CD38 HLA-DR% expression on CD4 and CD8 T-cells were log₁₀ transformed to achieve normality for analysis purposes. The regression coefficient (95% CI) and p-value are given for each variable.

7.5 Soluble tissue factor and ICAM-1 in PHI

Analysis of sTF and ICAM-1 were completed on 90 patients at SPARTAC week 0 and week 60, and 30 healthy controls. Due to samples haemolysis, tissue factor results were excluded for 3 samples at week 0, and for one patient at week 60, as particles released during haemolysis may be tissue factor-positive thus affecting results.

Absolute results for median values and IQR are given below in Table 7.5. sTF values were within the detection range of the assay, and within the normal range given by the kit manufacturer (25-57.8 pg/ml). ICAM-1 levels also fell within the detection range of the assay. In healthy controls ICAM-1 levels fell within the normal range as given by the kit manufacturer (100-307 ng/ml), but in HIV-1 infected subjects, ICAM-1 median levels were slightly above this range.

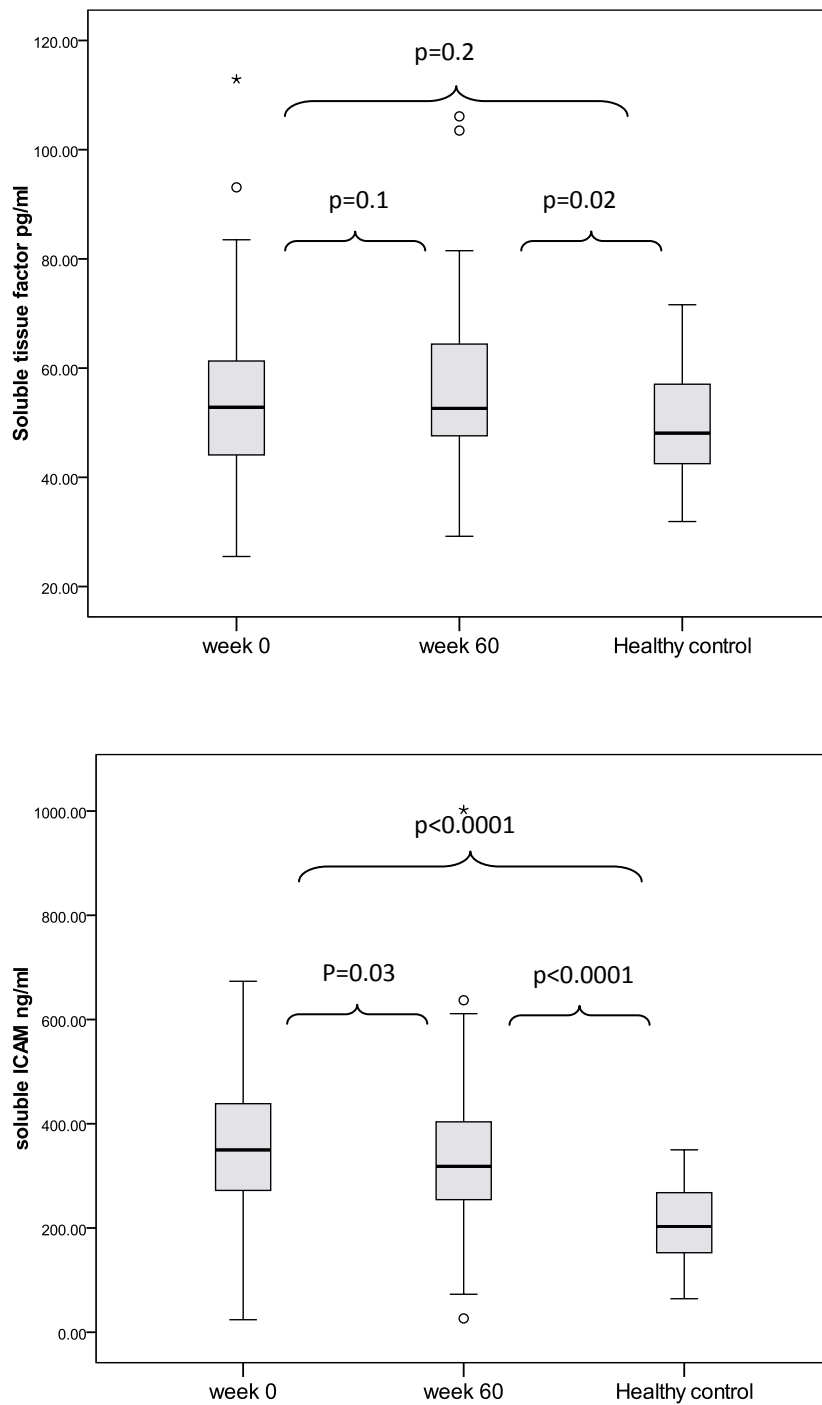
Box-plot graphs demonstrating sTF and ICAM-1 levels are shown in Figure 7.3. There was no significant change in sTF levels between SPARTAC week 0 and 60 ($p=0.1$). Levels of sTF were however were significantly higher at week 60 compared to healthy controls. ICAM-1 levels were higher in both early infection and week 60 compared with healthy controls and there was a significant decline from week 0 to week 60.

Table 7.5. Median (IQR) ICAM-1 and sTF results at week 0, week 60 and in healthy controls

Biomarker	Week 0 (n=90)		Week 60 (n=90)		Healthy control (n=30)	
	Median	[IQR]	Median	[IQR]	Median	[IQR]
sTF pg/ml*	53.1	[44.7, 61.4]	52.6	[47.4, 64.4]	48.0	[42.3, 55.8]
ICAM-1 ng/ml	349.7	[272.1, 438.5]	318.6	[254.3, 404.7]	212.4	[152.8, 272.4]

sTF and ICAM-1 shown in 87 and 90 SPARTAC participants respectively and in 30 healthy controls. *Due to sample haemolysis, sTF results from 3 samples were excluded at week 0 and 1 excluded at week 60.

Figure 7.3. sTF and ICAM-1 levels in SPARTAC participants at week 0 and week 60 and in healthy controls



Box-plots showing median, IQR and upper and lower adjacent values for sTF and ICAM-1 in SPARTAC participants at week 0 and week 60, and in healthy controls. Week 0 was compared to week 60 using Wilcoxon signed ranks test for paired samples. Mann-Whitney U tests were used to compare healthy controls to SPARTAC participants

7.6 Factors associated with sTF and ICAM-1 levels in PHI

sTF

The relationship between sTF and other baseline parameters including HIV RNA, CD4 cell count, duration of infection, was examined using linear regression (Table 7.6). sTF results were $\log_{10}$ transformed to normalise the distribution for analysis purposes. There was no relationship between sTF and age, BMI, blood pressure. sTF levels increased with increasing estimated days since seroconversion ($p=0.02$).

$\log_{10}$ sTF was inversely correlated with $\log_{10}$ HIV RNA. A one log increase in HIV RNA was associated with a 6% decrease in sTF (95% CI 1%, 11% $p=0.02$). The relationship between sTF levels and different VL strata is shown in Figure 7.4 and demonstrates a decrease in sTF with increasing VL strata. sTF was not related to CD4 cell count.

sTF levels were also inversely associated with IL-6 and there was a trend towards an inverse association with D-dimer. In addition, sTF was inversely correlated with T-cell activation as measured by CD8 CD38% expression and CD4 CD38 HLA-DR dual expression, and there was a trend towards inverse an association with CD8 CD38 HLA-DR% and CD4 HLA-DR expression. Thus it appears that in PHI, sTF is inversely associated with both levels of HIV-1 viraemia, and levels of T-cell activation and inflammation.

ICAM-1

The relationship between ICAM-1 and other baseline parameters is shown in Table 7.6. There was no relationship between sTF and age, BMI, blood pressure. There was a significant association between increasing levels of ICAM-1 and $\log_{10}$ HIV RNA; a one log increase in HIV RNA resulted in an increase in ICAM-1 of 35.4 ng/ml (95% CI 10.2, 60.7, $p=0.01$). The relationship between ICAM-1 and VL strata is shown in Figure 7.4 and there was an increase in ICAM-1 with every stratum. There was no relationship between ICAM-1 and CD4 cell count.

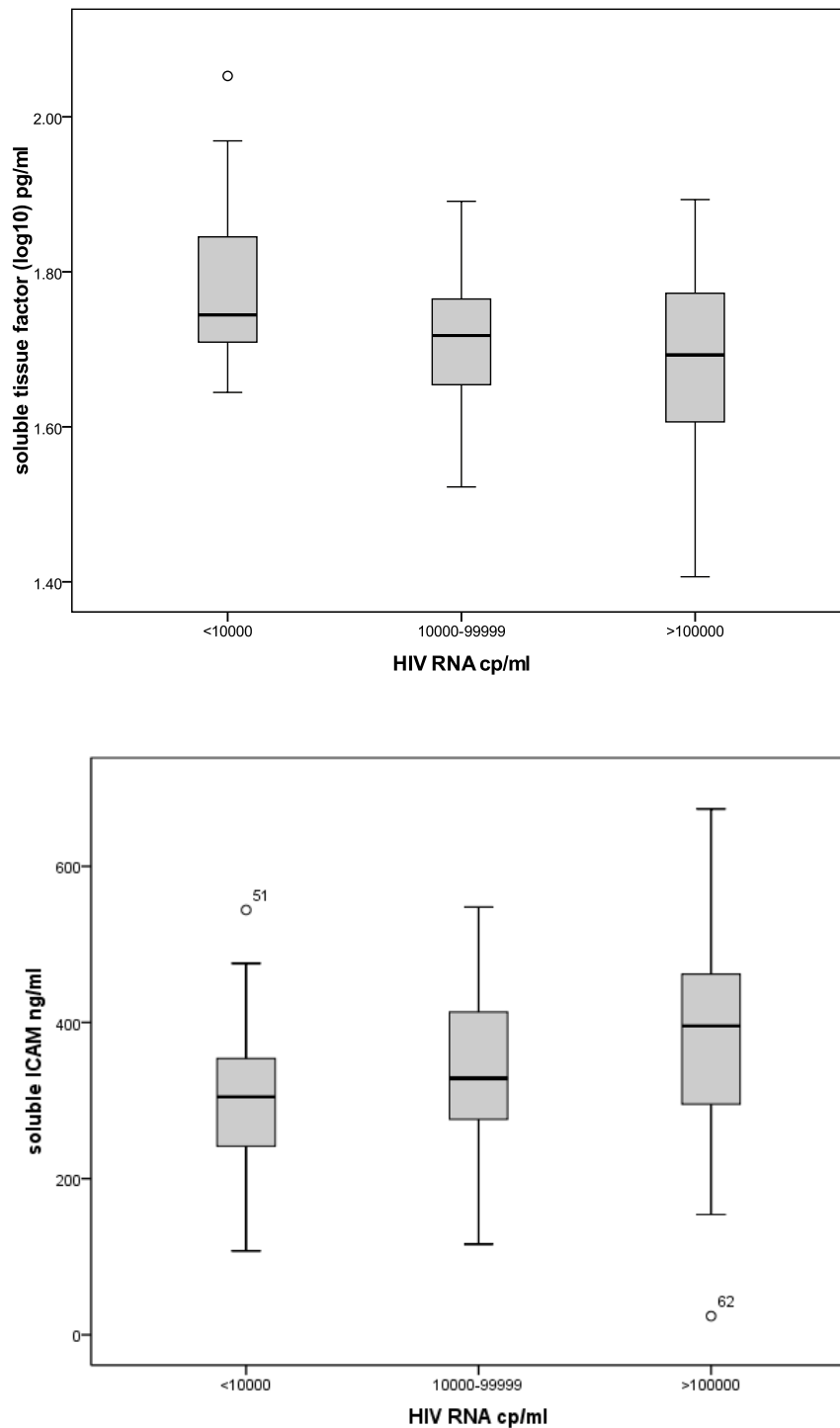
ICAM-1 was associated with markers of microbial translocation markers sCD14 ($p=0.02$) and LBP ($p=0.03$), and there was a trend for correlation with IL-6 ($p=0.05$). There was no association between ICAM-1 and markers of T-cell activation.

Table 7.6. Factors associated with sTF and ICAM-1 at in PHI

	Parameter	Coefficient	95 % CI	p-value
soluble tissue factor (log ₁₀)	HIV RNA copies/ml (+1 log)	-0.03	-0.05, -0.004	0.02
	days since seroconversion (+1 day)	0.001	0.000, 0.002	0.02
	IL-6 pg/ml(+ 1 log ₁₀)	-0.07	-0.13, -0.002	0.04
	D-dimer mg/L(+1 log ₁₀)	-0.08	-0.17, 0.015	0.09
	CD8 CD38% (+1%)	-0.001	-0.001, 0.000	0.05
	CD8 HLA-DR% (+1 log)	-0.06	-0.14, 0.03	0.17
	CD8 CD38+HLA-DR % (+1 log)	-0.06	-0.1, 0.008	0.08
	CD4 CD38% (+1%)	-0.001	-0.002, 0.001	0.3
	CD4 HLA-DR% (+1 log)	-0.08	-0.16, 0.005	0.07
	CD4 CD38+ HLADR% (+ 1 log)	-0.08	-0.15, -0.02	0.015
	LPS Binding Protein (+1 ng/ml)	-1.8	-4.2, 0.47	0.12
	sCD14 (+1x10 ⁶ pg/ml)	0.0001	-0.004, 0.05	0.85
	EndoCAb (1 log MMU)	0.006	-0.09, 0.11	0.9
ICAM-1	HIV RNA copies/ml (+1 log)	35.4	10.2, 60.7	0.01
	days since seroconversion (+1 day)	0.16	-0.6, 0.9	0.95
	IL-6 pg/ml(+ 1 log ₁₀)	66.0	-0.7, 132.6	0.052
	D-dimer mg/L(+1 log ₁₀)	79.9	-16.6, 176.4	0.1
	CD8 CD38% (+1%)	0.8	-0.5, 2.2	0.2
	CD8 HLA-DR (+1 log)	18.9	-77.6, 115.4	0.7
	CD8 CD38+HLA-DR% (+1 log)	29.3	-43.4, 102.1	0.4
	CD4 CD38 (+1%)	0.3	-1.1, 1.8	0.6
	CD4 HLA-DR %(+1 log)	38.1	-53, 129.9	0.4
	CD4 CD38+ HLADR %(+ 1 log)	52.2	-21.7, 126	0.16
	LPS Binding Protein (+1 ng/ml)	0.003	0.0003, 0.005	0.03
	sCD14 (x 10 ⁶ pg/ml)	0.58	0.1, 1.0	0.02
	EndoCAb (+ 1log MMU)	-53.1	-155.4, 49.2	0.3

Linear regression analysis of factors related to sTF (n=87) and ICAM-1 (n=90) in PHI. HIV RNA, sTF Factor, EndoCAb, IL-6, D-dimer, and HLA-DR% and CD38 HLA-DR% expression on CD4 and CD8 T-cells were log₁₀ transformed to achieve normality for analysis purposes. The regression coefficient (95% CI) and p-value are given for each variable.

Figure 7.4. Relationship between VL strata and ICAM-1 and sTF levels



Relationship between ICAM-1 and sTF with different VL strata are shown. Individuals are divided according to VL (ICAM-1: HIV RNA <10,000 n=23, 10,000-99,999 n=31 and >100,000 n=36; sTF: HIV RNA <10,000 n=21, 10,000-99,999 n=31 and >100,000 n=35). Box-plot graphs demonstrate median values, IQR and upper and lower adjacent values.

7.7. Summary of findings in Chapter 7

LBP and sCD14, both markers of bacterial LPS activity, were raised in HIV-1 infected individuals compared to healthy controls at the time of HIV-1 acquisition and remained raised at week 60. In PHI these two biomarkers directly correlated with HIV RNA. sCD14 also correlated positively with D-dimer and inversely with CD4 cell count, and LBP with both IL-6 and D-dimer. There was no correlation in PHI between markers of LPS activity and T-cell activation. At week 60, there was a significant association between all markers of LPS activity and T-cell activation: sCD14 and EndoCAb correlated with CD4 T-cell activation, and LBP correlated with CD8 T-cell activation. LBP and sCD14 also correlated with IL-6 and D-dimer.

In PHI, sTF inversely correlated with HIV RNA, IL-6 and T-cell activation. sTF was significantly raised compared to healthy controls at week 60. ICAM-1 was significantly raised compared to healthy controls at both time points and correlated with HIV RNA, sCD14 and LBP.

Discussion

Consistent with previous reports, we demonstrate raised levels of LPS activity in HIV-1 infected individuals. In a cross-sectional study examining individuals at different stages of HIV-1 infection, Brenchley et al. (2006) found that LPS levels increased as disease advanced, and were raised in chronic HIV-1 infection compared to HIV-negative controls and higher still in patients with AIDS. In our study, LBP and sCD14, surrogate markers of LPS activity, were raised in both PHI and at week 60 compared to healthy controls, suggesting that microbial translocation occurs in HIV-1 infected individuals at both PHI and early chronic infection. Brenchley et al. also examined markers of microbial translocation in acutely infected individuals within 4 weeks of seroconversion, and found that levels of sCD14 were raised, and EndoCAb levels low, but LPS and LBP were no different from healthy controls, and concluded that microbial translocation occurs at this early stage of disease but LPS is neutralised by EndoCAb. SPARTAC participants at baseline were less acutely infected than those described by Brenchley et al (within 6 months of seroconversion vs. 4 weeks), which may explain why we also saw changes in LBP, reflecting higher levels of microbial translocation at this later time point.

Consistent with findings from Brenchley et al. (2006), we saw a decline in EndoCAb levels from week 0 to week 60. In other conditions of chronic microbial translocation, such as inflammatory bowel disease, EndoCAb levels are increased, and Brenchley hypothesized that EndoCAb bind LPS to remove it from the circulation but are insufficiently replaced due to the dysfunctional B cell responses associated with HIV-1 infection (Titanji, et al. 2006). We did not see a difference in

EndoCAb levels compared with healthy controls, however, in our study, healthy controls had lower levels of EndoCAb than those reported by Brenchley et al, which may reflect cohort differences. In particular it should be noted that our healthy controls were not formally HIV-1 tested which may confound these results

sCD14 is secreted by monocytes and macrophages that have been stimulated via LPS and has been associated with increased mortality in HIV-1 infected cohorts (Lien, et al. 1998; Sandler, et al. 2010). We saw a direct correlation between sCD14 and HIV RNA levels in PHI, and inverse correlation between sCD14 and CD4 cell count. This is consistent with findings by Lien et al. in advanced disease but has not been previously demonstrated in PHI. We also saw correlations between sCD14 and D-dimer levels, indicating that monocyte activation in response to LPS may lead to activation of coagulation pathways, as hypothesised by Funderburg et al (2010). In addition LBP levels were correlated with D-dimer and IL-6 levels, as well as HIV RNA, indicating a relationship between LPS activity, inflammatory and coagulatory processes and HIV-1 replication. It is not clear from these data whether HIV-1 replication is a driver or consequence of these processes.

There was no association in PHI between surrogate markers of microbial translocation and T-cell activation. This is in contrast to week 60 where a direct correlation was seen between T-cell activation and markers of LPS activity in the same individuals, in agreement with findings described by Brenchley et al (2006) who showed that LPS levels correlated with CD38 HLA-DR expression on CD8 T-cells, and Jiang et al (2009), who found that bacterial rDNA levels also correlated with CD8 T-cell activation. The discrepancy between PHI and chronic infection may reflect different mechanisms driving T-cell activation in chronic compared with early infection, where, for example, the direct effects of high plasma viraemia may be the key driver of T-cell activation.

A limitation of our study is that due to logistic constraints we did not measure LPS directly, although several studies in HIV-1 infected cohorts have indicated that sCD14, LBP and EndoCAb are good indicators of LPS activity (Ancuta, et al. 2008; Brenchley, et al. 2006; Papasavvas, et al. 2009), and these are well-established markers of LPS activity in HIV-negative studies (Kitchens and Thompson 2005; Strutz, et al. 1999; Voss, et al. 2006). Although predictors of disease progression such as VL and CD4 cell count correlated with some of these markers, our data give little indication of the prognostic significance of these parameters. Once SPARTAC is unblinded an analysis of the predictive value of sCD14, LBP and EndoCAb, when measured both in PHI and at week 60 will be undertaken. The impact of intermittent ART in PHI on these markers will also be assessed. It is also possible that intervention with short-course ART in PHI, by controlling HIV-1 replication in the gut, may in turn limit markers of microbial translocation.

Recent studies have suggested that tissue factor (TF) activity is increased in chronic HIV-1 infection, and that monocyte TF expression is induced by LPS, and correlates with T-cell activation and D-dimer (Funderburg, et al. 2010b). Soluble TF is an easily measurable parameter that has been linked to cardiovascular events in HIV-1 infected individuals (Ford, et al. 2010). We did not see any relationship between sTF levels and markers of LPS activity. However, in contrast to our expectations we found an inverse relationship between sTF and HIV RNA, and an inverse association between sTF, IL-6, and markers of T-cell activation. A study in the pre-HAART era showed there was a selective reduction in LPS induced monocyte TF mRNA production in individuals with symptomatic HIV-1 infection, and that there was a continuum of diminished TF function in patients with asymptomatic disease, AIDS-related complex, and AIDS (Lathey, et al. 1990). The investigators went on to show a positive correlation between LPS stimulated TF expression and CD4 cell count, and a negative correlation between TF and plasma β 2 microglobulin and neopterin, consistent with our findings of a negative correlation between inflammatory and immune activation markers and sTF (Lathey, et al. 1992). Funderburg et al. (2010) also found that samples exposed to LPS in vitro showed increased monocyte TF expression, but samples exposed to IL-6 tended to decrease TF expression. The mechanism behind these findings, and the clinical consequences, are not clear. It is possible that inflammatory processes secondary to HIV-1 viraemia inhibit sTF production, for example as a negative feedback pathway. It should be noted that sTF is a surrogate marker only and the precise role and regulation of circulating sTF in blood remains unclear (Bogdanov, et al. 2003; Censarek, et al. 2007; Parhami-Seren, et al. 2006). Measuring monocyte TF expression and TF activity may help clarify the relationship between these markers; however samples required for these laboratory techniques were not available.

Levels of sTF increased with increasing time since estimated seroconversion. This could reflect resolving immune activation and high levels of HIV-1 viraemia associated with PHI. Conversely sTF levels may increase with duration of infection, supported by higher levels of sTF at week 60 compared to healthy controls.

Elevated levels of circulating adhesion molecules such as ICAM-1 and VCAM-1 predict future cardiac events in HIV-negative individuals (Hwang, et al. 1997; Ridker, et al. 2000a) and in chronic HIV-1 infection (Ford, et al. 2010). In this study, ICAM-1 levels were raised in both PHI and chronic HIV-1 infection compared to healthy controls. This has previously been demonstrated in chronic infection but not in PHI (Francisci, et al. 2009; Wolf, et al. 2002). There was a reduction in ICAM-1 levels from PHI to week 60 but whilst the study remains blinded it is not possible to determine whether this is due to the natural history of HIV-1 disease, or due to the receipt of short-course ART in some of the

week 60 population. We demonstrate a direct correlation between HIV RNA levels and ICAM-1, in keeping with previous reports showing a direct correlation between VCAM-1 and HIV RNA and a reduction in both ICAM-1 and VCAM-1 in response to ART (Wolf, et al. 2002). Melendez et al (2008) found that raised levels of circulating adhesion molecules were associated with inflammation in chronic HIV-1 infection. We demonstrate a weak association between ICAM-1 levels and IL-6 in PHI. In addition we showed a direct correlation between this marker of endothelial activation and sCD14 and LBP. These data suggest that endothelial dysfunction, as reflected by raised ICAM-1 levels, is related to macrophage activation and LPS activity and also to the magnitude of HIV-1 viraemia.

In conclusion, we demonstrate an association between surrogate markers of LPS activity, immune activation, inflammatory and coagulatory pathways, and endothelial activation. The interactions between HIV-1 and pathways of microbial translocation are complex and not fully understood, however it is likely that this process contributes to HIV-1-associated immune activation and inflammation and their subsequent complications.

Chapter 8: Discussion

This thesis has addressed four main goals in relation to PHI and the effect of short-course ART at this stage of infection. These were the assessment of:

- i. the degree of inflammation and immune activation in PHI and the relationship between surrogate markers of immune activation, inflammation and coagulation, and microbial translocation.
- ii. host and viral factors associated with these parameters.
- iii. the impact of commencing short-course ART in PHI on biomarkers of inflammation and coagulation.
- iv. the effects of discontinuing ART on viral rebound and biomarkers of inflammation and coagulation, and comparison with the SMART trial in chronic infection.

Assessment of markers of inflammation and immune activation in PHI

Markers of inflammation, coagulation, and endothelial activation, were elevated in HIV-1 infected individuals, both in PHI and 60 weeks later. In contrast to reports from the SMART study in chronic HIV-1 infection, which did not show a correlation between HIV RNA and levels of IL-6 or D-dimer (Baker, et al. 2010; Kuller, et al. 2008), in our study IL-6, D-dimer and ICAM directly correlated with plasma HIV RNA in PHI, confirming that HIV-1 replication is a key driver of inflammatory and coagulation pathways early in the course of infection. As these biomarkers are well established predictors of CVD in HIV-negative individuals (Ridker, et al. 1994; Ridker, et al. 1998; Ridker, et al. 2000b) and predictive of AIDS and non-AIDS adverse outcome in individuals with chronic HIV-1 infection (Ford, et al. 2010; Kuller, et al. 2008; Rodger, et al. 2009; Sipsas, et al. 2003), elevations of these biomarkers occurring early in HIV-1 infection may have significant implications for future disease course and outcome.

Deeks et al. (2004) showed that in individuals within a year of seroconversion, levels of T-cell activation, measured by CD38 expression on CD4 and CD8 T-cells, correlated with HIV RNA and were predictive of outcome. We confirmed that levels of T-cell activation were elevated when measured within six months of seroconversion, and were associated with HIV RNA levels and with levels of inflammatory and coagulation biomarkers. In addition, we have shown that PD-1 expression on both CD4 and CD8 T-cells was associated with both T-cell activation and with VL. This has not been previously demonstrated in PHI. Expression of PD-1 may occur to regulate T-cell activation, but in chronic HIV-1 infection it is associated with functional T-cell exhaustion, reduced viral control, increased tendency to apoptosis and faster CD4 T-cell decline (Day, et al. 2006; Holm, et al. 2008;

Petrovas, et al. 2006). Thus elevated PD-1 in PHI may have profound implications for future disease course.

We sought to verify previous findings suggesting that gut microbial translocation contributes to ongoing immune activation in HIV-1 infection. This was investigated by measuring surrogate markers of LPS activity. We confirmed findings from a previous study showing that surrogate markers of LPS activity are raised in HIV-1 infected individuals compared to healthy controls (Brenchley, et al. 2006), and demonstrated an association between LPS activity, and HIV RNA, IL-6 and D-dimer. We found that markers of LPS activity correlated with T-cell activation in chronic HIV-1 infection (SPARTAC week 60), but not in PHI (SPARTAC week 0), suggesting that microbial translocation may be a more important driver of immune activation in chronic infection than in PHI, where other factors such as high level viral replication are likely to predominate. These findings highlight the complex interplay between HIV-1 replication, immune activation, inflammation and LPS activity. However, while we and others have demonstrated a close relationship between these parameters, there remain few longitudinal data assessing microbial translocation in HIV-1 infection and hence its prognostic relevance remains uncertain. This will be investigated once the SPARTAC trial is unblinded.

Evaluation of host and viral factors associated with inflammation and immune activation

In addition to the level of HIV RNA, other viral factors and host characteristics determined the level of immune activation. We showed that X4 tropism is associated with T-cell activation in individuals with PHI, in keeping with previous data showing increased CD4 T-cell proliferation, measured by increased Ki67 expression, in individuals with X4 virus both one and 5 years after seroconversion (Hazenbergh, et al. 2003a). As the X4 receptor is mainly expressed on naive T-cells, increased activation, proliferation and apoptosis of these cells may lead to faster depletion of the naive CD4 T-cell pool and explain the accelerated disease progression known to be associated with X4 viruses (Hazenbergh, et al. 2003a).

This study has shown an independent association between HLA type and magnitude of T-cell activation and PD-1 expression which has not been previously demonstrated. There was an association between HLA B*57 positivity and reduced CD38 expression, and between HLA B*27 and reduced PD-1 expression as well as lower levels of fibrinolysis. These factors may contribute towards the delayed HIV-1 disease progression known to be associated with these HLA alleles, and, in addition, reduced PD-1 expression could reflect a mechanism of enhanced viral control in HLA B*27 positive individuals. These findings highlight the importance of host genetic factors in determining immunological response to HIV-1 infection.

We observed a significant correlation between increasing age and markers of inflammation, fibrinolysis, T-cell activation and T-cell exhaustion which could reflect a mechanism by which HIV-1 infection is associated with poorer prognosis in older individuals. Our data are in keeping with studies in HIV-negative individuals which show that aging is associated with raised levels of circulating pro-inflammatory cytokines, in particular IL-6 (Ershler and Keller 2000) and higher levels of T-cell activation, manifest by increasing HLA-DR expression (Czesnikiewicz-Guzik, et al. 2008). In addition, studies in a mouse model show increased PD-1 expression with increasing age (Channappanavar, et al. 2009). The many similarities between clinical and immunological manifestations of HIV-1 infection and those observed with age have led to the suggestion that persistent immune activation and inflammation lead to accelerated aging in HIV-1 infected individuals (Desai and Landay 2010). With advancing age, as with HIV-1, there is a generalised decline in immune competence to deal with infection and malignancy, and an increase in inflammation mediated disease processes such as CVD, neurocognitive dysfunction, and reduced bone mineral density. As HIV-1 infected individuals age, the clinical significance of these observations is likely to become more apparent, as patients are affected by increasing age in addition to the effects of HIV infection.

Assessment of the impact of commencing short-course ART in PHI on markers of inflammation and coagulation

There are no previous data on the effect of starting ART in PHI on inflammation and coagulation. In chronic infection, starting ART results in reductions in D-dimer (Baker, et al. 2010; Calmy, et al. 2009; Wolf, et al. 2002). Baker et al (2010) showed that IL-6 decreased compared to baseline levels in those achieving an undetectable HIV RNA level in chronic infection, but overall found no differences in IL-6 between treated and untreated individuals after six months of ART. In PHI, we saw reductions in IL-6 and D-dimer 12 weeks after commencing ART. As these biomarkers were correlated with HIV RNA levels in PHI, it is likely the decline in these biomarkers was a direct result of treatment. In addition, we saw greater reductions in IL-6 in individuals with higher CD4 cell counts indicating that starting ART at an earlier disease stage may lead to greater benefits in dampening inflammatory responses.

Assessment of the effects of discontinuing ART on these markers and comparison to the SMART trial

Whilst the SMART trial identified the risks of stopping ART in chronic HIV-1 infection irrespective of immunological recovery, the effect of interrupting ART following treatment in PHI has not previously

been investigated. In the SMART trial, stopping ART given during chronic infection was associated with a rise in both IL-6 and D-dimer four weeks after treatment interruption, which correlated with the magnitude of viral rebound. Viral rebound on stopping ART initiated in SPARTAC was significantly lower compared to SMART, which may reflect smaller reservoir size in PHI versus chronic infection in agreement with other reports (Strain, et al. 2005). However, we found that stopping ART given in PHI was associated with a significant rise in IL-6 and D-dimer, which rebounded to pre-treatment levels at 4 weeks. Although the magnitude of this rebound may be smaller than that observed in SMART at 4 weeks, the long-term trends in IL-6 and d-dimer after stopping ART have not yet been examined, and further rises in these biomarkers could be anticipated in parallel with increasing viral load.

The adverse events seen in SMART may reflect years of chronic inflammation, ART exposure and metabolic complications (Grunfeld, et al. 2008) and, given the short duration of HIV-1 infection in SPARTAC, treatment interruption following ART in PHI may not be associated with same adverse clinical events, regardless of magnitude of viral and inflammatory biomarker rebound. However, even in the absence of an observed adverse outcome in the short-term, long-term biomarker changes following ART discontinuation in PHI may be associated with significant long-term morbidity. Stopping ART commenced in PHI also has significant public health implications (Rieder, et al. 2010). The majority of individuals in SPARTAC and SMART, regardless of stage of infection, experienced viral rebound above those sufficient to pose a risk of onward HIV-1 transmission. Even if short-course ART is proven a safe and beneficial intervention compared to untreated individuals with PHI, long-term ART resulting in sustained suppression of plasma viraemia, immune activation and inflammatory responses may be a preferable intervention.

Clinical Implications

While the findings of the SPARTAC trial will inform the value of short-pulse ART in PHI, the trial does not address the long-term role of life-long immediate ART, nor will it enable clinicians to target therapy to individuals who will gain most benefit from immediate therapy. Although short-term ART is associated with HIV RNA and biomarker rebound on treatment discontinuation, commencing long-term ART in PHI may present overwhelming problems of acceptability, feasibility, toxicity, and cost. The important question of when to start long-term ART will be addressed in the START trial which compares early (CD4 cell count >500 cells/ μ l) versus deferred ART (CD4 cell count 350 cells/ μ l). The START trial will include cardiovascular and biomarker sub-studies, and will investigate the risk benefit analysis between ART-associated toxicities and complications relating to ART versus the impact of uncontrolled viral replication. However, although the START trial will include participants

with PHI, it is not specifically powered to examine the benefits of long-term treatment at the time of primary infection.

Although it is known that those with severe clinical manifestations during acute infection experience faster progression to AIDS (Lavreys, et al. 2006; Vanhems, et al. 1998), for most individuals it is difficult to predict the rate of disease progression at the time of PHI presentation. Development of a clinically relevant predictive algorithm, potentially combining indicators such as CD4 cell count, VL, viral tropism, HLA type, age, cardiovascular risk factors such as smoking, lipids and blood pressure, and biomarker results, could provide a useful tool for managing PHI. Measurement of inflammatory and coagulation biomarkers could be a useful strategy to assess individual risk and response to interventions, however, before such a strategy can be implemented, it is important to identify the threshold biomarker value at which intervention would be started and also confirm that the intervention reduces both the biomarker and also the clinical outcome in HIV-1 infected populations (Nixon and Landay 2010). Case control studies have shown that many of the biomarkers examined in this thesis, including IL-6, D-dimer, sTF and sCD14, are predictive of clinical outcome in chronic HIV-1 infection (Ford, et al. 2010; Kuller, et al. 2008; Sandler, et al. 2010); however the relationship between plasma biomarkers when measured in PHI and prognosis has not yet been established. Once the SPARTAC trial is unblinded, long-term clinical outcome data from a minimum of 3.5 years follow up will be available, allowing an assessment of the predictive value and clinical utility of these biomarkers in evaluating long-term outcomes, when measured within six months of seroconversion and in early chronic infection. Soluble plasma biomarkers are easier to measure than cellular markers of T-cell activation, and could be more easily translated into clinical practice. Interventions in high risk individuals could include starting ART, adjustment of modifiable risk factors such as smoking cessation and weight loss, as well as novel approaches to reduce inflammation and immune activation.

Although ART may help to reduce risk associated with inflammatory complications of HIV-1, treated individuals still have considerably higher levels of inflammatory and coagulation biomarkers compared to HIV-negative individuals, even among those with HIV RNA levels <400 copies/ml (Neuhaus, et al. 2010). The causes of ongoing inflammation and immune activation in ART treated individuals are likely to be multi-factorial but may include inflammatory processes triggered by specific ART agents (Lundgren 2008; Madden, et al. 2008), residual low level HIV-1 replication (Bonora, et al. 2009; Havlir, et al. 2003; Palmer, et al. 2008), or responses to other antigens, such as CMV (Hsue, et al. 2006; Naeger, et al. 2010). Recent strategies intensifying standard regimens with additional agents, such as raltegravir did not reduce low level HIV-1 replication (Gandhi, et al. 2010;

McMahon, et al. 2010) and had minimal effects on immune activation (Yukl, et al. 2010). Mixed results have been reported for the use of ART with potential anti-inflammatory properties, such as maraviroc, to dampen immune activation (Evering, et al. 2010; Funderburg, et al. 2010a; Wilkin, et al. 2010). Other interventions to limit the effects of immune activation and inflammation in HIV-1 infected individuals include non-ART agent such as statins, known to block pro-inflammatory signalling by inhibiting the endotoxin TLR-4 pathway (Stoll, et al. 2004), and other anti-inflammatory agents or aspirin (Tornero, et al. 2010).

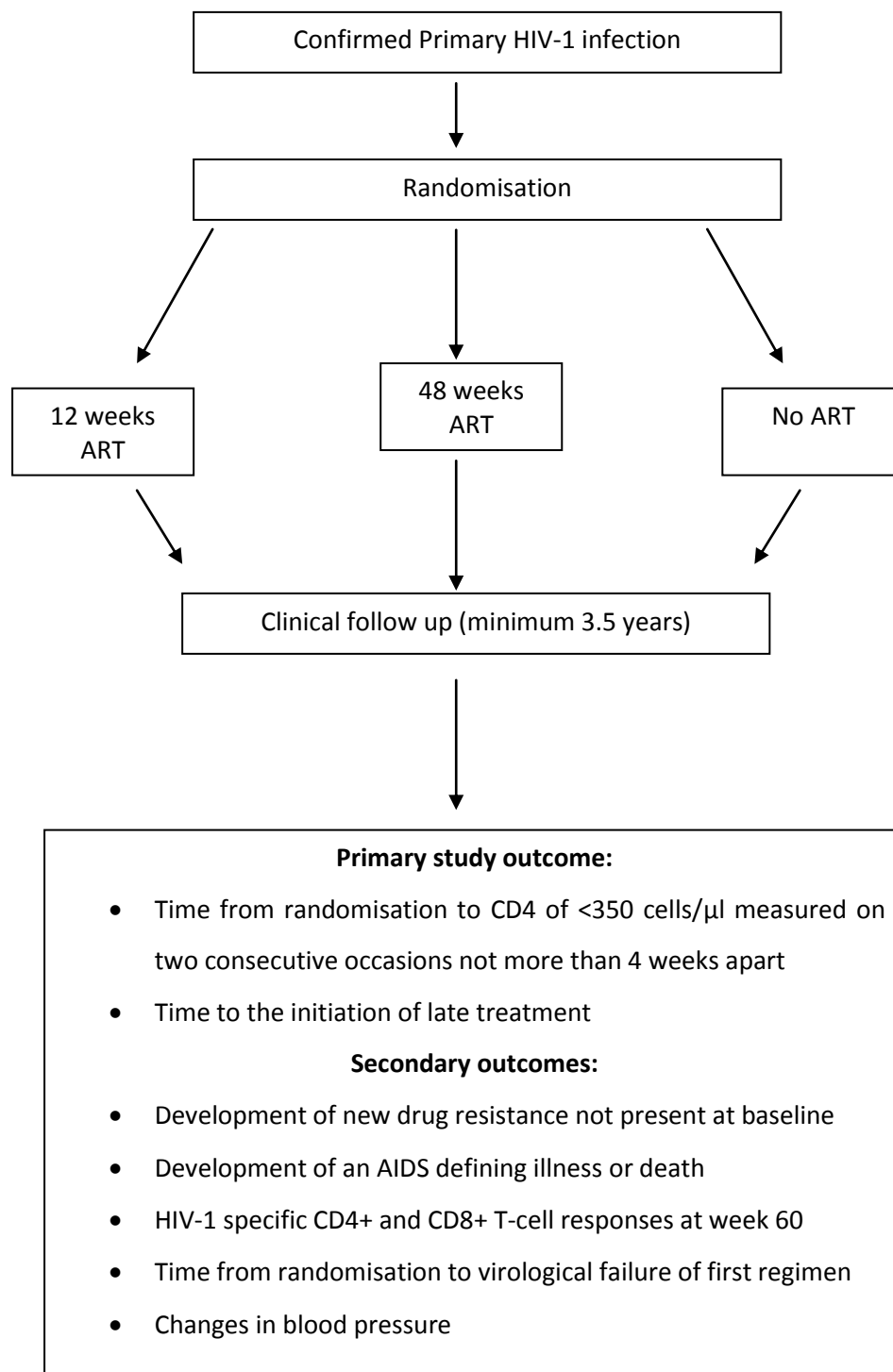
The main aim of SPARTAC is to determine the effects of short-course ART on long-term HIV-1 surrogate markers of clinical disease such as CD4 T-cell decline. The use of short-course ART in PHI, by influencing the immunological and virological events surrounding this phase of infection, may have a long-term impact after discontinuation of therapy, not just on clinical HIV-1 outcomes but also levels of inflammation and immune activation. However, to be effective, intervention in PHI may need to occur at a very early stage of acute infection, before an extensive viral reservoir has been established and massive immunological damage has taken place (Centlivre, et al. 2007; McMichael, et al. 2010). It has been hypothesised that intervention should be introduced before the development of peak viraemia, around 21-28 days after transmission (McMichael, et al. 2010). SPARTAC participants received intervention up to six months following seroconversion and may have missed this early window of opportunity. Identifying individuals with PHI poses a major challenge, and clinical and patient awareness as well as development of new incidence tests are crucial if individuals with very early infection are to be targeted. A benefit of SPARTAC is that, although it represents individuals at a later stage of PHI, if SPARTAC does show a positive result it should be possible to translate this strategy into clinical practice using currently available diagnostic tools. Although novel cross sectional incidence assay are currently in development there remain no reliable and easily accessible methods of identifying very early infection (Cohen, et al. 2010)

The main limitation of this thesis is that the SPARTAC study remains blinded at the time of writing. Future analyses will include assessing the predictive value of the biomarkers measured in this thesis, assessing the natural history of biomarker changes in untreated individuals, and assessing the effects of short-course ART across the three arms of SPARTAC. This study also has some key advantages. The bias associated with starting and stopping ART in many observational studies, including the pilot study described in this thesis, is limited by the randomised nature of SPARTAC, and the large numbers of participants included in the study means that the data are powered to clinically relevant endpoints.

Conclusion

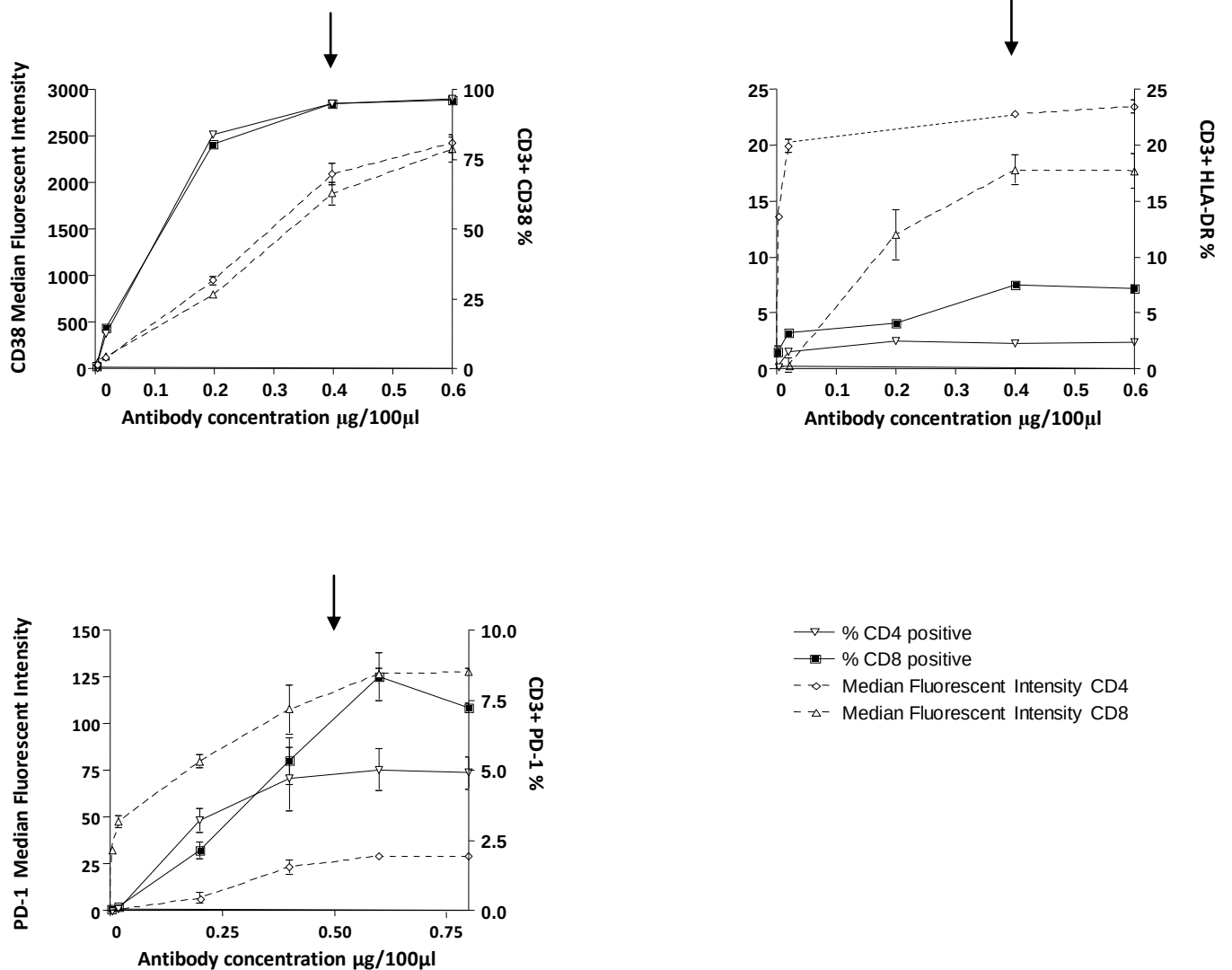
In conclusion, HIV-1 infection is a pro-inflammatory state characterised by chronic immune activation, microbial translocation, endothelial activation and activation of coagulation pathways. These processes play a key role in disease pathogenesis in HIV-1 infected individuals. The concurrence of viral and host factors contribute to the magnitude of immune activation, which itself is a driver of further viral replication. The biomarkers of inflammation, coagulation and immune activation measured in this thesis may provide additional prognostic information to identify individuals at high risk of HIV-1 disease progression or co-morbidities. By examining the natural history of these biomarkers, and understanding the effects of stopping and starting ART, it may be ultimately possible to target interventions designed at limiting these processes. As the HIV-1 infected population continues to age, and the impact of non-opportunistic disease becomes more apparent, an appreciation of these underlying pathogenic mechanisms is critical to enhance prevention, diagnosis and management of co-morbidities, and to optimise the use of current ART agents.

Appendix 1: Summary of SPARTAC study design



A summary of the SPARTAC trial design is shown. Patients with PHI were randomised to one of 3 study arms and followed up for a minimum of 3.5 years. Primary and secondary study endpoints are shown.

Appendix 2: Antibody titrations used to determine the optimal antibody concentrations for flow cytometry



The optimal antibody concentrations for flow cytometry were established using the antibody titrations shown above. Assays were run in triplicate and mean and standards deviation are plotted for each antibody concentration. Chosen concentrations are indicated by the arrow.

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