HIV persistence during antiretroviral therapy:

Characteristics of residual HIV-1 RNA in plasma

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Medicine

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Declaration of Authorship

I declare that this work is my own work except where indicated by references. Work done in collaboration with or with the assistance of others is specified and acknowledged.

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Acknowledgments

Contents

Figures

Tables

Abstract

Anti-retroviral therapy (ART) is highly effective in suppressing human immunodeficiency virus (HIV) and restoring immunocompetence. However, residual levels of HIV-1 RNA persist in patients with consistent viral suppression (viral load <50 copies/mL) and some immune functions are not reversed. Characterisation of residual viraemia (HIV-1 RNA <10 copies/mL) will facilitate to understand HIV persistence.

A systematic review of literature was conducted to identify predictors of low level viraemia (HIV-1 RNA = 10-200 copies/mL) in patients on successful ART. To investigate factors associated with residual viraemia, we recruited a cohort of stably treated patients on first line ART with a non-nucleoside reverse transcriptase inhibitor (NNRTI) or protease inhibitor (PI) based regimen and no evidence of virological failure. We measured HIV-1 RNA in plasma with our in-house single copy assay (SCA) and we studied total HIV-1 DNA in peripheral blood mononuclear cells (PBMC) and PD1 expression on memory CD4 and CD8 T populations. A subset of our study cohort was sampled twice during consecutive routine visits at the HIV clinic to perform a longitudinal analysis of HIV-1 RNA in plasma, total HIV-1 DNA in PBMC and drug levels.

Residual viraemia was identified in over one third (35.9%, n=19 of N=53) of subjects with a median of 3 copies/mL. In the longitudinal cohort (n=32), HIV-1 RNA presence in plasma was reproducible within an interval period of 4 months with 53% (n=17) having undetectable HIV-1 RNA at both time points and 25% (n=8) experiencing consistently detectable HIV-1 RNA. Total HIV-1 DNA in PBMC was strongly associated with residual viraemia (OR: 3.42, 95% CI: 1.32-8.83, p=0.011) in our study population. In the systematic review, low level viraemia was also associated with markers reflecting a bigger cellular reservoir including higher pre-ART viral load, total HIV-1 DNA in PBMC and shorter ART duration. Moreover, low level viraemia was more frequent with reduced adherence and with PI-based ART. The same trends were also seen in our cohort, but the number of patients on PI-based regimens (n=11) and with sub-optimal efavirenz levels (n=6) were small to allow statistical comparisons. Microbial translocation and immune activation were associated with low level viraemia in the review. On the contrary, our patients with undetectable HIV-1 RNA had higher PD1 expression on effector memory CD4 cells (median 43.9% vs. 33.7%, p=0.028),

effector memory CD8 cells (median 37.5% vs. 23%, p=0.022) and central memory CD8 cells (median 24.9% vs. 15.7%, p=0.034) compared to those with detectable HIV-1 RNA.

Our findings suggest that residual viraemia is probably clonal in our cohort of patients deriving mainly during activation of latently infected cells, which causes bursts of virus production. We noted that common factors were associated with both residual and low level viraemia. Although we consider residual and low level viraemia as two different entities, their sources may overlap. In this context, ongoing viral replication may drive residual viraemia in a small subset of patients. We also made an interesting observation as to PD1 expression on memory CD4 and CD8 subsets which warrants further investigation.

List of abbreviations

Ag: antigen

AIDS: acquired immunodeficiency syndrome

Amplicor: Roche Amplicor HIV-1 Monitor Test

APC: antigen-presenting cell

APOBEC: apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like

Aptima: Hologic Aptima HIV-1 Quant Dx assay

ART: anti-retroviral therapy

AZT: zidovudine

BC-CfE: British Columbia Centre for Excellence

BCR: B cell receptor

bDNA: branched-chain DNA

BHIVA: British HIV Association

BLQ: below limit of quantitation

bNAbs: broadly neutralising antibodies

BTLA: B and T lymphocyte attenuator

CAR: chimeric antigen receptor

cART: combined anti-retroviral therapy

CCR5: CC-chemokine receptor 5

CD4: cluster of differentiation 4

CD8: cluster of differentiation 8

CDC: Centers for Disease Control

cDNA: complementary DNA

CI: confidence interval

CNS: central nervous system

cpz: chimpanzees

CRF: circulating recombinant form

CTL: cytotoxic T lymphocyte

CTLA-4: cytotoxic T lymphocyte-associated protein 4

CXCR4: CXC-chemokine receptor 4

CYP3A4: cytochrome P450 3A4

CYP450: cytochrome P450

DHHS: United States Department of Health and Human Services

DMSO: dimethylsulphoxide

DNA: deoxyribonucleic acid

DRC: Democratic Republic of the Congo

DRV: darunavir

EACS: European AIDS Clinical Society

EDTA: ethylenediamine tetraacetic acid

EFV: efevirenz

ELISA: enzyme-linked immunosorbent-based assay

env: envelope

EPSRC: Engineering and Physical Sciences Research Council

FBC: fetal bovine serum

FDA: Food and Drug Administration

FTC: emtricitabine

gag: group-specific antigen

GALT: gut-associated lymphoid tissue

gp: glycoprotein

HAART: highly active anti-retroviral therapy

HBV: hepatitis B virus

HCV: hepatitis C virus

HIV: human immunodeficiency virus

HLA-B*5701: human leukocyte antigen B*5701

HPLC-MS: high-performance liquid chromatography–tandem mass spectrometry

HR: hazard ratio

HSV: herpes simplex virus

HTLV: human T cell leukaemia/lymphoma virus

IC: internal control

ICOS: inducible T cell co-stimulator

IFN: interferon

Ig: immunoglobulin

IL: interleukin

InSTI: integrase strand transfer inhibitor

IPDA: Intact Proviral DNA Assay

IQR: interquartile range

ITIM: immunoreceptor tyrosin-based inhibitory motif

ITISM: immunoreceptor tyrosine-based switch motif

iSCA: integrase single copy assay

kPCR: kinetic PCR

LBP: lipopolysaccharide-binding protein

LCMV: lymphocytic choriomeningitis virus

LLoQ: lower limit of quantitation

LLV: low level viraemia

LoD: limit of detection

LPV: lopinavir

LTR: long terminal repeat

LTRc DNA: LTR-containing circular DNA

M184V: methionine to valine substitution at amino acid position 184

MeSH: medline subject heading

MHC-I: major histocompatibility complex class I

mRNA: messenger ribonucleic acid

MS: multiple sclerosis

MSM: men who have sex with men

N/A: not applicable

NASBA: nucleic acid sequence-based amplification

ND: not detected

nef: negative regulator factor

NFAT: nuclear factor of activated T cells

NFATc1: nuclear factor of activated T cells calcineurin-dependent 1

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

NIHR CRN: National Institute for Health Research Clinical Research Network

NK: natural killer cells

NNRTI: non-nucleoside reverse transcriptase inhibitor

NRES: National Research Ethics Service

NRTI: nucleoside reverse transcriptase inhibitor

OR: odds ratio

ORF: open reading frame

PBMC: peripheral blood mononuclear cells

PBS: phosphate-buffered saline

PD1: programmed death 1

PD1-L1/2: PD1-ligand 1/2

PI: protease inhibitor

PI3K: phosphoinositide-3 kinase

pol: DNA polymerase

PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-Analyses

R5: CCR5 tropic

r: ritonavir

RD&I: Research Development and Innovation

RealTime: Abbott RealTi*me* HIV-1 Viral Load assay

REC: Research Ethics Committees

rev: regulator of expression of virion proteins

RLP: rilpivirine

RNA: ribonucleic acid

RPMI: Roswell Park Memorial Institute

RT: reverse transcriptase

RT-PCR: reverse transcriptase polymerase chain reaction

RV: residual viraemia

p: protein

S0: baseline sampling

S1: second sampling

SCA: single copy assay

SD: standard deviation

SE: standard error

SHP-1/2: Src homology 2 (SH2) domain-containing tyrosine phosphatase 1/2

SIV: simian immunodeficiency virus

SLE: systemic lupus erythematosus

T: temperature

$t_{1/2}$: half-time

T-20: enfuvirtide

TAF: tenofovir alafenamide fumarate

TaqMan v2: Roche COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0

tat: trans-activator of transcription

TCF1: T cell factor 1

TCR: T cell receptor

TDF: tenofovir disoproxil fumarate

Tfh: T follicular helper cell

TMA: transcription-mediated amplification

TND: target not detected

TNFα: tumour necrosis factor α

TRAIL: TNF-related apoptosis-inducing ligand

UD: undetectable

UK: United Kingdom

UNAIDS: Joint United Nations Programme on HIV/AIDS

URF: unique recombinant form

Versant kPCR: Siemens Diagnostics Versant HIV-1 RNA kinetic PCR assay

vif: viral infectivity factor

VL: viral load

VLLV: very low level viraemia

vpr: viral protein R

vpu: virus protein unique

WHO: World Health Organisation

X4: CXCR4 tropic

Xpert: Cepheid Xpert HIV-1 Viral Load assay

CHAPTER 1

Introduction

1.1 Human immunodeficiency virus (HIV)

1.1.1 Classification and origin

In 2019, there were 38.0 million people living with HIV and 690,000 deaths related to acquired immunodeficiency syndrome (AIDS) (UNAIDS, 2020). There are two types of HIV (HIV-1 and HIV-2) which along with the simian immunodeficiency viruses (SIVs) and the human T cell leukaemia/lymphoma viruses (HTLVs) belong to the genus of Lentivirus of the family of *Retroviridae* (ICTV, 2018). HIV-1 is the most pathogenic and easily transmitted type and the main cause of infections worldwide, while HIV-2 is restricted mainly to West Africa (Dougan et al., 2005). HIV-2 will not be further described as it is not part of this work. HIV-1 has four genetically distinct groups: major (M), outlier (O), nonmajor/ nonoutlier (N) and the recently discovered group P, which have derived from independent cross-species transmission events. HIV-1 group M is responsible for the HIV pandemic and has been closely related to SIVcpz strains, isolated from African chimpanzees in southeast Cameroon. It is yet unclear the exact mechanism of the zoonotic transmission, but it is believed that it was passed on to hunters through the bloodborne route around 1920. It was the transfer of the virus to the city of Kinshasa at the Democratic Republic of the Congo (DRC) and the spread through transport networks to other regions of sub-Saharan Africa that transformed an epidemic into a worldwide pandemic. Chimpazees were also the source of group N, whereas gorillas were the source of group O and P (Bbosa et al., 2019).

Viral genetic factors and socio-economic circumstances provided with a favourable advantage group M to spread exponentially around 1960 compared to the confined spread of group O to west-central Africa regions (Faria et al., 2014, Keele et al., 2006, Zhu et al., 1998). M has many subtypes or clades (A, B, C, D, F, G, H, J, K and L) from which subtype C

accounted for almost half (47%) of all HIV-1 infections in the period from 2010 to 2015 (Hemelaar et al., 2019). Subtypes emerge by genetical evolution (founder events) and differ in the amino acids in the envelope gene (env) and group-specific antigen (gag) gene by 30% and 15%, respectively, whereas the genetic distance within a subtype can be between 15% and 20%. Co-infection or super-infection with multiple strains in a subject can result in the emergence of a recombinant which has a mosaic genome composed of sequences from two or more distinct parental strains. Circulating recombinant forms (CRFs) are transmitted and spread within a population, whereas unique recombinant forms (URFs) are found only in an individual (Foley et al., 2018). There is significant increase in genetic diversity and prevalence of recombinants with over 100 CRFs reported to date (LANL, 2020). Subtype B dominates in Europe and America, whereas subtypes A, C and CRF02_AG are the most prevalent in Africa and CRF01_AE is mostly found in Asia (Bbosa et al., 2019). HIV diversity is constantly evolving with emergence of new strains and there has been an overall increase in the proportion of recombinant forms which accounted for almost a quarter (23%) of the global infections in the period between 2010 and 2015 (Hemelaar et al., 2019, Yamaguchi et al., 2020).

1.1.2 Structural and functional properties

HIV-1 is a round particle 100 nm in diameter which consists of three main structures: the envelope, the capsid and two molecules of single stranded ribonucleic acid (RNA). The envelope is an outer lipid bilayer membrane containing two viral glycoproteins (gp), the surface gp120 and the transmembrane gp41. Protein 24 (p24) is the main component of the conical capsid, which is covered by the matrix protein (p17). RNA is housed in two copies in the capsid along with important enzymes: protease, reverse transcriptase (RT), and integrase (Gelderblom, 1991). The HIV-1 genome is 9.2 kb in size and contains 9 open reading frames (ORF) encoding for 15 proteins. At the 5' end is a long terminal repeat (LTR) followed by the gag gene, which encodes for the matrix and capsid proteins, and DNA polymerase (pol) gene, which is transcribed into the viral enzymes. At the 3' end is also an LTR followed by the env gene encoding the precursor of envelope glycoproteins (gp160). ORFs are found between pol and the 3' LTR and encode regulatory and accessory proteins that are important for gene expression and virus production (Figure 1.1). Of note, negative regulator factor (nef) and virus protein unique (vpu) facilitate immune escape by downregulating the expression of major histocompatibility complex class I (MHC-I) and

cluster of differentiation 4 (CD4) receptor in host cells (Deeks et al., 2015, Ghosn et al., 2018).

1.1.3 Viral replication cycle

The human infectious dose of HIV-1 is estimated at 500-1,000 particles by any route of exposure (Zanetti et al., 2007). However, only one particle is required to enter a target cell and initiate recurrent cycles of replication for infection to be established.

Gp120 binds to the CD4 receptor expressed on T lymphocytes, monocytes, macrophages, dendritic cells and astrocytes. This initial interaction drives a conformational change of gp120 with exposure of the V3 loop and additional binding sites which engage mainly the CC-chemokine receptor 5 (CCR5) found on memory T lymphocytes, macrophages and dendritic cells or alternatively the CXC-chemokine receptor 4 (CXCR4) on memory and naïve T cells. Based on preferential coreceptor use, the viral strain is defined as CCR5 tropic (R5), CXCR4 tropic (X4) or dual tropic (R5/X4). Co-receptor engagement is of paramount importance in the establishment of HIV infection and the transmitted virus is almost invariably a R5, since mucosal CD4 T cells express high levels of CCR5. A homozygous deletion of the Δ32 region of CCR5 gene offers natural resistance to infection with HIV-1 (Allers and Schneider, 2015). During the course of infection, there may be a shift from CCR5-tropic to CXCR4-tropic in some individuals (Deeks et al., 2015).

Co-receptor engagement following the interaction between gp120 and the CD4 receptor results in fusion of viral gp41 into the cell membrane. Conformational changes within gp41 including a critical folding step and the formation of a six helical hairpin-like structure brings the host cell membrane and viral envelope in close proximity. Host and viral membranes fuse with the formation of a pore through which the capsid containing viral RNA and enzymes enters the cell (Engelman and Cherepanov, 2012).

The capsid shell then disassembles through a process called uncoating. This stage of the replication cycle is the most poorly understood. Three models propose that uncoating occurs immediately on viral entry, gradually as the virus moves from the entry point to the nucleus or before entering the nuclear pore (Campbell and Hope, 2015). Recent evidence shows that microtubule stabilisation is critical for virus capsid transport and release of its contents (Delaney et al., 2017).

Following uncoating reverse transcription of the HIV-1 RNA into double stranded deoxyribonucleic acid (DNA) occurs. RT has three enzymatic activities. First the RNA-dependent DNA polymerase uses the viral RNA as template to synthesise a complementary DNA (cDNA) strand. Then the RNA strand is degraded with the RNAse H function. Eventually, the DNA-dependent DNA polymerase converts the single stranded cDNA to double helix DNA (Bhagavan and Ha, 2015). Of note is that cDNA is not an exact replicate of the original RNA due to a high error rate of RT with a frequency of 10^{-4} to 10^{-5} and lack of proof-reading ability. The error-prone transcription process results in the generation of mutant variants called *quasispecies*. A portion will escape immunological protective mechanisms and will become dominant. Others will have an impaired replication machinery resulting in reduced fitness and will either vanish or replicate at low level (Malim and Emerman, 2001). RT is also responsible for the generation of recombinant variants by its ability to switch from one RNA template to another in cells infected with different viruses (Delviks-Frankenberry et al., 2011). Therefore, genetic diversity, a key characteristic of HIV infection and persistence, emerges mainly at the stage of cDNA generation. However, mutations are not only attributed to RT activity. The apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC) family, a host nucleic acid editing enzyme, adds to the genetic diversity by switching cytosine with thymine. Mutations occurring at other stages of the viral lifecycle, such as during viral gene transcription by host cellular RNA polymerase II, contribute less to genetic variation (Smyth et al., 2012).

cDNA accompanied by viral and cellular proteins called the pre-integration complex is transferred to the nucleus. The complex enters the nucleus through pores and HIV DNA is integrated in a random site of host DNA forming the provirus. Integrase catalyses the release of a dinucleotide from the 3' end of the HIV DNA exposing a conserved oligonucleotide region on the complementary strand (3' processing). During a second reaction (strand transfer), integrase assists the formation of a covalent bond between the exposed viral region and host nuclear DNA (Craigie, 2012).

The vast majority of cDNA is unintegrated (episomal DNA) and forms into a circle with one or two LTR regions (1- or 2-LTRc DNA). Linear episomal DNA survives for up to one month before being degraded, whereas LTRc DNAs are regarded as more stable. Circular DNAs have been recently reported to contribute to HIV protein expression, such as nef and trans-activator of transcription (tat). Although the levels of expression are very low, they are sufficient to trigger immune responses (Thierry et al., 2016).

In activated cells, viral gene expression is driven by host nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and nuclear factor of activated T cells (NFAT) and a promoter at 5' LTR region of the viral DNA (Pessler and Cron, 2004, Stroud et al., 2009). The integrated viral DNA is transcribed into genomic and messenger ribonucleic acid (mRNA) by cellular enzymes the same way as host genes. mRNA is translated into viral proteins at cellular ribosomes and the accruing proteins depend on the degree of splicing. Unspliced mRNA produces the gag and gag-pol polyprotein which contain structural proteins (p17, p24, nucleocapsid or p7 and p6) and enzymes (protease, RT and integrase), respectively. Partially spliced mRNAs encode envelope and accessory (viral infectivity factor (vif) and vpu) proteins and fully spliced transcripts encode the regulator of expression of virion proteins (rev), tat, viral protein R (vpr) and nef. Viral particles are transformed into mature and infectious virions by protease-mediated cleavage of the gag-pol and gag polyprotein transcripts sequentially into nine sites. This action yields all important enzymes for HIV replication including RT, integrase and protease and a structural transformation to form the conical capsid core. Genomic RNA and viral precursor proteins assemble in the cytoplasm. Envelope proteins are transported to the cellular membrane and the viral envelope is acquired during budding (Dunn et al., 2002, Pedersen et al., 2011).

1.1.4 Pathogenesis

HIV is transmitted through body fluids such as blood, genital secretions and breast milk. Blood transfusion and mother-to-child transmission from an HIV infected source have the highest risk of HIV acquisition (9,250 infections and 2,260 infections per 10,000 contacts, respectively). However, these risks have nearly been eliminated due to laboratory testing of donated blood products and use of prophylactic anti-retroviral therapy (ART), respectively. Sexual exposure is the most frequent mode of transmission with HIV acquisition among discordant heterosexual couples accounting for nearly 70% of all infections globally. Receptive anal intercourse carries the highest risk (138 infections per 10,000 contacts) followed by injection drug use with sharing equipment (63 infections per 10,000 contacts) (Patel et al., 2014). Preventive strategies include the use of condoms, pre- and post-exposure prophylaxis with ART, male circumcision and above all early diagnosis and treatment initiation of those infected (Crepaz et al., 2015, Marrazzo, 2017, Rodger et al., 2019, Rodger et al., 2016).

Effective transmission is instigated by a single founder virus, or a few genetic variants, which is fit enough to establish a productive infection. The virus spreads rapidly after transmission and establishes latent infection at the site of infection and then draining lymph nodes and eventually distal lymph nodes and mucosal-associated lymphoid tissue (Ananworanich et al., 2016, Keele et al., 2008a). The SIV macaque model has demonstrated that the latent reservoir, which is responsible for persistence despite effective treatment, is established in the first three days after infection (Whitney et al., 2014). HIV-1 RNA is detected in plasma around day 10 post infection (Fiebig I) and peaks in the following 2-4 weeks, coinciding with a transient reduction in CD4 T cell counts. Testing for p24 antigen can give a diagnosis of HIV around day 17 post infection (Fiebig II) (Fiebig et al., 2003). An immune response is initiated and seroconversion occurs with the clinical detection of antibodies against HIV around day 22 (Fiebig III). This is often accompanied by a non-specific clinical syndrome comprising fatigue, fever, rash, lymphadenopathy, headache and diarrhoea. Onset of immune responses is accompanied by a reduction in viraemia and partial recovery of CD4 cell counts.

At this point viral reservoirs are well-established within memory CD4 T cells, naïve CD4 T cells, cells of the monocyte and macrophage lineage and potentially other long-lived cells. Viral gene transcription is silenced in latently infected cells by complex mechanisms such as transition to a resting state and expression of immune checkpoints which down-regulate cellular activity (Ghosn et al., 2018).

The levels of HIV-1 RNA in plasma – described as the viral load – decline to a semi-stable level within the first 6 months of the infection. The viral set point varies among individuals from a few copies to 10^6 copies per mL of blood depending on viral and host factors. In untreated patients, the viral load set point is predictive of the rate of disease progression (Deeks et al., 2015). Primary HIV infection is followed by the clinically asymptomatic phase, which is characterised by ongoing virus replication and chronic immune activation. These processes gradually lead to a progressive exhaustion of immune responses which is reflected in a gradual decrease of the CD4 cell count at a rate predicted by the viral set point (Mellors et al., 1996). Eventually, severe immunodeficiency occurs, typically around 8 years from initial infection and once CD4 cell count drops below 200 cells/mL. At the final stage of AIDS there is an increase in viral load and a profound clinical deterioration characterised by recurrent infections, cancers, cachexia and eventually death (Pantaleo et al., 1993).

The lag in time between transmission and antibody seroconversion can result in missed diagnoses if antibody-based assays are used (Geretti, 2017). Fourth generation tests detect

p24 antigen and antibodies (immunoglobulin (Ig) M and G) decreasing the period between infection and a positive result to 2-3 weeks. Earlier diagnosis can be achieved with RNA detection in the plasma with reverse transcriptase polymerase chain reaction (RT-PCR) assays around 10 days after infection (Delaney et al., 2016) (Figure 1.2).

1.1.5 Anti-retroviral therapy

Since Food and Drug Administration (FDA) approval of zidovudine (AZT), the first anti-retroviral drug, in 1987, enormous advances have been achieved in HIV treatment, increasing substantially life expectancy of infected individuals (FDA, 2018, Mocroft et al., 2003, Samji et al., 2013). Most recent data reveal that timely diagnosis and lifelong adherence to current drugs ensure a life expectancy almost the same as the healthy population (Trickey et al., 2017). Combination ART has led to an era of clinically sustained virological suppression for patients who remain on therapy continuously and without interruptions (Geretti and Tsakiroglou, 2014). Yet, immunological damage that occurred early in the course of infection is not completely reversed and persists despite long-term treatment (Deeks et al., 2015). Seven classes of antiretrovirals block various steps of the viral replication cycle (Table 1.1).

Inhibition of HIV entry in host cells is achieved by compounds that interact with proteins on target cells or HIV particles which are involved in attachment, co-receptor binding and fusion (Kuritzkes, 2009). Maraviroc is a CCR5 antagonist that binds to the transmembrane region of the CCR5 preventing the interactions between the V3 loop of gp120 and the host cell co-receptor. Maraviroc is active against R5 virus and tropism should be determined prior to use. Yet, failure of maraviroc may occur if X4 or R5/X4 *quasispecies* pre-exist and become dominant under pharmacological pressure. Otherwise, mutations causing resistance to maraviroc in R5 virus are rare. It is metabolised by cytochrome P450 and dose adjustments are required with concurrent use of CYP450 inducers or inhibitors. Maraviroc is well-tolerated and the most frequent side effect is postural hypotension. It has been proposed to have immunomodulatory properties and, hence, trialled in inflammatory conditions, such as rheumatoid arthritis and non-alcoholic liver disease (Lewis et al., 2018). Enfuvirtide (T-20) is a fusion inhibitor targeting gp41. It is administered subcutaneously twice daily and injection site reactions are very common. The humanised monoclonal antibody ibalizumab is a post-attachment inhibitor which binds on CD4 receptors hindering interaction between CD4-bound gp120 and CCR5 or CXCR4 (Beccari et al., 2019, Kuritzkes, 2009). More entry

inhibitors (e.g. fostemsavir, leronlimab) are under development or in clinical trials (Cahn et al., 2018, Thompson, 2018). Guidelines reserve entry inhibitors for patients with multiple drug resistance or poor tolerability (DHHS, 2018, EACS, 2018).

Nucleoside and non-nucleoside reverse transcriptase inhibitors (NRTI and NNRTI) interact with RT inhibiting virus RNA conversion to cDNA (Table 1.1). NRTIs are nucleoside and nucleotide analogues which undergo phosphorylation by host enzymes into active triphosphate and diphosphate derivates, respectively. These active forms incorporate into nascent cDNA preventing further nucleotide addition and terminating the extension of the chain. Many NRTI resistance mutations impair virus fitness and antiviral activity is maintained. Also, cross-resistance among NRTIs does not always occur. A methionine to valine substitution at amino acid position 184 (M184V), for instance, reduces binding of the NRTI. M184V is selected by lamivudine, emtricitabine and abacavir causing high-level resistance to lamivudine and emtricitabine, but limited resistance to abacavir and increased susceptibility to tenofovir and zidovudine. Tenofovir is the most widely used NRTI and it is formulated as two prodrugs, tenofovir disoproxil fumarate (TDF) and the most recently developed tenofovir alafenamide fumarate (TAF). A combination of tenofovir with emtricitabine and a third agent is an effective first-line regimen. NRTI side effect profile includes peripheral neuropathy, lipoatrophy, pancreatitis and hepatic steatosis which are the consequences of mitochondrial toxicity and more frequent with older NRTIs such as stavudine (Lewis et al., 2003). Tenofovir can cause proximal tubulopathy with a broad spectrum of presentations (e.g. acute kidney injury, Fanconi syndrome, nephrogenic diabetes insipidus) particularly in patients with pre-existing kidney dysfunction. Also, patients on TDF may experience decreased bone mineral density (Bedimo et al., 2016). TAF has improved intracellular bioavailability and renal and bone toxicity profile compared to TDF (Sax et al., 2015), but appears to be associated with weight gain (Taramasso et al., 2020). Abacavir in combination with lamivudine and dolutegravir is an effective and frequently used regimen. Between 5% and 8% of patients develop a hypersensitive reaction which can be predicted by testing positive for human leukocyte antigen B*5701 (HLA-B*5701). Abacavir has also been associated with cardiovascular disease in patients with increased risk (Hetherington et al., 2001, Sabin et al., 2008).

NNRTIs bind to a pocket near the active site of RT causing a conformational change which impairs its enzymatic activity. NNRTIs have a low genetic barrier to resistance and significant cross-resistance. Older NNRTIs are nevirapine and efavirenz and more recent are etravirine and rilpivirine which were followed by doravirine. Efavirenz was the preferred third agent in

first-line ART but its central nervous system (CNS) toxicity and the introduction of integrase strand transfer inhibitors (InSTIs) resulted in being phased out. Rilpivirine, on the other hand, is better tolerated than efavirenz. Due to increased risk of virological failure and emergence of cross-resistance mutations with higher levels of HIV-1 RNA in plasma, rilpivirine is recommended only for patients with viral load less than 100,000 copies/mL (Sanford, 2012). Doravirine is expected to overcome these drawbacks and may retain activity in the presence of some resistant mutations (Cohen et al., 2013, Colombier and Molina, 2018). NNRTIs have a long plasma half-life and drug levels are maintained therapeutic if a dose is missed occasionally. However, resistance mutations are easily developed if triple ART is discontinued as they remain for longer periods in blood (Geretti et al., 2013b). Common side effects resulting in drug class switch are neuropsychiatric disorders (e.g. vivid dreams, confusion), lipid abnormalities, hepatoxicity, skin rash, nausea and prolonged QT interval.

InSTIs block integrase from joining the processed viral DNA ends in the host genome by binding to the pre-integration complex probably before it enters the nucleus (Pandey and Grandgenett, 2008). Second generation InSTIs, such as dolutegravir and bictegravir have a higher genetic barrier to resistance compared to raltegravir and elvitegravir (first generation InSTIs) and are currently the preferred choice for treatment initiation (Blanco et al., 2011, Doyle et al., 2015, Oliveira et al., 2018). Cabotegravir, an analogue of dolutegravir, is currently in phase 3 trials. Its long-acting nanoparticle injection formulation combined with long-acting rilpivirine is the most advanced long-acting regimen (Stellbrink and Hoffmann, 2018). Dolutegravir is currently the preferred third agent for first-line ART (DHHS, 2018, EACS, 2018), because comparative studies have demonstrated its superiority over efavirenz or boosted darunavir based regimens with improved safety profile and rapid viral load suppression (Osterholzer and Goldman, 2014). The most frequent side effects include weight gain, insomnia and neuropsychiatric disorders.

Protease inhibitors (PIs) stop particle maturation through competitive binding to the viral protease and prevention of subsequent cleavage of gag and gag-pol polypeptides (Voshavar, 2019). Ritonavir and cobicistat are CYP3A4 inhibitors and are used as pharmacokinetic enhancers for PIs (and elvitegravir), which are metabolised by CYP450 (Larson et al., 2014). Boosted darunavir and atazanavir are currently the recommended PIs used in combination regimens or as stand-alone drugs (DHHS, 2018). They may not be first-line options due to their increased risk of side effects and drug-drug interactions, but their high barrier to resistance makes them the cornerstone in patients with drug mutations and adherence

issues (Geretti et al., 2019). PIs have a marked profile of side effects including metabolic dysfunction (e.g. insulin resistance, hyperlipidaemia) and increased risk of cardiovascular disease, gastrointestinal side effects, hepatotoxicity, lipodystrophy and PR interval prolongation (Echecopar-Sabogal et al., 2018). Atazanavir and darunavir are probably the most well-tolerated PIs. However, atazanavir can cause renal toxicity and hyperbilirubinemia (Voshavar, 2019). Drug-drug interactions are also common with PIs. For instance, absorption is decreased with co-administration of stomach acid lowering drugs.

Standard ART usually consists of a combination of two NRTIs, referred to as the "backbone", and a third agent. Currently the preferred third agent is an InSTI (dolutegravir), which has replaced NNRTIs or PIs from first-line options (DHHS, 2018). Therapy choice depends on pharmacological and pharmacodynamic properties (e.g. PIs have high genetic barrier to resistance), individual circumstances (e.g. PIs are preferred if adherence is an issue), virological characteristics (e.g. NNRTI mutations causing drug resistance are frequent), tolerability (e.g. patients may become intolerant to efavirenz due to neurological toxicity) and ease of use (e.g. single tablet once daily formulations) (Yombi and Pozniak, 2016) .

Since ART is life-long, minimising drug exposure, risk of toxicity and cost are paramount aspects which make dual therapy appealing (Achhra et al., 2016). A meta-analysis of randomised controlled trials comparing dolutegravir/lamivudine with triple agent regimes for ART initiation revealed similar virological efficacy over a period of 48 weeks (Radford et al., 2019b). Two-drug combinations have also been tested as a simplification strategy in stably suppressed patients on triple ART (Achhra et al., 2016, Llibre et al., 2018). Dolutegravir/rilpivirine has been shown to be a good alternative for maintenance of suppression while lamivudine in combination with dolutegravir, boosted atazanavir or boosted darunavir is also supported by good evidence (Calza et al., 2018, Llibre et al., 2018, Radford et al., 2019a). European and North American guidelines have adapted dolutegravir/lamivudine and dolutegravir/rilpivirine for first line therapy and treatment switch, respectively, in selected adults (DHHS, 2018, EACS, 2018). Nonetheless, long-term data and larger studies are required to confirm the efficacy of various 2-drug combinations.

ART can successfully suppress viral replication, but it cannot eradicate HIV. A significant decrease in plasma HIV-1 RNA levels is noted to a level below 50 copies/mL within six months after ART initiation, with subsequent immune reconstitution (Maldarelli et al., 2007, Perelson et al., 1997). However, virus rebound to pre-treatment levels occurs nearly always in case of ART discontinuation (Bailey et al., 2006, Chun et al., 2010, Harrigan et al., 1999,

Jubault et al., 1998, Maggiolo et al., 2012, Palmer et al., 2008). The reason is that latently infected reservoirs established early during infection are not targeted by ART (Chun et al., 2011, Chun et al., 1997, Finzi et al., 1997, Palmer et al., 2011, Ruggiero et al., 2015, Shen and Siliciano, 2008b, Siliciano et al., 2003). Proliferation of HIV-harbouring cells maintains the reservoirs and it can be antigen-driven, homeostatic through cytokine pathways or due to activation of genes controlling cell cycle progression by integrated HIV sequences (Chomont et al., 2009, Maldarelli et al., 2014, Simonetti et al., 2016, Wagner et al., 2014, Wang et al., 2018).

ART preserves and partly restores immune functions, reducing chronic T cell activation and inflammation. As a result, the risk of both AIDS-defining and non-AIDS illnesses (e.g., cardiovascular disease) is minimised (Appay and Kelleher, 2016, Nou et al., 2016, Younas et al., 2016). Moreover, the absence of seroconversion among HIV discordant couples where the positive partner is under treatment indicates the important role of early ART initiation in transmission and control of the HIV pandemic (Anglemyer et al., 2011, Rodger et al., 2019). HIV infection in countries with widely available access to ART is a chronic, usually well-controlled disease, as long as compliance with therapy is maintained. In 2017, the United Kingdom (UK) met the Joint United Nations Programme on HIV/AIDS (UNAIDS) 90-90-90 target with 92% of infected people being diagnosed, 98% of people diagnosed with HIV being on treatment and 97% of those on treatment being suppressed (PHE, 2018).

Figure 1.1: HIV-1 structure at the level of genome and particle (Musumeci et al., 2015).
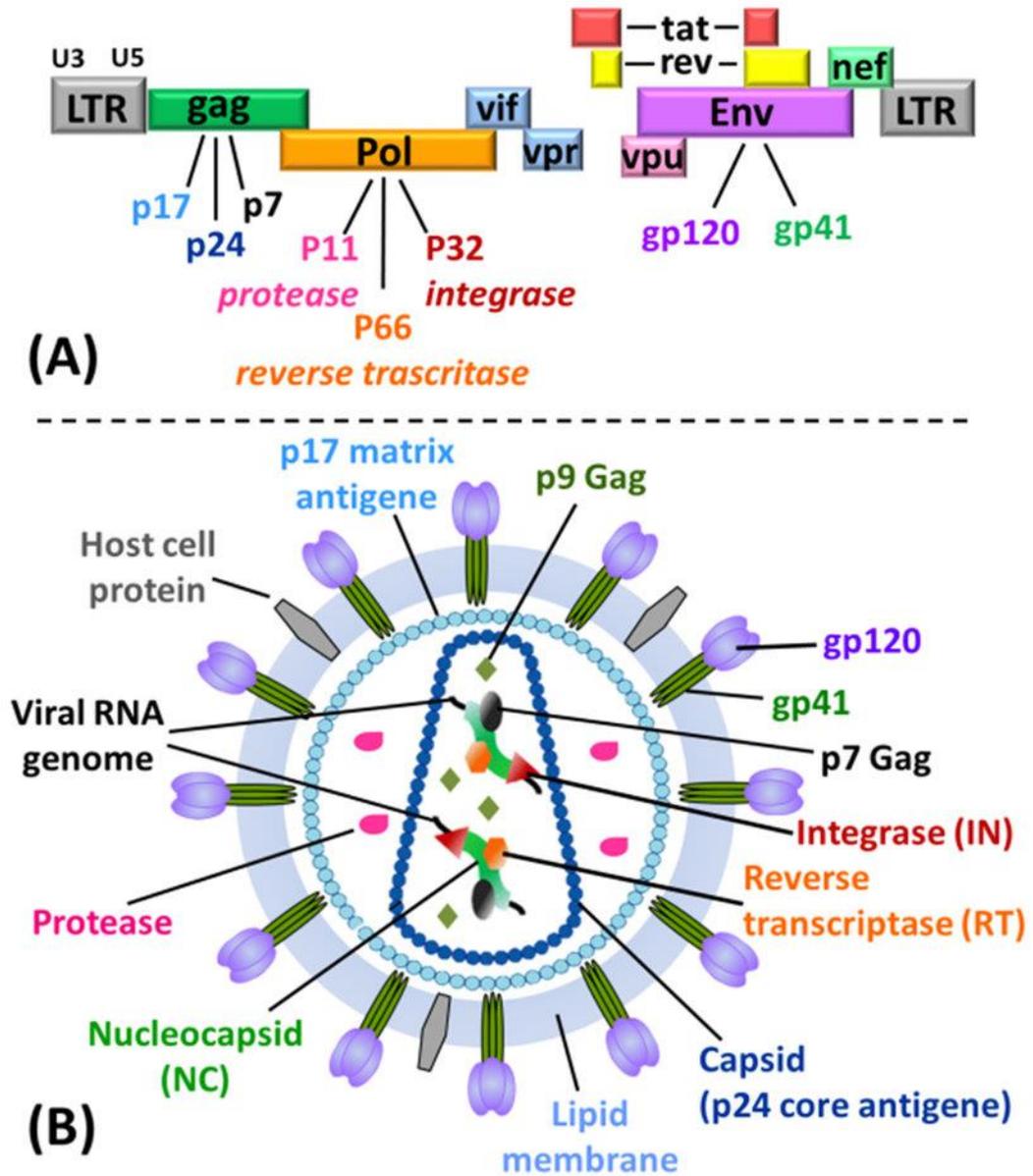
LTR: long terminal repeat; gag: group-specific antigen; p: protein; pol: DNA polymerase; vif: virion infectivity factor; vpr: viral protein R; tat: trans-activator of transcription; rev: regulator of expression of virion proteins; env: envelope; vpu: viral protein unique; gp: glycoprotein; nef: negative factor

Figure 1.2: Immune response to HIV infection (McMichael et al., 2010).
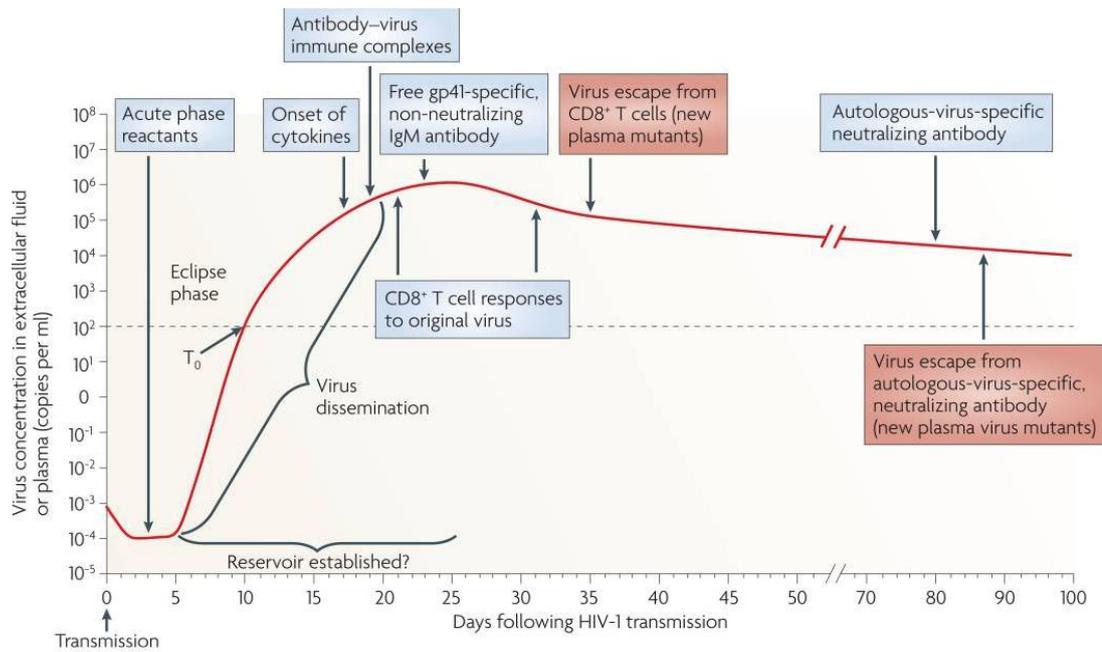
During the eclipse phase HIV-1 is replicating at the mucosal tissue and draining lymph nodes causing an increase in acute phase proteins. Around day 10 (T_0) HIV-1 RNA can be detected in the plasma and there is an interferon response to the spread of the virus. As HIV-1 RNA levels increase exponentially antibody and CD8 cytotoxic T lymphocyte responses take place which can control viral replication only partially due to rapid viral escape. Eventually complex virus-host interactions create a steady level of viraemia.

Table 1.1: Anti-retroviral classes and drugs (AIDSinfo, 2019).

Drug Class	Agents (other names and acronyms)	Trade name
Nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs)	abacavir (abacavir sulfate, ABC)	Ziagen
	emtricitabine (FTC)	Emtriva
	lamivudine (3TC)	Epivir
	tenofovir disoproxil fumarate (tenofovir DF, TDF)	Viread
	tenofovir alafenamide (TAF)	Vemlidy[1]
	zidovudine (azidothymidine, AZT, ZDV)	Retrovir
Non-nucleoside reverse transcriptase inhibitors (NNRTIs)	doravirine (DOR)	Pifeltro
	efavirenz (EFV)	Sustiva
	etravirine (ETR)	Intelence
	nevirapine (NVP)	Viramune
	rilpivirine (rilpivirine hydrochloride, RPV)	Edurant
Protease inhibitors (PIs)	atazanavir (atazanavir sulfate, ATV)	Reyataz
	darunavir (darunavir ethanolate, DRV)	Prezista
	fosamprenavir (fosamprenavir calcium, FOS-APV, FPV)	Lexiva
	lopinavir (LPV)	Kaletra[2]
	ritonavir (RTV, r)[3]	Norvir
	saquinavir (saquinavir mesylate, SQV)	Invirase
	tipranavir (TPV)	Aptivus
Fusion inhibitors	enfuvirtide (T-20)	Fuzeon
CCR5 antagonists	maraviroc (MVC)	Selzentry
Integrase strand transfer inhibitors (InSTIs)	bictegravir (bictegravir sodium, BIC)	Biktarvy[4]
	dolutegravir (dolutegravir sodium, DTG)	Tivicay
	elvitegravir (EVG)	Vitekta
	raltegravir (raltegravir potassium, RAL)	Isentress
Attachment inhibitors	ibalizumab (Hu5A8, IBA, Ibalizumab, TMB-355, TNX-355)	Trogarzo

Drug Class	Agents (other names and acronyms)	Trade name
Pharmacokinetic enhancers	cobicistat (COBI, c)	Tybost

1: Stand-alone TAF is not FDA approved for HIV but is approved for treating chronic HBV infection

2: Lopinavir in combination with ritovavir

3: Ritonavir is a PI which is used as a pharmacokinetic enhancer for other protease inhibitors

4: Biktarvy is a combination drug of bictegravir/emtricitabine/tenofovir alafenamide

1.2 Viral load monitoring

1.2.1 Viral load

Viral load, defined as HIV-1 RNA copies per mL of plasma, was early identified as a measurable predictor of clinical outcomes including rate of CD4 decrease, progression to AIDS and risk of death (Mellors et al., 1997). Viral load, in conjunction with CD4 counts, was originally used for prognosis and guided ART initiation and monitoring (Saag, 1997, Wei et al., 1995, Young et al., 2015). The target was to suppress viral load at levels below the assay quantification limits which were around 400 copies/mL for first generation assays (Churchill and Weber, 1999, Saag et al., 1996). Quantification of HIV-1 RNA levels in plasma can predict treatment failure earlier than CD4 count and it is not required to be performed more than twice annually in stable patients (BHIVA, 2019, DHHS, 2018, EACS, 2018, Geretti, 2009, Young et al., 2015). Eventually viral load became the gold standard and only surrogate marker for monitoring long-term clinical response to therapy (BHIVA, 2019, DHHS, 2018, EACS, 2018) and CD4 count measurements are performed less frequently. Viral load serves as an indicator of virus control and risk of transmission (Quinn et al., 2000, Rodger et al., 2019, Rodger et al., 2016).

Samples can be easily obtained, transported and processed on automated platforms. The half-life of HIV in plasma at body temperature is two days, but this increases at lower temperatures (Moudgil and Daar, 1993). Hence, transport of whole blood in ethylenediamine tetraacetic acid (EDTA) tubes allows 72 hours of RNA stability at room temperature with minimal degradation (Bonner et al., 2014). However, samples from patients with viraemia <200 copies/mL should be processed within 4-8 hours after collection to avoid cell lysis. Blood cells infected with HIV-1 can release viral DNA upon lysis and if the assay employs total nucleic acid extraction method, contaminant HIV-1 DNA can be measured along with RNA during the amplification step (Fernandes et al., 2010).

Technical characteristics and accuracy of clinical assays has improved significantly over time with lower limits of quantification reducing from 400 copies/mL with first generation assays to 50 copies/mL with second generations assays and to 40 and 20 copies with the most popular third generation assays traded by Abbott (RealTi*me* HIV-1 Viral Load assay, which will be referred to as RealTime in this work) and Roche (COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0, which will be referred to as TaqMan v2), respectively (Doyle and

Geretti, 2012). Four major technologies have been developed for viral load measuring: RT-PCR, nucleic acid sequence-based amplification (NASBA), branched-chain DNA (bDNA) and the most recently FDA approved transcription-mediated amplification (TMA). Currently real-time RT-PCR and TMA remain in commercial use and will be described in more details below, whereas NASBA and bDNA are phased out. Overall commercial viral load assays have similar accuracy, quantitation range and technical characteristics (Wang et al., 2010). Studies comparing the two most widely used third-generation assays (the RealTime and TaqMan v2.0) show good correlation for all HIV-1 subtypes. However, discordances occur for low level viraemia and around the lower quantitation limit (Doyle and Geretti, 2012).

1.2.2 Commercial assays used in clinical settings

1.2.2.1 RT-PCR

FDA approved the Roche Amplicor HIV-1 Monitor Test (referred to as Amplicor) for quantitation of viral load in the summer of 1996 (FDA, 2018). Since then RT-PCR for HIV-1 RNA detection and quantitation is the gold standard clinical tool for viral load monitoring in a two-step enzymatic process (RT and PCR step). Primers, nucleotides, enzymes and template RNA are mixed together and undergo one thermal cycle of annealing and extending that derives the cDNA. Purified HIV-1 RNA is used as template to be converted to a more stable form of cDNA. Then cDNA is amplified through recurrent thermal cycles (30-40) of template denaturation (temperature (T) $>90^{0}$C), primer annealing (T: 50-75^{0}C) and elongation (T: 72-78^{0}C) to exponentially produce the amplicon of a DNA region (Mackay et al., 2002). Primers choice is probably the most important aspect in design, as they should achieve template specificity, thermodynamic stability and functional agreement in the mixture (Farrell, 2010). Quantitation of the amplicon happens as a fluorescence signal accumulates with nucleotide consumption (real-time). Meaningful results accrue from absolute or relative quantitation, where a standard curve or a control sample are used as reference, respectively (Farrell, 2010b). Assays report a lower limit of quantitation (LLoQ) and a limit of detection (LoD) (CLSI, 2012). LLoQ is the lowest concentration of HIV-1 RNA that can be reliably quantified within a total error of usually 1 log copies/mL. The LLoQ is determined by testing serial dilutions of the 3rd HIV-1 World Health Organisation (WHO) International Standard (subtype B) and it is verified across subtypes and groups. The highest LLoQ for all subtypes and groups is chosen. The LoD is the concentration of HIV-1 RNA that is detected with at least 95% probability and it is determined in a similar way as the LLoQ

through Probit analysis. A value is reported for samples with HIV-1 RNA levels equal or above the LLoQ and a qualitative result ("target detected") is returned for samples with viral load between the LLoQ and LoD. Viral load is reported as "target not detected" if it is below the LoD. Real-time RT-PCR reduces process time, has good sensitivity and precision and minimises contamination risk (Mackay et al., 2002).

The Amplicor assay utilised end-point RT-PCR technology by targeting the gag region of HIV-1 genome with a single enzyme for both the conversion and amplification steps (Mulder et al., 1994). It had an upper limit of quantitation at 10,000,000 copies/mL and a LLoQ at 400 copies/mL with version 1.5 or 50 copies/mL with the ultrasensitive assay (Sun et al., 1998). The introduction of the COBAS® AmpliPrep instrument upgraded the assay (COBAS® AmpliPrep/COBAS® Amplicor HIV-1 Monitor Test, version 1.5) by performing a fully automated HIV-1 RNA extraction and purification from plasma samples (COBAS AmpliPrep platform) (Germer et al., 2007). End-point assays dominated at clinical laboratories for over a decade until 2008 when the replacement by real-time RT-PCR started (de Mendoza and Soriano, 2009, Holodniy, 2006). Yet, most data demonstrating ART efficacy and the predictive role of viral load suppression accrued with the utilisation of the Amplicor technology.

The COBAS® rival AmpliPrep/TaqMan® HIV-1 Test utilised real-time RT-PCR technology with earlier versions targeting highly conserved regions within the gag gene and a LLoQ at 40 copies/mL. However, studies showed significant bias in reported values with mean differences exceeding $1 \log_{10}$ copies/mL when compared with viral load testing by other real-time platforms such as the RealTime (Wirden et al., 2011). Roche identified that genetic variability for group M and non-B subtypes resulted in primer or probe mismatches and underestimation. Hence, they upgraded to version 2.0 which incorporates a dual target strategy amplifying highly conserved gag and LTR sequences and achieving a LLoQ at 20 copies/mL (Roche Molecular Systems, 2018, Schumacher et al., 2007). TaqMan probes (25-30 nucleotides) are single stranded and carry a fluorescence reporter at 5' end and a quencher absorber at 3' end. The probe hybridizes with its complimentary DNA sequence and it is cleaved by a DNA polymerase which disassociates the fluorescence reporter from the quencher with signal emission. During an effective process the signal aggregates with a rate proportional to the amount of the original target RNA and the machine records the cycle at which it overcomes background (C_T). The C_T values are used in a calculation with the C_T values from internal standards (and competitive control as indicator of PCR inhibition) to report viral load in copies/mL (Liegler TJ and RM, 2009).

Abbott entered commercial competition with the RealTime which also adapts a fully automated extraction process to purify RNA from whole blood samples (m2000sp platform). The principle in methodology is similar to Roche assays with conversion of RNA to cDNA, amplification and fluorescent detection. However, Abbott technology targets a highly conserved integrase region within the pol gene. Abbott probe is also partially double stranded with reporter and quencher being on each strand and signal emission occurring upon uncoupling. The rationale is to allow hybridization at lower temperatures with tolerance to mismatches and genetic variability (Johanson et al., 2001). Also, Abbott uses a non-competitive internal control (hydroxypyruvate reductase of pumpkin) to indicate PCR inhibition events. As to method of quantitation, a stored standard curve, calibrated at each run, is used in a linear regression model with the input of the C_T value for each sample to return the quantity of HIV-1 RNA in copies/mL. The LLoQ is 40 copies/mL (Abbott, 2018, Tang et al., 2007).

The Versant HIV-1 RNA kinetic PCR assay by Siemens Diagnostics (referred to as Versant kPCR) employs a real-time (or kinetic) PCR method to target a highly conserved region within the pol gene utilising TaqMan probes which emit signal upon unfolding during the amplification step. It involves automated sample preparation, an internal control and two external quantitation standards. LLoQ is 37 copies/mL (Ruelle et al., 2009).

The Xpert® HIV-1 Viral Load assay by Cepheid (referred to as Xpert) prequalified by WHO in 2017. Xpert is based on real-time RT-PCR targeting a region within LTR and it detects group M (subtypes A, B, C, D, AE, F, G, H, AB, AG, J, and K) and groups N and O. Its LLoQ is 40 copies/mL and its upper limit is 10,00,000 copies/mL. It requires minimal training (fully automated) and moderate infrastructure (single cartridge containing all reagents and internal controls) to provide rapid (within 90 minutes) and accurate results. The same platform can also be used for testing for a variety of infections including tuberculosis and hepatitis B and C. A systematic review (12 studies included) showed good performance of the Xpert assay compared to current reference tests (Nash et al., 2018). It is a reliable tool for viral load monitoring and detection of treatment failure in the clinic including resource-limited settings (Sacks et al., 2019, Villa et al., 2020).

1.2.2.2 TMA

The Hologic Aptima HIV-1 Quant Dx assay (referred to as Aptima) received FDA approval for viral load monitoring in 2016. It is a fully automated process (Panther system) involving target capture, TMA and fluorescent detection. With the exception of the TMA step the rest

of the process has many similarities with RT-PCR methods. TMA is also a transcription-mediated nucleic acid amplification method, but with some important differences. HIV-1 RNA is isolated from plasma samples and captured by oligonucleotides targeting a highly conserved region of the HIV-1 genome. The hybridised RNA is separated with magnetic microparticles and a magnetic field. Purified viral RNA is converted to DNA by a reverse transcriptase, which is used as template for the production of multiple RNA copies by a T7 RNA polymerase. Two target regions within pol and LTR are amplified. Single-stranded nucleic acid torches hybridise to the RNA amplicon in real-time and as result the quencher moves away from the fluorophore allowing for the signal to emit. An internal control is present at each reaction to ensure amplification efficiency and quantitation of target RNA. The concentration of the HIV sample and the internal control are determined on a calibration curve. The linear range of quantitation is between 30 and 10,000,000 copies/mL and LoD is around 12 copies/mL with some variation across subtypes and groups (e.g. 17 copies/mL for subtype G). Equally the LLoQ is 10 copies/mL for B subtypes and from 10 to 30 copies/mL for non-B subtypes (Hologic, 2017). Contrary to RT-PCR, the whole process takes place at the same temperature. Comparative studies have shown that the Aptima assay is precise, accurate, sensitive and concordant with widely used assays including the RealTime and the TaqMan v2.0 (Longo et al., 2018, Wiesmann et al., 2018).

1.2.3 Historical assays

1.2.3.1 NASBA

NASBA methodology comprises of iso-thermal amplification of RNA and detection of target sequences (gag originally and then LTR) based on electro-chemiluminescence (end-point) or molecular beacon (real-time) technologies (Deiman et al., 2002). The NucliSens HIV-1 QT assay was commercialised by bio Mérieux in 1995. A second generation assay with a quantitation range of 176 to 3,470,000 copies/mL received FDA approval in 2001 (Ginocchio et al., 2003). The NucliSens EasyQ HIV-1 version 2.0 was an update incorporating an automated process and molecular beacons for target quantitation (Xu et al., 2010). According to literature the NASBA technology is as accurate as PCR, if not more sensitive, for the quantitation of HIV-1 RNA levels (Ginocchio et al., 2003, Mourez et al., 2015, Notermans et al., 2000, Shepard et al., 2000). However, interlaboratory variability has been reported more frequently than other assays probably due to free choice of extraction protocols and starting sample volume and greater inter-subtype variability (Senechal and James, 2012).

1.2.3.2 bDNA

The representative of bDNA technology was the Versant HIV-1 RNA 3.0 assay by Siemens Medical Solutions (previous versions known as Quantiplex). HIV RNA is not purified but released with a lysis step and captured on a microwell coated with oligonucleotides with homology to conserved regions of the pol gene. Another set of nucleotides target HIV RNA and amplify the signal by binding at multiple sites. There is no transcription of viral RNA. Detection is by chemiluminescence and determined from a standard curve. The range of quantitation is 75 to 500,000 copies/mL (Collins et al., 1997, Liegler TJ and RM, 2009). Under-quantification has been shown with the Siemens bDNA assay and reports demonstrate consistently lower values compared to other technologies (Senechal and James, 2012).

1.2.3.3 ELISA

Enzyme-linked immunosorbent-based assays (ELISA), such as ExaVir RT (Cavidi) were inexpensive and simple assays used at resource limited settings (Boni et al., 1997, Labbett et al., 2009, Pascual et al., 2002, Stevens et al., 2005). The ExaVir RT assay measured RT activity on a poly-A oligonucleotide template with an incorporated alkaline phosphatase colorimetric assay. RT activity correlated with signal intensity. Version 3.0 had a LLoQ corresponding to viral load of 200 copies/mL (Labbett et al., 2009).

1.2.3 Ultrasensitive HIV-1 RNA assays used in research settings

Ultrasensitive assays with a cut-off as low as a single copy, or near single copy, of HIV-1 RNA per mL of plasma have been developed for research purposes (Palmer et al., 2008). They require large volumes of sample which is either concentrated or tested in replicates. Samples are usually intensively processed before quantitation and operator-led over automated steps analogy varies (Table 1.2). Commercial platforms or in-house RT-PCR technology targeting highly conserved regions of the HIV-1 genome are usually utilised to measure residual virus presence (Amendola et al., 2011, Hatano et al., 2009, Margot et al., 2018, Palmer et al., 2003, Yukl et al., 2011).

Ultrasensitive assays attempt to shed light on viral kinetics during suppressive ART and predict the risk of virological failure in patients with residual levels of virus presence in the blood or other compartments which are not detected with commercial assays (Palmer et al., 2008). First-generation single copy assays (SCA) targeted HIV-1 gag with higher rates of primer mismatch and larger volumes of sample (7 mL). A subsequent SCA targeted a highly

conserved region of integrase (iSCA) in x3 concentrated samples from an original volume of 3 mL (Cillo et al., 2014). Based on the iSCA a clinical study showed that all samples from patients on suppressive ART reported as "target not detected" with the TaqMan v2.0 had at least 1 copy of HIV RNA. Interestingly, samples with viral loads above 20 copies/mL appeared to measure lower with the iSCA indicating some overestimation with the Roche platform (Margot et al., 2018). An update version of iSCA (v2.0), which uses a greater proportion of total extracted nucleic acid for HIV-1 RNA quantitation, increased the number of samples with detected HIV-1 RNA by almost one third and more than doubled the amount of copies/mL (Tosiano et al., 2019). Automated platforms testing multiple replicates with single copy sensitivity are currently under development (Bakkour et al., 2019).

Lack of standardisation of SCA results in significant variation in methodology, input plasma volume and detection limit. Hence, findings may be conflicting and non-comparable. Of note, commercial assays report discrepant results towards their LLoQ even within the same platform (Doyle and Geretti, 2012, Ruelle et al., 2012). Nevirapine compared to efavirenz and lopinavir/ritonavir appeared to have a virological advantage in patients with HIV-1 RNA in plasma less than 2 copies/mL as measured with a modified protocol of the Amplicor v1.5 (Bonora et al., 2009). A SCA-based study also supported the claim that nevirapine may be better than efavirenz in suppressing virus replication (Haim-Boukobza et al., 2011). No such correlation, though, was found in a study comparing nevirapine with PI-based therapy utilizing a modified version of the RealTime assay (Kiselinova et al., 2015).

Table 1.2: Characteristics of representative single copy assays.

Reference	(Palmer et al., 2003)	(Yukl et al., 2011)	(Hatano et al., 2009)
Type of sample	Plasma	Plasma	Plasma
Input sample volume, mL	7	30	2
Method of sample concentration	Ultracentrifugation (170,000 ×g, 30 min, 4°C, Sorvall T-1270 rotor)	Centrifugation (47,810 xg, 3 hrs, 4°C, Sorvall RC6 SH3000 rotor) with density cushion (OptiPrep Density Gradient Medium)	N/A
Number of replicates	N/A	N/A	4
Internal control	RCAS	Abbott IC	Aptima IC
RNA extraction method	Manual/operator-based	Automated	Semiautomated
Assay	In-house two step RT-PCR (quantitative)	Abbott RealTime HIV-1 Viral Load (quantitative)	Gen-Probe Aptima HIV-1 (semiquantitative)
Target region on HIV genome	gag	pol integrase	polymerase and LTR regions[1]
Standard	Plasmid pQP1-derived transcripts generate a standard curve at each run	Abbott calibrator kit generate and store a standard curve which is validated at each run	Positive and negative Aptima calibrators defining a signal-to-cutoff (S/Co) ratio at each run
Measurement of HIV-1 RNA copies/mL	Interpolation of Ct values from x3	Interpolation of Ct values from x1	Matching of S/Co ratios from x4 replicates to

Reference	(Palmer et al., 2003)	(Yukl et al., 2011)	(Hatano et al., 2009)
	replicates in a linear regression model	replicate in a linear regression model	predefined viral load values derived from in vitro validation experiments

RCAS: replication-competent avian sarcoma; N/A: not applicable; IC: internal control; LTR: long terminal repeat

1: (Giachetti et al., 2002)

1.3 HIV-1 RNA presence in plasma during effective therapy

1.3.1 Viral kinetics during suppressive antiretroviral therapy

ART suppresses viral replication to plasma HIV-1 RNA levels below the LLoQ of commercial assays (20 and 40 copies/mL) within 3-6 months of therapy initiation (Boender et al., 2015, Doyle et al., 2012, Tanner et al., 2016). However, ART does not eradicate HIV-1 from latently infected cells. Viral decay following ART initiation occurs in 4 phases (Palmer et al., 2008, Riddler et al., 2016). Phase one and two are very dynamic stages during which turnover of virus population and productively infected cells is suppressed and plasma HIV-1 RNA decreases below 50 copies/mL (Wei et al., 1995). First there is a rapid decline in plasma viraemia with a half-life of 1-2 days, which is the effect of a significant reduction in infected activated CD4 T cells. A second phase of slower decay (half-life 2-3 weeks) follows because of clearance of long-lived cells harbouring and producing HIV. Phase three and four are more stochastic and the cellular source associated with them involves latently infected cells with self-renewal capacity. The third phase is dominated by a very slow decay (39 weeks half-life) due to decrease of latently infected cells that become sporadically activated, such as resting memory CD4 T-cells. During the fourth phase viraemia declines at an extremely low rate (0.026 $\log_{10}$ per year) with a half-life of at least 11.5 years (95% CI 6.2-83 years) (Riddler et al., 2016). Although a similar half-time ($t_{1/2}$) (13 years) has been estimated for the decay of the total cell-associated HIV-1 DNA (Gandhi et al., 2017), the half-life of productively infected cells is not known (Jacobs et al., 2019).

Residual viral RNA in the plasma between 1 and 3 copies/mL is quantified with ultra-sensitive assays in the majority of patients despite optimal ART (Cillo et al., 2014, Maggiolo et al., 2012, Margot et al., 2018, Palmer et al., 2008). It has been previously reported that patients on a boosted lopinavir based regimen and with suppressed viral loads less than 50 copies/mL for 7 years have at least one sample with detectable HIV-1 RNA measured with a SCA (Palmer et al., 2008). Although most patients on stable ART experience at least intermittently quantifiable HIV-1 RNA levels, the exact proportion of patients with detectable levels with SCAs varies among different studies (Cillo et al., 2014, Gandhi et al., 2010, Hofstra et al., 2014, Kiselinova et al., 2015, Margot et al., 2018, Tosiano et al., 2019). It is unclear if the reason for these discrepancies lies with study population demographics,

viral characteristics, immune system capacity to control viral replication along with ART, SCA complexity and technical limitations or a combination of the above.

The exact mechanism of residual HIV-1 RNA presence in the plasma is unclear. Ongoing cycles of viral replication may supply circulation with viruses (Lorenzo-Redondo et al., 2016, Rothenberger et al., 2019, Sahu, 2015). This model is supported by evidence showing that drug concentrations vary among tissues and ART penetration may be sub-optimal at sites which are rich in reservoirs such as lymph nodes (Di Mascio et al., 2009, Estes et al., 2017, Fletcher et al., 2014, Lee et al., 2020). Alternatively, clone-specific T cell activation triggered by antigen recognition or local inflammatory processes may cause small bursts of viral production which are immediately suppressed by ART (Shen and Siliciano, 2008b). Of note, phylogenetic studies have not revealed genetic evolution which is a hallmark of viral replication (Bailey et al., 2006, Josefsson et al., 2013, Kearney et al., 2014, Kieffer et al., 2004). Hence, ongoing viral replication should be rare and residual virus should not derive from new infection cycles (Bachmann et al., 2019, Bozzi et al., 2019, Joos et al., 2008, Palmer et al., 2011, Rosenbloom et al., 2017, Sigal et al., 2011). Moreover, integration sites were identical in infected cells, whereas integration during new infections occurs at different sites of the host genome (Cohn et al., 2015, Wagner et al., 2014).

Pharmacological interventions also argue that intermittent clonal-driven virus production is the main mechanism of residual viraemia. Analytical treatment interruption studies have demonstrated that viral rebounds are populated by genetically identical viruses (De Scheerder et al., 2019, van Zyl et al., 2018). On the other end of the spectrum, ART intensification by the addition of one or more potent drugs such as raltegravir, maraviroc, efavirenz or a boosted PI, did not have an effect on residual HIV-1 RNA levels implying that current treatment regimens achieve maximal efficiency in suppressing viral replication (Chaillon et al., 2018, Dinoso et al., 2009, Gandhi et al., 2010, Hatano et al., 2011, Hatano et al., 2013, McMahon et al., 2010, Puertas et al., 2014, Rasmussen et al., 2018). Interestingly, though, measurement of different outcomes in intensification studies shows a decrease in immune activation and transient increase in viral episomal DNA (Buzon et al., 2010, Yukl et al., 2010). Pharmacological features and immune functions, yet to be revealed, may be the reason for these seemingly conflicting findings. Although bursts of viral production appear to be the most prevalent view on presence of residual HIV-1 RNA in plasma, ongoing viral replication may also be relevant in some patients depending on host and virus characteristics (Darcis and Moutschen, 2017, Nightingale et al., 2016).

1.3.2 Low level viraemia and residual viraemia

Plasma viral load is undoubtedly the most accurate surrogate marker of ART efficacy as indicated by viral suppression (HIV-1 RNA <50 copies/mL). Although guidelines recommend a definition for viral rebound as viral load above 200 copies/mL on two occasions, new evidence suggests that this threshold may need revision (BHIVA, 2019). There is no consensus on the definition of residual viraemia, and subsequently low level viraemia (Doyle and Geretti, 2012). In this work we have adapted the cut-off at 10 copies/mL and we defined residual viraemia when HIV-1 RNA levels in the plasma are less than 10 copies/mL and low level viraemia when HIV-1 RNA levels are between 10 and 200 copies/mL.

It is common clinical practice to use a threshold of 50 copies/mL to define viral suppression However, viral load between 50 and 200 copies/mL is also acceptable in selected patients and the North American guidelines use 200 copies/mL as cut-off of therapy success (BHIVA, 2019, DHHS, 2018). These thresholds are based on the risk for viral rebound which is stratified by the level of viraemia. A quarter of patients who have achieved suppression with first-line ART will experience viral rebound with at least one viral load between 50 and 400 copies/mL in the first year after suppression. Almost a quarter of them will have persistent viraemia (median 162 copies/mL) with a two-fold increase in the risk of virological failure (>400 copies/mL) (Geretti et al., 2008). In another study patients on ART for at least one year were stratified according to their level of persistent viraemia in three categories including patients who did not achieve suppression. Authors demonstrated that the risk of virological failure (>1,000 copies/mL) was increased by five times with viraemia 500-999 copies/mL and doubled with viraemia 50-199 and 200-499 copies/mL compared to patients with suppressed viral loads (<50 copies/mL) when adjusting for date of HIV infection and use of tenofovir, emtricitabine, and efavirenz. Although almost half patients with viraemia 50-199 copies/mL did not achieve virological suppression before their viraemic episode, findings remained interesting (HR 1.52 from 2.61) when the analysis was restricted to only those with previous suppression (Laprise et al., 2013). HIV-1 RNA levels less than 50 copies/mL have also been shown to favour virological rebound in stably treated patients (Doyle et al., 2012, Gianotti et al., 2015, Hofstra et al., 2014, Maggiolo et al., 2012). Doyle and colleagues were first to suggest a revisit of clinical thresholds as to treatment efficacy since they observed an increased risk for viral rebound >50 and >400 copies/mL in patients with viraemia between 10 and 40 copies/mL and between 40 and 50 copies/mL, respectively. Hence, a cut-off at 10 copies/mL is the most appropriate relevant threshold

not associated with viral rebound (Doyle et al., 2012, Gianotti et al., 2015, Hofstra et al., 2014, Maggiolo et al., 2012).

Residual viraemia less than 10 copies/mL is non-dynamic and does not predict further increases in viraemia. Viruses appear to have a clonal origin with no genetic evolution over time (Bailey et al., 2006, Hermankova et al., 2001, Kieffer et al., 2004, Nettles et al., 2005, Tobin et al., 2005, Shen and Siliciano, 2008a). Lack of genetic evolution also precludes emergence of drug resistance (Doyle and Geretti, 2012). Fully reconstructed viruses from the plasma of a patient under suppressive ART revealed that half of them were replication competent and the other half carried mutations corresponding to decreased viral fitness due to defects of the replication machinery (Sahu et al., 2010). However, replication-competent virions may not necessarily infect new cells due to their very low levels and ART pressure (Wang et al., 2018).

The impact of residual viraemia on the balance of immune activation and tolerance is not clear. Chronic antigenaemia drives immune exhaustion following recurrent cycles of immune proliferation and activation (Kuchroo et al., 2014). The immune system has acquired memory to HIV antigens and even small amounts of HIV particles during effective ART may activate immune mechanisms. Effective clearance of the antigen results in undetectable levels of HIV-1 RNA in plasma (Hatano, 2013, Younas et al., 2016). Immune activation is expected to be proportional to antigenaemia and current methods may not be able to measure it in cases of residual viraemia (Yukl et al., 2010). Instead the focus should be on HIV-1 specific immunity, as in elite controllers who are able to suppress viraemia at the absence of ART and maintain normal immune activation but increased HIV-1 specific immunity (Bello et al., 2009). Indeed, HIV-specific CD8 T cell responses in elite controllers demonstrate increased antiviral efficacy compared to progressors (Collins et al., 2020).

Determinants of residual viraemia are elusive with potential unknown host and virological factors influencing frequency and size of viral presence in plasma. Previously, latent reservoir size, ART duration, pre-ART viraemia and age have been associated with residual viraemia but literature may be contradictory for some parameters (Chun et al., 2011, Kiselinova et al., 2015, Palmer et al., 2008, Zheng et al., 2013). Large scale studies and new techniques are required to clarify the field of sustained residual viraemia despite ART.

Third-generation assays have a LLoQ between 20 and 40 copies/mL, but their LoD is even lower and down to ~3.5 and 10 copies/mL with the TaqMan v2.0 and RealTime, respectively (Abbott, 2018, Amendola et al., 2014, Doyle and Geretti, 2012). However, there is lack of

concordance the lower the detection limit is set (Amendola et al., 2014, Karasi et al., 2011). Due to remarkable assay variability at low levels of viraemia, study outcomes should be treated with caution and in the context of the method of measurement applied. For instance, findings based on the TaqMan v2.0 imply that viraemia between 20 and 50 copies/mL was not associated with virological failure (Charpentier et al., 2012). A recent study, though, re-tested samples (stored aliquot) with viral load between 50 and 200 copies/mL and found that 42% of them had a repeat viral load less than 50 copies/mL underscoring intra-assay variability (White et al., 2018). Assay characteristics and evidence from comparison studies should be considered to interpret these reports.

1.3.3 Source of residual viraemia

HIV can effectively infect activated cells, a small fraction of which escape the cytopathic effect of viral replication and revert to resting memory cells within a very short time window from the original transmission event (Siliciano et al., 2003, Swiggard et al., 2005, Zhou et al., 2005). This long-lived reservoir is established early after transmission and it is maintained despite pharmacological and immune pressure (Archin et al., 2012, Bukrinsky et al., 1991, Chun et al., 1998). However, only a small fraction of this reservoir (2-10%) contains full-length, intact proviruses which are replication competent (Bruner et al., 2019, Ho et al., 2013, Hiener et al., 2017). This small subset has the potential to cause viral rebound upon treatment discontinuation and remains the supreme obstacle for a cure. During suppressive ART, the majority of these proviruses is localised in T follicular helper cells (Tfh) at the germinal centres of lymph nodes (Banga et al., 2016, Estes et al., 2017).

Two mechanisms have been suggested to maintain the HIV-1 reservoir during ART: homeostatic CD4 T cell proliferation and residual viral replication (Chomont et al., 2009, Fletcher et al., 2014, Frenkel et al., 2003, Grossman et al., 1999, Kearney et al., 2014, Lorenzo-Redondo et al., 2016, McManus WR et al., 2018, Rosenbloom et al., 2017). We have already discussed that new infections are very rare in patients with residual viraemia.

HIV-1 DNA is mainly found in resting CD4 T cells in suppressed patients. Other cells such as monocytes, macrophages and haematopoietic stem cells contain HIV-1 DNA to a lesser extend (Mitchell et al., 2019). Cells harbouring HIV-1 DNA circulate throughout the body and may reside at lymph nodes, gut-associated lymphoid tissue (GALT), cerebrospinal fluid, adipose tissue and the urethra (Chun et al., 2008, Couturier et al., 2015, Ganor et al., 2019,

Nightingale et al., 2016, Perreau et al., 2013b). Residual viraemia is sourced from some of these cells and tissues which may vary between patients and over time within an individual (Jacobs et al., 2019). Intensive sampling and single genome sequencing suggested that residual viraemia originates from memory CD4 T cells which reside in lymphoid-rich tissues or traffic in circulation (Bailey et al., 2006). It has been demonstrated that effector and transitional memory CD4 T cells (CD4+/CD45RA-/CCR7-/CD27- and CD27+, respectively) accommodate proviral sequences which are closely related with viral sequences in residual viraemia (Puertas et al., 2016). However, there are also predominant identical sequences that are underrepresented in resting CD4 T cells. These probably derive from clonal species of virus which do not undergo any changes in their sequence for up to 3 years. The type of cell harbouring these viruses should have proliferative capacity regardless of activation status, such as a progenitor of the monocyte-macrophage lineage (Shen and Siliciano, 2008b).

1.4 Programmed death 1 in the pathogenesis of HIV infection

1.4.1 Immune response and HIV escape

HIV remains "invisible" in the 10 days after transmission, as demonstrated by the absence of viraemia and HIV-specific immune responses (eclipse phase) (Fiebig et al., 2003, McMichael et al., 2010). According to findings from the simian model, in the first week post transmission the first cells to become infected are mucosal resting memory CD4 T cells along with submucosal dendritic cells. The latter transfer HIV to local lymph nodes where activated T cells and follicular dendritic cells secrete pro-inflammatory cytokines such as interleukin-1 (IL-1), IL-6 and tumour necrosis factor α (TNFα) offering an ideal environment for host cell infection and spread of virus particles in great numbers (Perreau et al., 2013a). Vigorous replication results in apoptosis of infected and bystander cells with release of immunosuppressive microparticles such as TNF-related apoptosis-inducing ligand (TRAIL) (Gasper-Smith et al., 2008). Although acute phase cytokines peak towards the end of the eclipse phase and before viraemia, the first HIV-specific CD8 T cell response is recorded to coincide with the peak of viral RNA in the plasma about 3 weeks post infection (Borrow et al., 1994, Stacey et al., 2009). The exact timing of the onset of HIV-specific CD4 T cell responses is unclear and probably precede CD8 activation, but their function is impaired with the predominance of inhibitory pathways triggered by HIV (D'Souza et al., 2007, Gloster et al., 2004, Perreau et al., 2013b).

Plasma viraemia at the end of the eclipse phase assists HIV spread to distant lymphoid-rich tissues such as the GALT, where the rate of CD4 T cell depletion is four times higher than the rate of CD4 infection (Brenchley et al., 2004). Around 3 weeks post infection plasma viraemia peaks, viral reservoirs have been established and HIV-specific CD8 responses are triggered (Figure 1.2). It is only later that humoral immunity with broadly neutralising antibodies (bNAbs) develops (Huber and Trkola, 2007). However, under the selective pressure of antibodies, HIV rapidly evolves by mutating the env gene to escape neutralisation. Moreover, gp120 and gp41 demonstrate conformational changes and their regions are heavily glycosylated protecting them from interactions with circulating antibodies. Yet, over the course of infection, potent bNAbs with cross-reactivity are developed in "elite neutralizers". These have unusual specifics such as somatic hypermutation and very short

or long antigen-binding loops making their adaptation for a vaccine challenging (Landais and Moore, 2018, Richman et al., 2003, Wei et al., 2003).

Cytotoxic CD8 T cell driven immunity appears to be the most important function to control HIV dissemination. The initial CD8 response against key targets such as the env and nef HIV proteins is effective with reduction in viraemia. However, virus escape mutants occur rapidly under immune pressure, with sequentially mutated epitopes triggering recurrent cycles of CD8 activation (Arcia et al., 2016, Goonetilleke et al., 2009, Keele et al., 2008b). Continued epitope recognition and lack of CD4 T cell help, due to their depletion and functional impairment, cause exhaustion of cytotoxic T lymphocytes (CTL) allowing virus persistence (Blattman et al., 2009, Petrovas et al., 2006, Wherry, 2011, Zhang et al., 2007).

Immune escape is achieved via multiple pathways which affect the interactions between MHC-I/HIV-1 epitope/T cell receptor (TCR) complex. First the antigenic epitope on gp120 has a variable region with significant diversity among strains. Transformation of RNA to DNA produces antigenic diversity due to the innate tendency of RT to create single-nucleotide mutations and strand recombination. Furthermore, HIV proteins, such as nef and vpu, and presence of non-immunogenic glycans on epitopes have a direct effect on host recognition through down-regulation of MHC-I expression (Oldstone, 1997, Wei et al., 2003). Escape variants are not always effective to produce cell infection. Defects that impair replication and cell invasion have a significant impact on their fitness to survive (Kent et al., 2005).

Latent infection is also an escape mechanism from both immunological and pharmacological clearance. Latency is characterised by a resting state of the infected cell and increased expression of immune checkpoints, such as the programmed death 1 (PD1) and cytotoxic T lymphocyte-associated protein 4 (CTLA-4) (Chomont et al., 2009). Indeed, virus production requires HIV-1 DNA transcription which largely depends on an increase of host transcription factors in the nucleus following T cell activation. These pathways are suppressed by immune checkpoints (Deeks et al., 2016).

1.4.2 Structure, genome, ligands and role of PD1

In 1992, the RNA of a novel inducer of programmed cell death was isolated from apoptotic murine cell lines and was named PD1 (or CD279) (Ishida et al., 1992). Further studies revealed that PD1 and its ligands play a pivotal immunoregulatory role by inducing T cell anergy (Jin et al., 2011).

PD1 is a 55-kDa monomer cell surface protein of the CD28 superfamily expressed in CD4 and CD8 T cells, B cells, natural killer (NK) cells, monocytes and dendritic cells. It has an extracellular Ig-like variable domain, a transmembrane region and a cytoplasmic tail containing an immunoreceptor tyrosin-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM) (Figure 1.3). PDCD1 on chromosome 2 is the gene that encodes PD1 and consists of 5 exons. Alternative splicing results in 4 variants with similar properties as the full length isoform except from the PD1Δex3 transcript that lacks the transmembrane region and thus is in soluble form (Ishida et al., 1992, Jin et al., 2011, Nielsen et al., 2005).

PD1 ligands are PD-L1 (B7-H1 or CD274) and PD-L2 (B7-DC or CD273). PD-L1 is constitutively expressed on a variety of non-lymphoid tissues including parenchymal organs, such as the heart, lung and placenta. T and B cells also express negligible amounts of PD-L1 which are significantly increased upon activation (Dong et al., 1999). On the other hand, PD-L2 expression is confined to professional antigen-presenting cells (APC), such as macrophages, dendritic cells, bone-marrow derived mast cells and resting peritoneal B1 cells, and it is interferon-γ dependant. The binding affinity of PD-L2 to PD1 is two to six folds higher than that of PD-L1, and it appears to have an additional receptor with a costimulatory role for T cell activation (Shin et al., 2003, Tseng et al., 2001, Youngnak et al., 2003). PD-L1 and PD-L2 induced effects appear to be distinct (Dyck and Mills, 2017a). A murine model of malaria demonstrated a protective role of PD-L2 in preventing PD-L1 induced exhaustion and establishing effective CD4 T cell immunity with better pathogen clearance (Karunarathne et al., 2016). Also, mice lacking PD-L2, but not PD-L1, developed an excessive immune response to cutaneous leishmaniasis with marked production of pathogen-specific antibodies, while PD-L1(-/-) mice were resistant to disease (Liang et al., 2006).

Immune activation begins with a primary signal generated from an antigen (Ag) binding to a TCR. The primary response will be either enhanced or suppressed depending on accessory signals derived from co-receptors such as the B7-recognising immunoreceptors (CD28, CTLA-4, ICOS, PD1 and BTLA). The balance between a positive (CD28 and ICOS) and a negative (CTLA-4, PD1 and BTLA) stimulation ensures a controlled immune response and determines the fate of the cell (Bretscher, 1999, Freeman et al., 2000, Ishida et al., 1992, Nielsen et al., 2005, Okazaki et al., 2013). Clonal expansion is enhanced or diminished depending on the original signal and immune checkpoints, maintaining a balance between activation and tolerance (Fife and Bluestone, 2008, Wang et al., 2018).

PD1 and CTLA-4 are the most well described immune checkpoints and are targeted with inhibitors in cancer therapy with the aim to boost cytotoxic effector T cell responses (Dyck and Mills, 2017b). PD1 expression plays a central role in an inhibitory regulatory pathway on both recently activated and memory T cells (Figure 1.4). A similar effect but less prominent is observed on naïve T cells during a primary immune response (Agata et al., 1996, Cai et al., 2004). The PD1 pathway regulates a broad spectrum of immune functions including immunological tolerance, regulation of T cell, B cell and NK cell proliferation and effector functions. TCR or B cell receptor (BCR) mediated cell activation induces PD1 expression. During chronic infection there is increased expression of PD1 on virus-specific T cells. Its ligands are also expressed at high levels by various tumour cells implying that cancer and viruses exploit PD1 pathway properties to their survival benefit (Jin et al., 2011).

1.4.3 Molecular mechanism

TCR-mediated calcium influx activates the nuclear factor of activated T cells calcineurin-dependent 1 (NFATc1) which initiates expression of PD1 (Oestreich et al., 2008). In addition, γ-chain associated cytokines (e.g. IL-2) and type I interferons (e.g. IFN-α) synergistically up-regulate PD1 transcription. It has been reported that cytokine-stimulated expression of PD1 may be antigen-independent during chronic infection (Cho et al., 2008, Kinter et al., 2008, Terawaki et al., 2011). Once PD1 integrates in the cell membrane it sustains its position making it an easy target for quantification with flow cytometry.

PD1 interaction with its ligands results in phosphorylation of its intracellular tyrosines and their association with Src homology 2 (SH2) domain-containing tyrosine phosphatase 1 and 2 (SHP-1, SHP-2). SHP-1 and -2 inactivate key signalling molecules of the antigen recognition cascade. Dephosphorylation of phosphoinositide-3 kinase (PI3K) inhibits Akt phosphorylation (Parry et al., 2005, Sheppard et al., 2004) and the secretion of cytokines (IFN-γ, TNFα, IL-2, Bcl-xL) implicated in effector cell functions, cell expansion, differentiation and survival. While inhibition of Erk by binding SHP-2 results in decreased proliferation (Butte et al., 2007, Keir et al., 2008).

1.4.4 Function

The PD1 pathway inhibits the effector functions of lymphocytes and preserves survival of target cells such as infected or cancerous cells. This signifies two sides of the same coin:

immunotolerance and immunopathology. PD1 plays a crucial role in prevention of immune over-activation securing balanced immune responses and peripheral tolerance. It has been demonstrated that PDCD1 knockout mice develop autoimmunity and PDCD1 polymorphisms in human are associated with autoimmune diseases such as systemic lupus erythematosus (SLE), type 1 diabetes, Grave's disease and multiple sclerosis (MS) (Okazaki and Honjo, 2007). Cancerous and infected cells exploit the physiological inhibition to escape immunity and establish malignancies and chronic infections, respectively. Antigen-specific T cells during chronic viral infections suffer an impaired function and PD1 is implicated to play a pivotal role in the establishment of this state of anergy (Barber et al., 2006, Boni et al., 2007, Day et al., 2006, D'Souza et al., 2007, Okazaki et al., 2013, Radziewicz et al., 2007). Virus-specific CD8 T cells become gradually exhausted originally by loss of their cytotoxicity and IL-2 production which is followed by loss of TNFα production and their proliferative potential (Freeman et al., 2006). Models of lymphocytic choriomeningitis virus (LCMV) infection have indicated that knock down of PD1 expression may result in a rapid clearance of the pathogen preventing chronic infection, but the effect of excessive T cell activation on healthy tissue is deleterious (Barber et al., 2006).

High and persistent expression of PD1 has been observed in virus-specific immune cells during chronic infection as a result of demethylation in the promoter region of the PDCD1 gene (Youngblood et al., 2011). Hence, a state of phenotypic and functional senescence persists. Although cells are seemingly too "exhausted" to proliferate or produce cytokines, their transfer to an infection-naïve murine recipient experiencing acute infection triggers cell expansion and control of infection (Utzschneider et al., 2013). These data imply that at least a fraction of the exhausted virus-specific CD8 T cells is not terminally differentiated and retains unaffected memory properties of proliferation and cytokine production upon antigen presentation. Virus-infected cells seem to express PD1 to prevent an overwhelming chronic immune response (Utzschneider et al., 2013).

Similarly, some tumours ensure their survival by expressing high levels of PD-L1 which interacts with PD1 on cytotoxic T cells inhibiting their effector function. PD-L1 is also upregulated in various chronic viral infections. For instance, increased PD-L1 expression is demonstrated on bronchial and alveolar epithelial cells after their infection with respiratory syncytial virus, human hepatocytes infected with adenovirus, hepatocytes and circulating monocytes of patients with hepatitis B virus (HBV) infection, dendritic cells infected with rhinoviruses and macrophages infected with the herpes simplex virus (HSV) type 1. In agreement PD1 expression was increased at the acute phase of hepatitis C virus (HCV)

infection and either decreased in patients with virus clearance or persisted in those with chronic infection (Trautmann et al., 2007). Murine models also imply that the same strategy of immune escape, by increased expression of PD-L1, is adapted by HIV-infected cells (Akhmetzyanova et al., 2015).

1.4.5 PD1/PD1 ligand blockade

Interferon α, β and γ increase PD-L1 expression during early innate immune responses to infection (Freeman et al., 2006). Early data showed that transient blockade of PD1/ligand interaction restored the normal functions of LCMV-specific CD8 T cells (Barber et al., 2006). Similarly, monoclonal antibodies targeting the PD1/PD-L1 pathway are employed successfully in cancer therapy because they have shown to enhance cytotoxic T lymphocyte responses resulting in tumour regression (Hirano et al., 2005, Ohigashi et al., 2005). Although PD-L1 blockade overall improves cytotoxic functions, most experiments were conducted on T cell lines and replication of findings in primary T cells is required (Trautmann et al., 2007).

Checkpoint inhibitors have shown to suppress viral load in chronic infections with HBV, HCV and HIV by reversing exhaustion in virus specific T cells (Cox et al., 2017). Nivolumab, an anti-PD1 monoclonal antibody, was shown to reduce significantly viraemia in a chimpanzee model and a small subset of patients with HCV (Fuller et al., 2013, Gardiner et al., 2013). Responses were enhanced when two different inhibitory pathways were blocked. Liver-derived CD8 T cells from chronically infected HCV patients had their functionality effectively restored once both the PD1 and CTLA-4 pathways were pharmacologically inhibited (Nakamoto et al., 2009). Studies in humanised mouse models and macaques have reported lymphocyte reconstitution with regard to both T and B cell functions and decrease in viral load with PD1 blockade (Finnefrock et al., 2009, Seung et al., 2013, Velu et al., 2009).

Immune checkpoint inhibitors have been used in HIV positive patients with advanced malignancies and a recent systematic review has demonstrated their safety and efficacy (Cook and Kim, 2019). However, reports on virological and immunological parameters of the HIV reservoir were inconsistent. Nivolumab in lung cancer patients has been shown to cause a decrease in HIV-1 DNA and an increase in viral-specific CD8 T cell activity (Guihot et al., 2018, Le Garff et al., 2017), whereas there was no effect in other studies (Gay et al., 2017, Lavolé et al., 2018, Scully et al., 2018). Administration of a single dose of nivolumab to an HIV-infected patient with metastatic melanoma on ipilimumab (anti-CTLA-4) increased cell-associated HIV RNA in CD4 T cells, but there were no changes to total HIV-1 DNA or viraemia (Evans et al., 2018). Of note, the sample sizes of these studies were small and their methodologies differed. Currently there a few ongoing clinical trials that will shed more light on the efficacy of checkpoint inhibitors in HIV infection (Chen et al., 2020). Combination therapy may also improve outcomes (Van der Sluis et al., 2020, Wykes and Lewin, 2018).

1.4.6 PD1 and HIV

A hallmark of HIV infection is chronic immune activation which is coupled with increases in checkpoint molecules. Antigen presence and persistence consist the main drives for the upregulation of several checkpoint receptors in T cell populations. PD1 expression is increased during HIV-1 infection on specific CD4 and CD8 T cells, Tfh cells, follicular CD8 T cells and HIV-1 infected CD4 T cells (Chen et al., 2020).

Chronic persistent infection results in the unresponsiveness of cytotoxic CD8 T cells to infected cells. Exhausted CD8 T cells first lose their ability to produce IL-2 and be cytotoxic and then TNFα and IFN-γ production. Studies based on the LCMV model of infection demonstrated that epitope-specific CD8 T cells seem to have a phenotype of chronic activation with indefinitely impaired antigen-effector functions. This status of anergy was found predominately in CD4 (-/-) knockout mice implicating the critical role of CD4 T-helper cells in the function of cytotoxic CD8 T cells during infection clearance. Moreover, they were incapable of producing long lived memory subsets which persist at the absence of antigen (Wherry and Ahmed, 2004, Zajac et al., 1998). This functional impairment is also demonstrated in HIV infection (Sperk et al., 2018).

The PD1 pathway suppresses cytokine driven T cell proliferation and induces apoptosis during later stages of activation (effector phase) by inhibiting TCR-induced signals (Fife and Bluestone, 2008, Keir et al., 2008). Lack of CD4 helper cells and prolonged immune activation (both present in HIV infection) result in increased PD1 expression (Buchbinder and Desai, 2016). PD1 expression prior to ART commencement correlated with viral DNA levels and it was a predictor of time to rebound after treatment suspension (Hurst et al., 2015). Therefore, inhibitory pathways such as the PD1 have been proposed as responsible for suboptimal immune reconstitution in chronic HIV infection.

Day *et al.* and Trautmann *et al.* showed that PD1 expression on HIV-specific CD8 T cells was higher than on CMV-specific CD8 T cells and the degree of expression varied among HIV epitope-specific CD8 T cells from a single individual. Moreover, antigenaemia positively correlated with PD1 expression on HIV-specific or total CD8 T cells and total CD4 T cells. Further characterisation of HIV-specific CD8 T cells revealed that they were pre-terminally differentiated, with additional high expression of CD27 and low expression of CD28, CCR7 and CD45RA. Their cytotoxic (TNFα and IL-2 production) and proliferative functions were impaired, but restored with blockade of the PD1/PD-L1 axis (Day et al., 2006, Trautmann et al., 2006). Petrovas *et al.* took one step further and demonstrated that decreased cytokine

production was an indirect effect of high PD1 expression on CD8 T cells which resulted from a defect in their proliferative capacity. Increased levels of PD1 expression can induce cell apoptosis independently of other factors (Petrovas et al., 2006). During chronic untreated infection PD1 expression on HIV-specific CD4 cells is up-regulated in strong correlation with viral load and it is twice higher than on HIV-specific CD8 cells. The same study also observed that viral suppression with ART causes a more prominent decrease of PD1 expression on HIV-specific CD4 T cells, but still the expression remains higher than on HIV-specific CD8 T cells. Simultaneous measurements in lymph node derived T cells demonstrated similar findings except from that PD1 expression is much higher compared to blood circulating T cells. This is related with increased expression of PD-L1 on APC reflecting the high levels of viral replication and antigen presence. PD-L1 blockade increased HIV-specific CD4 T cell proliferation, but not cytokine production. Interestingly, IFN-γ-producing cells demonstrated higher levels of PD1 expression compared to IL-2 producing cells (D'Souza et al., 2007).

Eradication strategies focus on the clearance of quiescent virus harboured in resting cells. Blockade of checkpoint pathways appears to reverse latency, as indicated by enhancement of immune responses against HIV and changes in markers of reservoirs, raising promises for a functional cure (Fromentin et al., 2019, Sperk et al., 2018). Based on the principles of chimeric antigen receptor (CAR) T-cell therapy for cancer treatment, viral reservoirs can be potentially reduced with ex vivo expansion and re-introduction of HIV-specific CD8 T cells which are negative for PD1 and other markers of impaired effector function (Chapuis et al., 2011, Miliotou and Papadopoulou, 2018, Porter et al., 2011, Terme et al., 2008). Similarly, but in vivo, a strategy called "shock and kill" involves stimulating activation of resting reservoirs in order to induce an immune response sufficient to clear them (Shan et al., 2012). This approach may be assisted by the blockage of inhibitory pathways such as PD1 (Amancha et al., 2013, Finnefrock et al., 2009, Freeman et al., 2006). Achieving a HIV cure is likely to require a combination of more than one strategy.

Figure 1.3: Schematic representation of PD1 interaction with its ligand (Sharpe and Freeman, 2002).
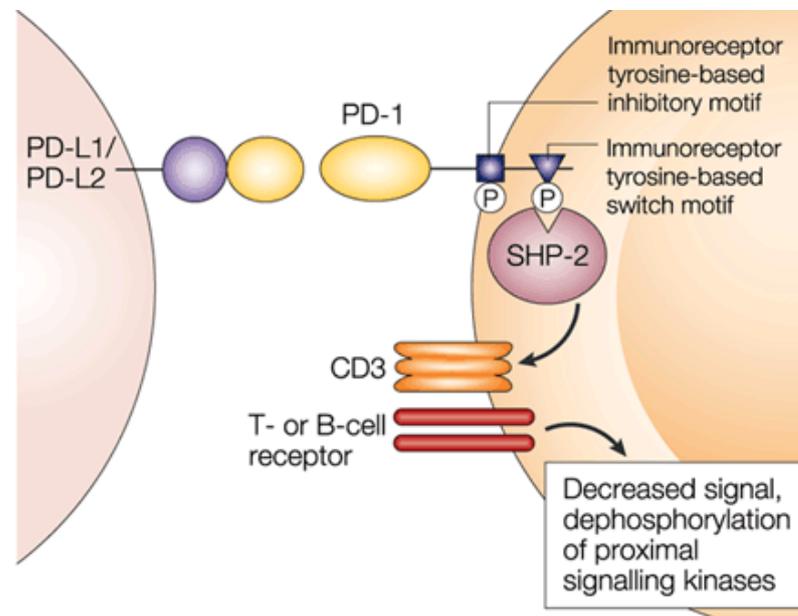
The cytoplasmic tail of PD-1 contains two tyrosines: the immunoreceptor tyrosin-based inhibitory motif (ITIM) and the immunoreceptor tyrosine-based switch motif (ITSM). Ligation of T cell receptor (TCR) and PD-1 can lead to tyrosine phosphorylation (P) and activation of Src homology 2-containing phosphotyrosine phosphatase (SHP-2), resulting in dephosphorylation of signalling molecules and reduced cytokine mRNA synthesis

Figure 1.4: The PD1 pathway illustrated (Buchbinder and Desai, 2016).
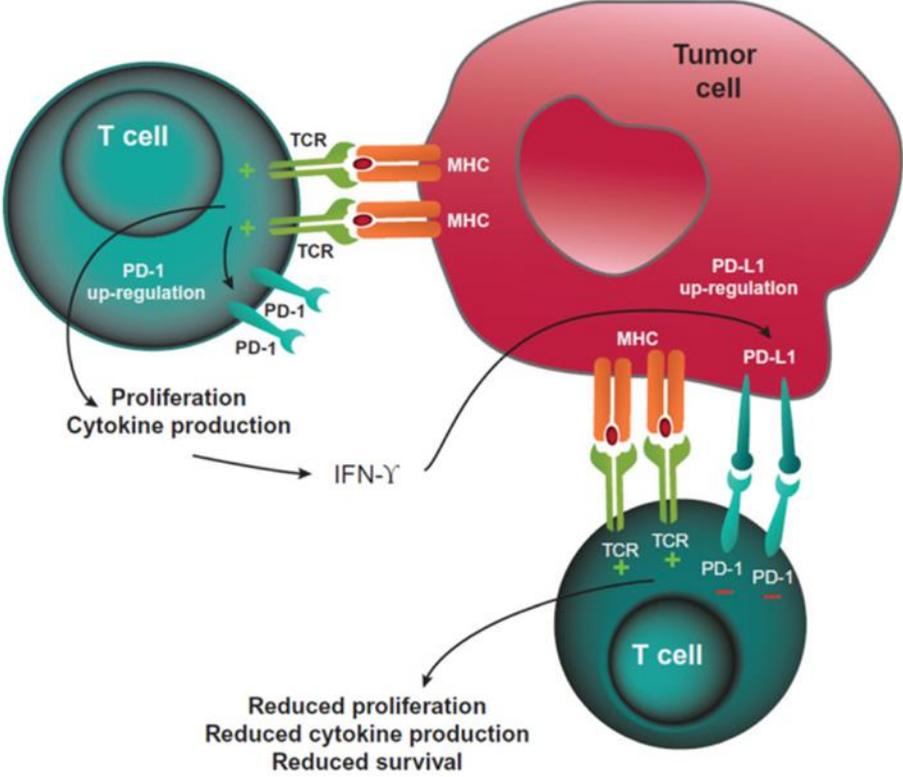
1.5   Aims and objectives

1.5.1 Aim

This work aims to investigate factors associated with residual viraemia defined as HIV-1 RNA in plasma less than 10 copies/mL during ART.

1.5.2 Objectives

1.5.2.1 Predictors of the presence of HIV-1 RNA in plasma between 10 and 200 copies/mL (low level viraemia) despite effective ART will be identified through a systematic review of current literature.

1.5.2.2 We will measure virological (total HIV-1 DNA in peripheral blood mononuclear cells (PBMC)) and immunological (PD1 expression on circulating memory CD4 and CD8 T cells) parameters and determine their association with residual viraemia, defined as HIV-1 RNA in plasma less than 10 copies/mL in successfully treated patients.

1.5.2.3 HIV-1 RNA in plasma, total HIV-1 DNA in PBMC and drug levels will be measured during two consecutive clinical visits to describe their reproducibility and their relation.

CHAPTER 2

A systematic review of factors associated with low level viraemia in HIV-positive patients on effective antiretroviral therapy

2.1 Introduction

Low level viraemia (LLV) is the presence of HIV-1 RNA in plasma despite effective ART which is detected by commercial assays and may reach a few hundred copies (Geretti et al., 2008). After a sharp decline in viral load (VL) following ART commencement and in the absence of treatment interruptions, VL remains suppressed (<50 copies/mL) in most patients. Inability to achieve VL suppression or virological rebound after an initial response may indicate treatment failure and a possible requirement for ART modification. VL thresholds defining virological failure vary from 50 to 1,000 copies/mL with most recommendations adapting a cut-off at 200 copies/mL (Table 2.1). Although guidelines suggest switch of treatment for confirmed viraemia above 200 copies/mL, some authors recommend considering regimen changes in cases of persistent VL results between 50 and 200 copies (Thompson et al., 2012). It remains unclear whether LLV ranging from a "target detected" result to 200 copies/mL is associated with an increased risk of resistance mutations (Gallien et al., 2011, Jordan et al., 2013, Malet et al., 2012, Swenson et al., 2014b, Taiwo et al., 2011) and immune activation (Schuler et al., 2013, Stephan et al., 2013, Zheng et al., 2014). Discordances in study design and definitions are main reasons for the conflicting findings.

Intermittent episodes of LLV which are preceded and followed by an undetectable VL measurement are referred to as viral blips (Di Mascio et al., 2003, Grennan et al., 2012, Greub et al., 2002, Mira et al., 2002, Nettles and Kieffer, 2006, Sklar et al., 2002). In a cohort from the Netherlands, episodic LLV between 50 and 1,000 copies/mL did not instigate therapy changes or clinical deterioration, whereas viraemia above 1,000 copies/mL was linked with drug resistance (van Sighem et al., 2008). At least one fifth of fully suppressed patients experience viral blips (Greub et al., 2002, van Sighem et al., 2008). Blips of low magnitude, such as less than 500 copies/mL as suggested by Grennan *et al.* (2012), do not predispose to virological failure or rebound. They may represent normal biological variation in patients with viraemia below or around the assay LLoQ, statistical variation due to assay variability at lower HIV-1 RNA levels, or methodological artefacts. On the contrary, viral blips of higher magnitude have been associated with virological failure (Easterbrook et al., 2002, Grennan et al., 2012, Moore et al., 2002, Nettles and Kieffer, 2006, Nettles et al., 2005). Persistent LLV, confirmed with at least two consecutive measurements, has been related with an increased risk for virological failure when above 200 copies/mL (Bernal et al., 2018, Boillat-Blanco et al., 2015). However, some studies reported an association between virological failure and any level or frequency of confirmed LLV identified with commercial assays (Laprise et al., 2013, Leierer et al., 2016, Pernas et al., 2016).

The range of LLV varies in literature depending on the assay used and the country of study (Appendix, Table A.1). Current commercial assays are based on the technologies of real-time RT-PCR and TMA achieving a LLoQ between 20 and 40 copies/mL. Introduction over time of more sensitive real-time assays increased the number of patients experiencing LLV on suppressive treatment who had previously undetectable VL (Do et al., 2011). Nevertheless, ultra-sensitive experimental methods have revealed that the effect of ART is suppressive and not eliminatory with residual HIV-1 RNA around 3 copies/mL persisting in patients with undetectable VL (Shen and Siliciano, 2008b, Bailey et al., 2006).

More than three quarters of stably suppressed patients with viral load below 50 copies/mL experience residual viraemia when tested with an ultrasensitive single copy assay (Palmer et al., 2008). Although the mechanism of this residual viraemia is unclear, it has been suggested that virus is primarily produced upon activation of chronically infected cells and, to a lesser or even negligible extent, during cycles of viral replication (Anderson et al., 2011, Palmer et al., 2011, Shen and Siliciano, 2008b). As to the significance of residual viraemia, it has been demonstrated that the risk for viral rebound is gradually increasing with levels of HIV-1 RNA above approximately 10 copies/ml (Doyle and Geretti, 2012, Doyle et al., 2012,

Gianotti et al., 2015, Hofstra et al., 2014, Maggiolo et al., 2012). Current commercial VL assays have a LoD of around 10 copies/mL, reporting a qualitative "target detected" signal between the LLoQ and the LoD. In several studies, patients with "target detected" results by such commercial assays, were found to be at increased risk of virological rebound compared to those with "target not detected" (Doyle and Geretti, 2012, Doyle et al., 2012, Gianotti et al., 2015, Henrich et al., 2012, Hofstra et al., 2014, Pugliese et al., 2013). In contrast, HIV-1 RNA levels below this threshold appear to be stable and non-evolving (Shen and Siliciano, 2008b, Shen and Siliciano, 2008a).

LLV is a grey area causing confusion and nervousness as to clinical management. Our knowledge of probable predictors of LLV is even more limited since most studies adapt a cross-sectional observational design rather than an investigation of pre-defined causal relationships. Pre-ART conditions, ART-related factors and microbial translocation, as well as assay technical characteristics are proposed to contribute to LLV. We conducted a systematic review in order to summarise our current knowledge of clinical, virological and immunological factors associated with viral rebound post original suppression. Considering the LoD of commercial assays and the threshold of virological failure recommended by most guidelines, we defined LLV in treated patients as VL between 10 and 200 copies/mL.

Table 2.1: Definitions of virological failure according to current guidelines.

Guidance	Definition of virological failure	Reference
WHO	>1,000 copies/mL on two consecutive measurements (3-6 months apart)	(WHO, 2017)
DHHS	>200 copies/mL[1]	(DHHS, 2018)
EACS	>200 copies/mL 6 months post first-line ART[1] >50 copies/mL on two consecutive measurements (1-2 months apart) in previously suppressed patients	(EACS, 2018)
BHIVA	>200 copies/mL on two consecutive measurements	(BHIVA, 2019)
BC-CfE	>250 copies/mL on two consecutive measurements	(British Columbia Centre for Excellence in HIV/AIDS, 2015)

1: Although the definition of virological failure does not specify the number of measurements required, it is recommended that treatment switch is considered with confirmed virological failure implying that a second measurement is needed.

WHO: World Health Organisation; DHHS: U.S. Department of Health and Human Services; EACS: European AIDS Clinical Society; BHIVA: British HIV Association; BC-CfE: British Columbia Centre for Excellence.

2.2 Methods

2.2.1 Search strategy and review methodology

Three main databases, Medline (interrogated with Ovid and PubMed), Cochrane Library and Scopus were searched from 1st January 2008 to 16th August 2018 for studies identifying factors associated with LLV. We included work that adapted a definition for LLV as VL rebound between "target detected" and 200 copies/mL or 20 and 200 copies/mL for studies using a qualitative or quantitative lower cut-off, respectively. We also screened studies with broader definitions for subgroup analysis and we considered including those which reported that 95% of subjects with LLV had a VL within our pre-defined range. We used the median (or mean) and confidence interval (or standard deviation) of VL where the percentage was not mentioned. We chose 2008 as a starting point to capture mainly studies utilizing third generation assays which report VL values below 50 copies/mL. Our search strategy was developed using Medline and adapted for the other databases (Table 2.2). It was based on five main terms and their alternative keywords: HIV, viral load, ART, low level viraemia and predictors.

Virological blips were excluded as long as they were confirmed with the acquisition of three consecutive measurements, whereas studies using single measurement of VL and not providing confirmation of LLV were included (Table 2.3). Abstracts from meetings would be included if they contained sufficient information and their findings were not published in another work. Scripts in English were checked for eligibility. The references listed in the articles from the final selection were also queried for relevant records.

2.2.2 Data extraction and quality assessment

Citations were exported to EndNote X7 manager and the results from the three databases were merged. An EndNote command identifying duplicates (or triplicates) was followed by manual recognition and extraction of the same articles appearing in more than one database. Then titles and abstracts were carefully reviewed for relevance and eligibility (Table 2.3). In most cases the information on LLV was inadequate and a search of the main text was performed focusing on the 200 copies/mL cut-off (first stage of selection). A

detailed read of the remaining articles followed (second stage of selection). Only studies showing positive associations between a factor and LLV were included.

The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) checklist was followed except from the points referring to meta-analysis conduct as this is a qualitative review. Being part of a thesis, abstract is not provided, but we will consider adding one in case of publishing in a peer reviewed journal. This work is registered with the PROSPERO international database (registration No: CRD42019132516).

2.2.3 Data analysis and synthesis

Studies were summarised according to study design. We extracted all factors demonstrating positive association with LLV from each publication. A template was created in excel to contain key characteristics of the selected studies including time period, country, primary outcome, sample size, definition of LLV, VL assay, ART regimen and suggested predictors. A second template was populated with information on each potential predictor including patient groups, descriptive statistics and test or model. Common categories and subcategories were identified to include relevant factors. For each factor we presented the number of studies reporting a significant association with LLV and details on the analysis performed by authors. The templates were also used to determine study heterogeneity and if a quantitative synthesis could be performed.

A detailed description of findings within the context of the selected studies was generated. We presented factors associated with LLV in author-reported multivariate models with odds ratios (OR) and 95% confidence intervals (CI), as well as the co-variates that the models were adjusted for. Medians and interquartile ranges (IQR) or means and standard deviations (SD) were used for continuous parameters which were identified with less robust statistical methods than multivariate analysis. Categorical variables were presented with percentages. The statistical information for each factor was extracted from the publications based on the most robust method used. For instance, if a parameter was significant in the multivariate analysis, we did not include the univariate analysis. We generated tables reporting study characteristics including definition of LLV, assay for VL measurement, factors associated with LLV and statistical methods. Factors identified by author-reported multivariate models were summarised in a forest plot-like figure.

Table 2.2: Search strategy.

Search	Query
#1: MeSH	(HIV/ OR HIV-1/) AND (RNA, viral/ OR Viral Load/ OR Viremia/) AND (Anti-retroviral Agents OR Anti-HIV Agents OR Antiretroviral Therapy, Highly Active OR HIV Integrase Inhibitors OR HIV Protease Inhibitors OR Reverse Transcriptase inhibitors) AND (Risk Factors/)
#2: ab.ti	(HIV) AND (anti?retroviral therapy OR ART OR cART OR HAART) AND (low level vir?emia) AND (predictors OR determinants OR factors)
#3: mp	(HIV) AND (low level vir?emia) AND (anti?retroviral therapy)
#4	#1 OR #2 OR #3
#5	Limit #4 to (humans AND yr= "2008-Current")

MeSH: Medline subject headings; ab.ti: abstract.title; ART: anti-retroviral therapy; cART: combined anti-retroviral therapy; HAART: highly active anti-retroviral therapy; mp: keyword; yr: year

Table 2.3: Eligibility criteria.

Inclusion criteria	Exclusion criteria
1. HIV-1 infected adult patients (≥18 years of age)	1. Not specifying VL assay, LLV range or ART duration
2. Treated with ART for at least 6 months and achieving original suppression	2. Not achieving suppression within 6 months of ART commencement
3. VL measured with commercial assays	3. Utilisation of experimental in-house or modified assays
4. LLV clearly defined as VL between 20 and 200 copies/mL or "target detected" and 200 copies/mL or at least 95% of the LLV group having VL within these ranges	4. LLV >200 copies/mL
	5. Isolated viral blips (VL sequence: ND -> 20-200 copies/mL -> ND)
5. Original publications dated from 01/01/2008 to 16/08/2018	6. Reviews and poster abstracts if original studies published in peer-reviewed journal already included

ART: anti-retroviral therapy; VL: viral load; LLV: low level viraemia; ND: not detected

2.3 Results

2.3.1 Study characteristics and heterogeneity

Term search returned 639 publications including 165 duplicates which were removed (Figure 2.1). An original screening of 474 unique articles was conducted to review their relevance to the objective of this work and their eligibility as to the predefined inclusion/exclusion criteria. The original language of nine papers was not English but their titles and abstracts being in English contained adequate information to satisfy our exclusion criteria, such as defining LLV above 200 copies/mL and being reviews. A detailed examination of all sections (stage two) identified that more than half of records (28/43) were ineligible. The majority of excluded studies at the second review stage failed to fulfil our definition of LLV with less than 95% of patients ranging within 20 and 200 copies/mL (n = 7) or lack of clarity in reporting the portion of patients with VL within our range (n = 3). The second most frequent reason for exclusion was the utilisation of a modified protocol of a commercial assay aiming to increase sensitivity (n = 6). Other less frequent reasons were the allowance of isolated blips (n = 3), poor virological response to ART at 6- or 12-months post therapy commencement (n = 3) and a review. Query of references contributed 3 papers. In total 15 relevant publications are presented in this systematic review which are grouped as studies identifying patient parameters associated with LLV (n=8) (Table 2.4) and work comparing commercial assays (n=7) (Table 2.5).

Several factors have been associated with LLV in published multivariate and univariate models. These can be categorised as pre-ART, ART-related, virological, immunological and methodological due to assay variability. There were differences among authors in the way that a factor was treated (e.g. ordinal vs. continuous variable) and these were outlined under each category. Factors were presented along with the statistical method which was used by authors and differences in study design if they were identified in more than one work.

Definition of LLV, as to the VL range and confirmation on repeated measurements, varied significantly. Only two studies used our definition of LLV (20-200 copies/mL) with a single measurement of VL, while three adapted a VL range between 20 and 50 copies/mL as determined at one or multiple occasions. Three studies adapted a definition of LLV of detected HIV-1 RNA but less than the LLoQ of the assay used. Most authors (5 out of 8 articles) compared the group with LLV to a group of patients with reported VL as "target not

detected" (Table 2.4). All parameters that were associated with LLV in published multivariate models are presented in Figure 2.2. Articles comparing the performance of commercial platforms identified variability around a cut-off (Table 2.6). This threshold varied between the LLoQ of assays and 50 copies/mL (Table 2.5). The definition of some variables was also different among authors. Hence, heterogeneity did not allow pooling of data and performance of meta-analysis. Instead a realist synthesis has been adapted (Bearman and Dawson, 2013).

2.3.2 Patient parameters associated with low level viraemia

2.3.2.1 Pre-ART factors

Three studies reported that a higher VL prior to ART initiation was a predictor of LLV post therapy commencement. Pre-ART VL was treated as both ordinal and continuous variable. As ordinal there were two thresholds applied at 100,000 (or 5 $\log_{10}$) copies/mL (Martin-Blondel et al., 2012) and at 10,000 (or 4 $\log_{10}$) copies/mL (Leierer et al., 2015). Pre-ART VL higher than 100,000 copies/mL was strongly associated with LLV between 20 and 200 copies/mL in the published (Leierer et al., 2015) multivariate analysis (Table 2.7). VL between 9,999 and 100,000 copies/mL also exhibited a significant, but weaker, association with LLV (Table 2.7). As a continuous variable, the median VL (IQR) for the group with LLV and the group with undetected VL was 5.13 (4.87-5.70) and 4.97 (4.65-5.29) $\log_{10}$ copies/mL, respectively (Pascual-Pareja et al., 2010).

Patients with LLV between 20 and 200 copies/mL (single measurement of VL) had a diagnosis of HIV for a median of 18 years (median, IQR: 14-20) compared to 10 years (IQR: 6-16) in patients with undetected VL (Table 2.7). Charpentier and colleagues reported that 53% of patients with confirmed LLV between 20 and 50 copies/mL had a diagnosis of CDC stage B/C at the time of first-line ART initiation compared to 30% in patients with undetectable VL. The difference was significant in a univariate, but not in the multivariate analysis. The same study also reported that the LLV group had an older age (median: 47, IQR: 43-53) compared to undetectable controls (median 44 years; IQR: 38-50) (Table 2.7).

2.3.2.2 ART-related factors

Two groups demonstrated that PI-based ART combinations were independently associated with LLV between 20 and 50 (confirmed LLV) or 20 and 200 copies/mL (Table 2.7). Falasca *et al.* reported a positive association between confirmed LLV up to 200 copies/mL and use of InSTI (Table 2.7). The authors added that half their patients receiving an InSTI were also on a PI. NRTI-sparing combinations were associated with LLV between 20 and 200 copies/mL in a study by Reus and colleagues (Table 2.7).

Shorter ART duration was associated with LLV in two published multivariate logistic regressions adjusting for different co-founding factors as shown in Table 2.7. One study treated ART duration as an ordinal variable (<9 and >18 months), whereas the other study as a continuous (median: 15 months in the group with LLV and median: 25 months in the group with undetected VL). LLV was also associated with shorter duration of viral suppression (<50 copies/mL) less than 1 year (Table 2.7). Difference in ART duration was not significant in studies with treatment duration for more than one and a half years.

Previous ART interruption longer than 8 days and at least 6 months prior to study inclusion was associated with LLV between 20 and 200 copies/mL as determined with a single measurement in a multivariate model (Table 2.7). The presence of clinical symptomatology or AIDS-defining conditions (CDC stage B/C) during stable ART was also related to LLV between 20 and 50 copies/mL (Table 2.7).

2.3.2.3 Virological factors

Infection with HIV non-B subtype (40% AG, 20% A, 17% G, 7% AE, 3% AB, 3% CRF & 3% F) was a predictor of LLV less than 50 copies/mL in a study from France (Table 2.7).

A study found that higher levels of HIV-1 DNA in PBMC were associated with confirmed LLV below 200 copies/mL. This work stratified LLV into measurable VL between the LLoQ (37 copies/mL) and 200 copies/mL and very low level viraemia (VLLV) reported as "target detected" below 37 copies/mL. Total HIV-1 DNA in PBMC was higher in the group with LLV (median: 3.05 log copies/10^6 PBMC; IQR: 3.0–3.36) than VLLV (median: 2.87 log copies/10^6 PBMC; IQR: 2.53–3.18) and undetectable VL (median: 2.59 log copies/10^6 PBMC; IQR: 2.25–2.88). A logistic regression model adjusting for several clinical and immunological co-founding factors, as shown in Table 2.7, confirmed the relation between HIV-1 DNA load and LLV or VLLV.

Charpentier and colleagues demonstrated that more frequent episodes of transient viraemia (blip) between 50 copies/mL and 1,000 copies/mL were an independent predictor of persistent LLV from 20 to 50 copies/mL. Similarly, higher levels of HIV-1 RNA during earlier ART regimens were significantly associated with LLV during current ART, but this factor was not tested in the multivariate model (Table 2.7). Of note, only 6% (n=38) of their study population experienced LLV.

2.3.2.4 Immunological factors

Immune activation and microbial translocation are potential determinants or consequence of LLV. Lower CD4 count prior to initiation of current regimen and current CD4 count were significantly associated with LLV between 20 and 50 copies/mL and below 200 copies/mL, respectively, but did not maintain significance in the multivariate analysis (Table 2.7). Immune activation was also observed in a group of patients with detectable but unquantifiable VL <37 copies/mL. CD14+/CD16+/CD163+, indicating activated monocytes, were expressed with higher frequencies in the LLV group compared to patients with undetectable VL. The same work also reported an association of LLV with higher levels of lipopolysaccharide-binding protein (LBP) (Table 2.7). A similar study, setting a higher limit for LLV at 200 copies/mL, demonstrated a significant association between soluble CD14, a marker of microbial translocation, and LLV in a logistic regression model. The same work also revealed a relation between the inflammatory marker TNFα and LLV, which did not reach statistical significance in the model (Table 2.7). Moreover, Reus *et al.* showed that increased bacterial ribosomal DNA circulated in the blood of patients with VL from 20 to 200 copies/mL. An analysis of variance indicated IL-6 levels were also higher in the group of patients with LLV compared to undetectable controls (Table 2.7).

2.3.3 Assay variability

Current knowledge as to LLV is founded on second and third generation assays the performance of which is compared in 7 studies (Tables 2.5 and 2.6). VL was measured in parallel with three real-time assays and the Amplicor v1.5 assay in a large cohort (n=391) of HIV-positive patients with a wide range of viraemia from target not detected (TND) to >100,000 copies/mL. The frequencies of LLV between 50 and 200 copies/mL with the RealTime, TaqMan v2.0 and TaqMan v1.0 were 11.5%, 8.2% and 9.5%, respectively, compared to 6.4% reported with the Amplicor v1.5 (Do et al., 2011). Overall agreement at

the cut-off of 50 copies/mL between the Amplicor v1.5 and the two assays currently in use (RealTime and TaqMan v2.0) was reported between 87% and 92% and kappa statistics (0.14) showed only slight agreement for LLV between the LLoQ and 200 copies/mL (Table 2.8). The most discrepant results were demonstrated in the comparison of the TaqMan v1.0 and Amplicor v1.5 with 73% (598/819) of samples being discordant at the cut-off of 50 copies/mL and kappa coefficients less than 0.20 (Table 2.8).

Higher level of discordance has been observed between current and previous versions of real-time assays. In one study, 27% (63/230) of samples that were measured as <50 copies/mL with the RealTime assay, were above 50 copies/mL with the TaqMan v1.0 (Table 2.8). Yan *et al.* reported fair agreement of the two methods (kappa coefficient = 0.38) for LLV between the LLoQ and 200 copies/mL (Table 2.8). Comparing the TaqMan v1.0 and v2.0 in 69 paired clinical samples with VL <200 copies/mL, 41% were quantified with a mean difference of 1 copy/mL and 9% were undetectable with both assays (Table 2.8). One study compared the Versant kPCR v1.0 with the RealTime and TaqMan v2.0 and found that 9.9% (6/61) and 8.2% (5/61) of samples, respectively, were discordant at the cut-off of 50 copies/mL (Table 2.8).

Discrepancies are reported with currently used real-time assays around their LLoQ. Leierer *et al.* conducted a multi-centre study with 78% of VL samples measured using the TaqMan v2.0 and 22% with the RealTime. In a multivariate analysis they found that the probability of having LLV between 20 and 200 copies/mL vs VL <20 copies/mL was lower if the RealTime was utilised (Table 2.7). Karasi *et al.* tested 328 clinical specimens with VL <200 copies/mL, including samples with TND, with both methods. They demonstrated 91% overall agreement (95% CI: 88-93) at the threshold of 50 copies/mL and 2 copies/mL mean difference of quantifiable VL. Overall, 60% (196/328) and 46% (150/328) of samples were undetectable with the RealTime and TaqMan v2.0 assay, respectively (Table 2.8). Two studies showed that around 13% (73/569 and 7/59) of samples that were <50 copies/ml by RealTime were above 50 copies/mL using the TaMan v2.0 (Table 2.8).

Figure 2.1: Flowchart of study selection.

Literature search: 16/08/2018

Databases: Medline/Ovid, Medline/PubMed), Cochrane Library and Scopus

Terms: HIV, viral load, ART, low level viraemia and predictors

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639 records identified → 163 duplicates removed

476 records → 433 studies excluded on initial screening (LIST REASONS)

- LLV defined above 200 copies/mL
- Non-clinical experimental studies or animal models
- VL not measured with commercial assays
- Reviews

43 records → 28 studies excluded on secondary screening

- 7: LLV definition not within 20-200 copies/mL with <95% having a VL 20-200 copies/mL
- 3: LLV definition not within 20-200 copies/mL and median/mean VL not mentioned
- 6: Modified protocol of a commercial assay
- 3: inability to achieve virological suppression 6 months post ART commencement
- 3: allowing isolated blips in the control or case group
- 1: no clear definition of LLV or accessible 95% of determinant VL
- 1: <6 months on ART
- 1: review study (references screened)
- 1: abstract from an included study
- 1: abstract not specifying the assay used
- 1: 8 case studies not powered to report associations

15 records:

- 8 present positive associations with LLV
- 7 compare commercial assays

ART: anti-retroviral therapy; LLV: low level viraemia; VL: viral load

Figure 2.2: Factors associated with low level viraemia in published multivariate models[1]
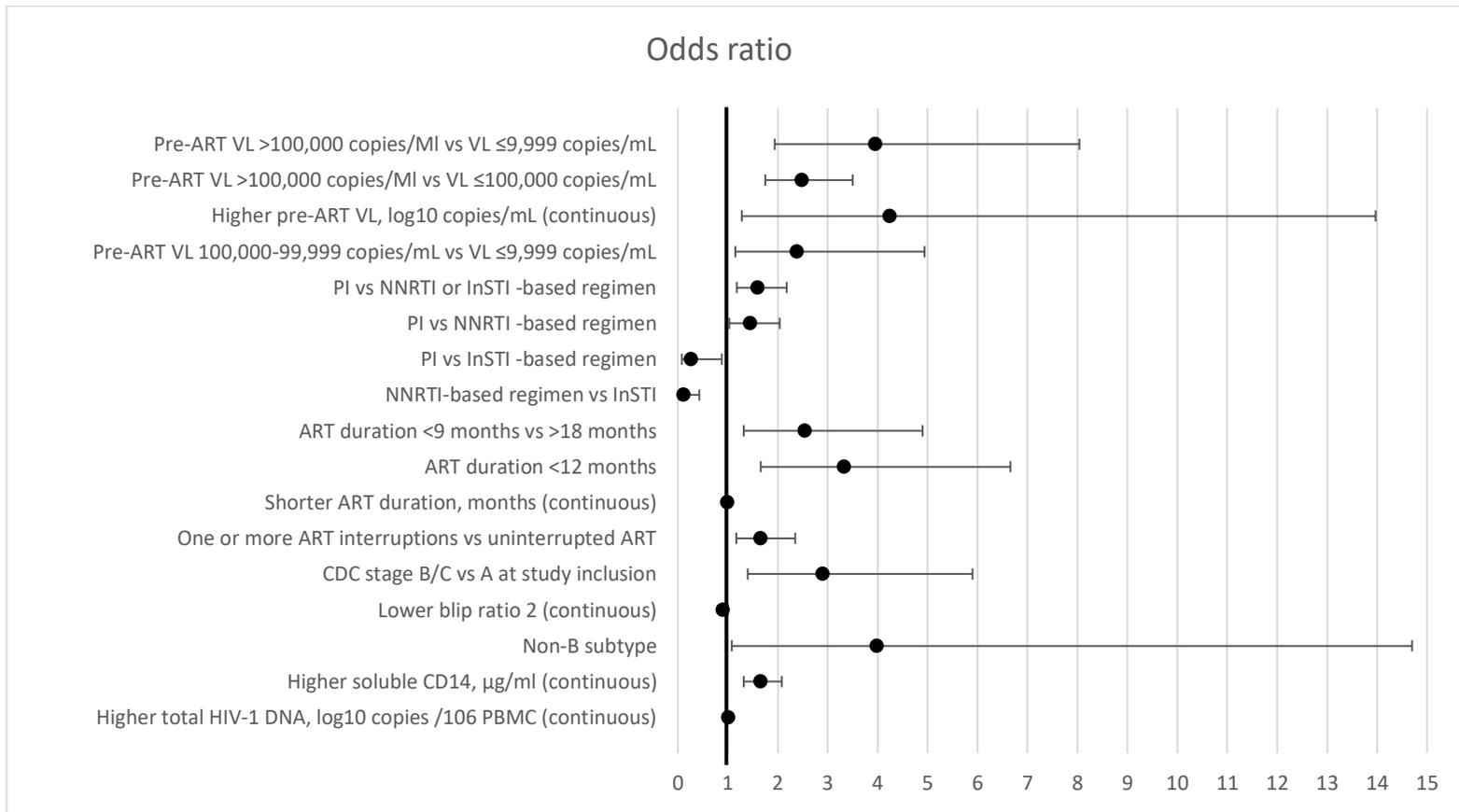
1: Only variables that remained associated with LLV after adjustment are presented

2: Blip ratio was defined as the proportion of VL values >50 copies/mL to the number of VL measurements for the period from initiation of current ART until study inclusion

ART: anti-retroviral therapy; VL: viral load; PI: protease inhibitor; NNRTI: non-nucleoside reverse transcriptase inhibitor; InSTI: integrase strand transfer inhibitor; CDC: Centres for Disease Control; PBMC: peripheral blood mononuclear cell

Table 2.4: Characteristics of studies identifying patient parameters associated with low level viraemia.

Reference	Study type (country; period)	ART regimen (%)	Assay (LLoQ, copies/mL)	Definition of control group	Definition of LLV, copies/mL	VL determinants[1]
(Leierer et al., 2015)[3]	Multi-centre (x5) retrospective (Austria; 2012-2013)	PI-based (43); NNRTI- or InSTI-based (57)	TaqMan v2.0 (20) RealTime (40)	Below the LLoQ	20-200	1
(Martin-Blondel et al., 2012)	Cross-sectional (France; 2011)	PI-based (43); NNRTI-based (45); raltegravir-based (12)	TaqMan v2.0 (20)	Target not detected	20-50 (LLV) and target detected below the LLoQ (VLLV)	LLV/VLLV: 1-2 Control: 2
(Charpentier et al., 2012)[2]	Longitudinal retrospective (France; 2009-2010)	PI-based (49); NNRTI-based (34); INI-based (6); Other (11)	TaqMan v2.0 (20)	Target not detected	20-50	LLV: ≥2 Control: ≥3 (median = 4 in both groups)

Reference	Study type (country; period)	ART regimen (%)	Assay (LLoQ, copies/mL)	Definition of control group	Definition of LLV, copies/mL	VL determinants[1]
(Pascual-Pareja et al., 2010)	Cross-sectional (Spain; 2009)	PI-based (55); NNRTI-based (45)	TaqMan v2.0 (20)	Below the LLoQ	20-49	1
(Saison et al., 2013)	Longitudinal prospective (France; 2009-2010)	PI-based (89); NNRTI-based (7); raltegravir (2); T20 (2)	RealTime (40)	Target not detected	< 50 including target detected below the LLoQ	LLV: ≥1 Controls: all (median = 8 in both groups)
(Torres et al., 2014)[3]	Cross-sectional (Spain; 2011-2012)	PI-monotherapy (67); PI-based triple therapy (33)	Versant kPCR v3.0 (37)	Target not detected	Target detected below the LLoQ	1
(Falasca et al., 2017)	Longitudinal prospective (Italy; 2-years)	PI-, NNRTI- & INI-based and other (not specified)	Versant kPCR v1.0 (37)	Target not detected	37-200 (LLV) and target detected below the LLoQ (VLLV)	LLV: ≥2 VLLV: ≥3 Controls: all

Reference	Study type (country; period)	ART regimen (%)	Assay (LLoQ, copies/mL)	Definition of control group	Definition of LLV, copies/mL	VL determinants[1]
(Reus et al., 2013)	Cross-sectional (Spain; 2011)	PI (52); NNRTI (67); NRTI (86) Other (19)	TaqMan v2.0 (20)	Below the LLoQ	20-200	1

1: Number of VL measurements required to define the groups

2: This study also has a prospective follow-up period (2010-2011) aiming to assess virological outcomes, such as virological failure, which is not included in this analysis.

3: The study objective is to present the impact of ritonavir-boosted PI monotherapy (darunavir or lopinavir) on immune activation, inflammation and microbial translocation compared to PI-based triple therapy. In this review we include a sub-analysis comparing activated monocytes (CD14+CD16+CD163+ cells) and lipopolysaccharide-binding protein (LBP) as a marker of microbial translocation in the LLV and control group.

ART: anti-retroviral therapy; LLoQ: lower limit of quantitation; LLV: low level viraemia; VL: viral load; PI: protease inhibitor; NNRTI: non-nucleoside reverse transcriptase inhibitor; InSTI: integrase strand transfer inhibitor; TaqMan v2.0: Roche COBAS AmpliPrep/COBAS TaqMan version 2.0; RealTime: Abbott RealTime HIV-1 Viral Load assay; VLLV: very low level viraemia; NRTI: nucleoside reverse transcriptase inhibitor; Versant kPCR v3.0: Siemens Versant HIV-1 RNA v3.0; Versant kPCR v1.0: Siemens Versant HIV-1 RNA kPCR v1.0

Table 2.5: Characteristics of studies comparing the performance of commercial assays.

Reference	Study type (country; period)	Assays (LLoQ, copies/mL)	Definition of LLV, copies/mL (VL determinants[1])
(Do et al., 2011)	Cross-sectional multi-centre (x3) (USA; 2007-2008)	TaqMan v1.0 (40) TaqMan v2.0 (20) RealTime (40) Amplicor v1.5 (50)	<50 measured using the Amplicor v1.5 (1)
(Lima et al., 2009)	Retrospective (Canada; 2006-2008)	TaqMan v1.0 (40) Amplicor v1.5 (50)	No definition
(Swenson et al., 2014)	Multi-site (x14) cross-sectional (International)	TaqMan v1.0 (40) TaqMan v2.0 (20) RealTime (40) Amplicor v1.5 (50)	<1,000 (1)
(Yan et al., 2010)	Cross-sectional (Australia; 2008-2009)	TaqMan v1.0 (40) RealTime (40) Amplicor v1.5 (50)	0-200 (not specified)
(Wojewoda et al., 2013)	Cross-sectional (USA)	TaqMan v1.0 (48)[2] TaqMan v2.0 (20)	<200 (1)

Reference	Study type (country; period)	Assays (LLoQ, copies/mL)	Definition of LLV, copies/mL (VL determinants[1])
(Karasi et al., 2011)	Cross-sectional (Luxembourg & Rwanda; 2009)	RealTime (40) TaqMan v2.0 (20) Amplicor v1.5 (50)	<200 including unquantifiable samples and target not detected (1)
(Ruelle et al., 2012)	Cross-sectional (Belgium)	Versant kPCR v1.0 (37) TaqMan v2.0 (20) Realtime (40)	<50 and target detected below the LLoQ (1)

1: Number of VL measurements required to define the groups

2: Wojewoda et al. report a LLoQ for the TaqMan v1.0 of 48 copies/mL in their publication.

LLoQ: lower limit of quantitation; LLV: low level viraemia; VL: viral load; TaqMan v1.0: Roche COBAS AmpliPrep/COBAS TaqMan HIV-1 Test v1.0; TaqMan v2.0: Roche COBAS AmpliPrep/COBAS TaqMan HIV-1 Test v2.0; Abbott: Abbott RealTime HIV-1 Viral Load assay; Amplicor v1.5: Roche COBAS Amplicor HIV-1 Monitor Test, v1.5; Versant kPCR v1.0: Siemens Versant HIV-1 RNA 1.0 kPCR

Table 2.6: Characteristics of commercial assays utilised by the selected studies.

Assay	Lower limit of quantitation, copies/mL	Assay characteristics	Reference
Roche COBAS AmpliPrep/ COBAS TaqMan HIV-1 Test v2.0 (RT)	20	Dual-target of gag and LTR regions Quantitation based on an internal standard Group M (A-H & CRF01-AE) and O subtypes	(Roche Molecular Systems, 2018)
Abbott RealTime HIV-1 Viral Load (RT)	40	Targets the pol integrase region Quantitation based on a calibration curve Group M (A-G, CFR01-AE & CRF02-AG), O and N subtypes	(Abbott, 2018)
Roche COBAS AmpliPrep/ COBAS TaqMan HIV-1 Test v1.0 (RT)	40	Targets a highly conserved gag region Quantitation based on an internal standard Group M (A-H & CRF01-AE) subtypes	(Roche Molecular Systems, 2007, Swenson et al., 2014)
Roche COBAS Amplicor HIV-1 Monitor Test v1.5 (end-point)	50	Targets a highly conserved gag region Quantitation based on an internal standard Non-B subtypes amplified with reduced efficiency	(Roche Diagnostic Systems, 1996)
Siemens Versant HIV-1 RNA 1 - 3 (kPCR)	37	Targets a highly conserved pol integrase region Quantitation based on 2 calibrators	(Siemens, 2016)

Assay	Lower limit of quantitation, copies/mL	Assay characteristics	Reference
		Group M (A-H & CRF02-AG) and O subtypes	

RT: real-time; LTR: long terminal repeat; CRF: circulating recombinant form, kPCR: kinetic PCR

Table 2.7: Patient factors associated with low level viraemia.

Reference	Groups (n)	Statistical method used by authors	Parameters associated with LLV	Reported OR (95% CI) or median (IQR) or mean (SD) or as otherwise stated and p-value
(Martin-Blondel et al., 2012)	LLV (296) VLLV (617) TND (479)	Multivariate logistic regressions (x2) Outcome: TND vs LLV (1) or VLLV (2) Co-variates: time since HIV diagnosis, duration of ART, zenith VL, duration of suppression (<50 copies/mL), current CD4 count, zenith VL and NNRTI-based regimen (ref: PI).	Zenith VL <5 $\log_{10}$ copies/mL	(1): 2.48 (1.75–3.50); p <0.001 (2): 1.51 (1.15–1.99); P = 0.003
			NNRTI-based regimen	(1): 1.45 (1.03–2.04); P = 0.03 (2): N/K [3]
			Current CD4 count <500 cells/mm^3	(1): 1.25 (0.86-1.80); P = N/K[3] (2): 1.44 (1.08–1.92); P = 0.01
			Duration of viral suppression >1 year	(1): 3.33 (1.66–6.66); P = 0.0006 (2): 1.56 (0.83-2.94); P = N/K[3]
			Duration of viral suppression >2 years	(1): 3.23 (1.64-6.67); P = not reported (2): 2.33 (1.20–4.54); P = 0.01
		Analysis of variance (ANOVA)	Time since HIV diagnosis	LLV: 14 years (7) VLLV: 13 years (7) TND: 15 years (7)

Reference	Groups (n)	Statistical method used by authors	Parameters associated with LLV	Reported OR (95% CI) or median (IQR) or mean (SD) or as otherwise stated and p-value
				P = 0.02
(Pascual-Pareja et al., 2010)	LLV (21) BLQ (62)	Multivariate regression with a forward stepwise selection	Pre-ART VL	4.24 (1.28–13.97) P = 0.018
		Outcome: LLV Co-variates: pre-ART VL and duration of ART	ART duration	0.987 (0.974–1) P = 0.044
(Leierer et al., 2015)	LLV (222) BLQ (1,972) Sensitivity analysis (S): LLV (199) BLQ (1,497)	Multivariate logistic regressions x2: VL measured with the TaqMan v2.0 or RealTime assay and VL measured with the TaqMan v2.0 assay only (S) Outcome: LLV Co-variates (ref): age (>50 years), HIV transmission mode (MSM), ART interruptions (no interruptions), VL assay (TaqMan v2.0), ART duration (>18 months), ART regimen	Pre-ART VL ≥ 100,000 (a) and 10,000-99,999 copies/mL (b)	(a): 4.19 (2.07–8.49); p = N/K (S)(a): 3.95 (1.94-8.04); p = N/K (b): 2.52 (1.23–5.19); p = N/K (S) (b): 2.38 (1.15-4.94); p = N/K
			Female heterosexuals	0.62 (0.41–0.95); p = N/K (S): 0.67 (0.43-1.04); p = N/K
			PI-based ART	1.54 (1.15–2.06); p = N/K (S): 1.60 (1.18-2.18); p = N/K
			ART duration < 9 months	2.59 (1.38–4.86); p = N/K

Reference	Groups (n)	Statistical method used by authors	Parameters associated with LLV	Reported OR (95% CI) or median (IQR) or mean (SD) or as otherwise stated and p-value
		(NNRTI- or InSTI- based regimens) and pre-ART VL (≤9,999 copies/mL)		(S): 2.54 (1.32-4.90); p = N/K
			ART interruptions	1.69 (1.22–2.34); p = N/K (S): 1.66 (1.17-2.35); p = N/K
			RealTime assay	0.33 (0.21–0.51); p = N/K (S): N/A
(Charpentier et al., 2012)	LLV (38) TND (618)	Multivariate logistic regression using a backward elimination Outcome: LLV Co-variates: age at inclusion, CDC stage at inclusion (B/C vs A), CDC stage at initiation of first-line ART (B/C vs A), VL at initiation of current ART, CD4 count at initiation of current ART, duration of current ART and blip ratio[2].	CDC stage B/C at study inclusion	2.9 (1.4–5.9) P = 0.003
			Blip ratio[2]	0.9 (0.9–1.0) P = 0.001

Reference	Groups (n)	Statistical method used by authors	Parameters associated with LLV	Reported OR (95% CI) or median (IQR) or mean (SD) or as otherwise stated and p-value
		Wilcoxon	Age at study at study inclusion	LLV: 47 years (43-53) TND: 44 years (38-50) P = 0.04
		Fisher's exact test	CDC stage B/C at initiation of first-line ART	LLV: 53% TND: 30% P = 0.006
		Wilcoxon	VL at initiation of current ART	LLV: 2.0 $\log_{10}$ copies/mL (1.7-4.5) TND: 1.7 $\log_{10}$ copies/mL (1.7-3.4) P = 0.02
		Wilcoxon	CD4 count at initiation of current ART	LLV: 254 cells/mm^3 (142-583) TND: 402 cells/mm^3 (262-587) P = 0.02
(Reus et al., 2013)	LLV (13) BLQ (39)	Mann-Whitney	Time since HIV diagnosis	LLV: 18 years (14-20) BLQ: 10 years (6-16)

Reference	Groups (n)	Statistical method used by authors	Parameters associated with LLV	Reported OR (95% CI) or median (IQR) or mean (SD) or as otherwise stated and p-value
				P < 0.01
		χ^2	NRTI-sparing regimens	LLV: 69% BLQ: 92% P < 0.05
		Mann-Whitney	IL-6	LLV: 90 pg/mL (81–154) BLQ: 79 pg/mL (61–105) P <0.05
		χ^2	Ribosomal bacterial DNA (16S rDNA) in blood	LLV: 46% BLQ: 18% p <0.05
(Falasca et al., 2017)	LLV (95) VLLV (133) TND (113)	Multivariate logistic regression Outcome: LLV or combined LLV and RV Co-variates: age, sex, therapy combination (ref: INI-based regimens), duration of therapy, CDC stage (ref: A), years since diagnosis, co-infection with HBV and HCV,	PI-based regimens	0.26 (0.08-0.88)[1] P = 0.030
			NNRTI-based regimens	0.11 (0.03-0.43)[1] P = 0.001
			HIV-1 DNA in PBMC	1.01 (1.00–1.02)[1] P = 0.003

Reference	Groups (n)	Statistical method used by authors	Parameters associated with LLV	Reported OR (95% CI) or median (IQR) or mean (SD) or as otherwise stated and p-value
		HIV-1 DNA in PBMC, soluble CD14, IL-6 and TNFα.	Soluble CD14	1.66 (1.32–2.08)[1] P < 0.001
		Mann-Whitney with Bonferroni correction for LLV vs TND (1) and LLV vs TND (2)	TNFα	LLV: 25.6 pg/mL (22.7–30.1) VLLV: 23 pg/mL (18.2-26.2) TND: 15.5 pg/mL (13.1-21) P = 0.02 (1) and P = 0.046 (2)
		Kruskal-Wallis for VLLV vs TND	CD4 count	LLV: 565 cells/mm^3 (356-925) VLLV: 565 cells/mm^3 (303-795) TND: 641 cells/mm^3 (523-888) P = 0.017
(Saison et al., 2013)	LLV (44) TND (13)	Multivariate Cox regression model Outcome: TND 2 years post virological suppression Co-variates: age at HIV diagnosis and non-B subtype	B subtype	HR (95% CI): 3.98 (1.08–14.7) P = 0.037

Reference	Groups (n)	Statistical method used by authors	Parameters associated with LLV	Reported OR (95% CI) or median (IQR) or mean (SD) or as otherwise stated and p-value
(Torres et al., 2014)	LLV (17) TND (39)	Mann-Whitney	Activated monocytes in blood (CD14+/CD16+/CD163+)	LLV: 13.65% (4.02-22.17) TND: 7.65 (4.21-13.31) P = 0.029
		Mann-Whitney	Lipopolysaccharide-binding protein (LBP)	LLV: 9,964 ng/mL (8,452-13,213) TND: 8,574 ng/mL (7,820-9,548) P = 0.025

1: Proportional odds ratio of LLV vs combined RV and TND and combined LLV and RV vs TND.

2: Blip ratio was defined as the proportion of VL values >50 copies/mL to the number of VL measurements for the period from initiation of current ART until study inclusion

3: Not presented because not statistically significant or p >0.05.

LLV: low level viraemia; 95% CI: 95% confidence interval; IQR: interquartile; SD: standard deviation; VLLV: very low level viraemia; TND: target not detected; VL: viral load; NNRTI: non-nucleoside reverse transcriptase inhibitor; PI: protease inhibitor; BLQ: below limit of quantitation; ART: anti-retroviral therapy; TaqMan v2.0: Roche COBAS AmpliPrep/COBAS TaqMan HIV-1 Test v2.0; Abbott: Abbott RealTime HIV-1 Viral Load assay; MSM: men who have sex with men; InSTI: integrase strand transfer inhibitor; CDC: Centers for Disease Control; NRTI: nucleoside reverse transcriptase inhibitor; HBV: hepatitis B virus; HCV: hepatitis C virus; PBMC: peripheral blood mononuclear cells; IL-6: interleukin 6; TNFα: tumour necrosis factor aplha

Table 2.8: Comparison of performance of assays.

Reference	Comparisons	No of paired samples	VL range of samples, copies/mL	Comparison strategy	Results
(Do et al., 2011)	Amplicor v1.5 vs: TaqMan v1.0 (1) or TaqMan v2.0 (2) or RealTime (3)	391	TND - >100,000	Concordance analysis at 50 copies/mL cut-off with percent agreement and McNemar's test (p-value)	Overall agreement % (95% CI); p-value: (1): 91.8 (88.6-94.3); 0.0004 (2): 92.1 (88.9-94.5); 0.0196 (3): 90.5 (87.2-93.3); 0.2498
		(1): 19 (2): 20 (3): 14	50-200	Mean difference	log_{10} copies/mL (SE); 95% CI: (1): 0.225 (0.0931); 0.030-0.421 (2): 0.303 (0.1307); 0.030-0.577 (3): 0.316 (0.1074); 0.084-0.548
(Lima et al., 2009)	TaqMan v1.0 vs Amplicor v1.5	159	40-100	Analysis at 50 copies/mL cut-off with percent agreement and Cohen's Kappa	- 45% overall agreement - kappa statistic 0.19 (95% CI: 0.10-0.29) indicating slight agreement - mean difference log_{10} copies/mL (SD): 0.04 (0.01)

Reference	Comparisons	No of paired samples	VL range of samples, copies/mL	Comparison strategy	Results
					-0.46 sensitivity, 0.88 specificity, 0.94 positive predictive value, 0.29 negative predictive value
(Swenson et al., 2014)	RealTime vs TaqMan v1.0 (1) RealTime vs TaqMan v2.0 (2) Amplicor v1.5 vs TaqMan v1.0 (3) Amplicor v1.5 vs TaqMan v2.0 (4)	(1): 230 (2): 569 (3): 819 (4): 172	< 50 measured using the RealTime, (1)-(2) or Amplicor v1.5, (3)-(4)	Discordance at 50 copies/mL with presentation of percentages and absolute numbers	% (n) of samples with VL >50 copies/mL measured using the TaqMan v1.0 or v2.0 (1): 27 (63) (2): 13 (73) (3): 73 (598) (4): 6 (10)
(Yan et al., 2010)	Amplicor v1.5 vs TaqMan v1.0 (1) Amplicor v1.5 vs RealTime (2) TaqMan v1.0 vs RealTime (3)	(1): 103 (2): 133 (3): 166	LLoQ - 200	Cohen's kappa coefficient	Kappa coefficient (degree of agreement; 95% CI) (1): = 0.16 (slight: -0.03-0.35) (2): 0.14 (slight; 0.06-0.38) (3): 0.38 (fair; 0.22-0.53)

Reference	Comparisons	No of paired samples	VL range of samples, copies/mL	Comparison strategy	Results
(Wojewoda et al., 2013)	TaqMan v2.0 vs TaqMan v1.0	69	<200 copies/mL, including TND, as measured with the TaqMan v1	Frequencies	- 49% (n = 34) were quantifiable with TaqMan v1.0 and 54% (n = 37) with TaqMan v2.0 - 41% (n = 28) were quantifiable with both assays with mean difference -0.096 $\log_{10}$ copies/mL - 9% (n = 6) were TND with both assays
(Karasi et al., 2011)	TaqMan v2.0 vs RealTime (1) TaqMan v2.0 vs Amplicor v1.5 (2) RealTime vs Amplicor v1.5 (3)	(1): 328 (2): 309 (3): 209	<200 copies/mL including TND	Concordance analysis at 50 copies/mL with percent agreement and Wilsons efficient-score method (95% CI)	Overall agreement, % (95% CI): (1): 90.9% (87.9-93.1) (2): 88.3 (N/A) (3): 87.1 (N/A)
(Ruelle et al., 2012)	RealTime (1) vs TaqMan v2.0 (2) vs Versant kPCR v1.0 (3)	61	Detected VL <50 copies/mL	Frequencies of VL >50 copies/mL	% (n) (1): 3.3 (2) (2): 14.8 (9) (3): 6.6 (4)

VL: viral load; TaqMan v2.0: Roche COBAS AmpliPrep/COBAS TaqMan HIV-1 Test v2.0; RealTime: Abbott RealTime HIV-1 Viral Load assay; Amplicor v1.5: Roche COBAS Amplicor HIV-1 Monitor Test, v1.5; TaqMan v1.0: Roche COBAS AmpliPrep/COBAS TaqMan HIV-1 Test v2.0; TND: target not detected; 95% CI: 95% confidence interval; SE: standard error; SD: standard deviation; LLoQ: lower limit of quantitation; Versant kPCR v1.0: Siemens Versant HIV-1 RNA 1.0 kPCR

2.4 Discussion

Higher pre-ART VL, particularly above 5 $\log_{10}$ copies/mL, time since HIV diagnosis and CDC stage B/C have been associated with LLV in the published literature. The most consistent association was with pre-ART VL, which was identified as a strong predictor in three independent multivariate models that adjusted for variables including time since HIV diagnosis, duration of ART, ART combination, CD4 count, age, HIV mode of transmission and ART interruptions (Leierer et al., 2015, Martin-Blondel et al., 2012, Pascual-Pareja et al., 2010). Pre-ART VL has also been described as a predictor of the level of residual viraemia (Maldarelli et al., 2007, Palmer et al., 2008). These findings seem to reflect a larger cellular compartment harbouring integrated virus which was established prior to therapy commencement.

Falasca and colleagues measured HIV-1 DNA in PBMC and they found that they were independently and positively associated with LLV in a multivariate analysis (Falasca et al., 2017). However, it was unclear whether viral replication drove the increase in total HIV-1 DNA or higher proviral load caused LLV. The frequency of HIV infection in CD4 T cells residing in the periphery, such as the GALT, was higher than in circulating CD4 T cells and cross-infection between the two compartments was recorded (Chun et al., 2008). Virus production may take place in the GALT with virus shedding in the blood. Parallel measurement of the size of reservoirs and HIV-1 RNA level in the periphery is important, but also more challenging.

Shorter duration of viral suppression also related with LLV in three independent multivariate analysis (Leierer et al., 2015, Martin-Blondel et al., 2012, Pascual-Pareja et al., 2010). A rapid decline in VL post ART initiation (first and second phase of viral decay) is followed by a third and fourth phase of viral decay (Palmer et al., 2008). The third phase represents a cellular compartment with a half-life of 39 months causing a decrease in HIV-1 RNA levels below 50 copies/mL during the first couple of years of ART. The fourth phase reflects a stable reservoir with proliferative capacity that replenishes residual viraemia. Indeed, in one study using two cut-offs for ART duration one year of suppressive ART was less strongly associated with LLV below the LLoQ than 2 years (Martin-Blondel et al., 2012).

Patients on PI-based regimens also appeared to be more likely to experience LLV (Leierer et al., 2015, Martin-Blondel et al., 2012). PIs have a high genetic barrier to resistance and they

are preferred by clinicians in cases of suspected non-adherence issues. Although the selected studies did not report or adjust their models for adherence, a sensitivity analysis revealed that groups of patients at higher risk of non-compliance, such as intravenous drug users, were more likely to be on PI-based ART (Leierer et al., 2015). Similarly, NRTI-sparing regimens and use of InSTI were associated with LLV (Falasca et al., 2017, Reus et al., 2013). These combinations were not first-line options at the time of recruitment for most studies and they were possibly used to target mutated viruses that were either transmitted or evolved during periods of treatment interruptions. As to the association of LLV with non-B subtypes it is likely to reflect factors other than the actual virus sequence and subtype. Socio-economic considerations, health care access and delays in HIV diagnosis may cluster with non-B subtypes in some settings (Saison et al., 2013). Moreover, most patients (51 out of 57) were on PI-based ART which was not accounted for in the analysis. A review investigating LLV up to 500 copies/mL reported poor adherence as a risk factor (Ryscavage et al., 2014). Adherence is difficult to quantify, because it is a parameter based on patient-reporting and its accuracy may be questioned. However, it is an obvious cause of LLV and virological failure in a similar way as ART interruptions were independently associated with subsequent LLV (Leierer et al., 2015).

Markers of microbial translocation including soluble CD14, ribosomal bacterial DNA in blood, LBP and activated monocytes, were positively associated with LLV (Falasca et al., 2017, Reus et al., 2013, Torres et al., 2014). Increased levels of the inflammatory markers IL-6 and TNFα in patients with LLV may also be the result of increased microbial translocation (Falasca et al., 2017, Reus et al., 2013). CD4 depletion in the GALT during early infection causes microbial translocation, which declines with the reduction of VL following therapy initiation, but does not normalise (Jiang et al., 2009). Residual viral replication at the intestinal mucosa may cause compromisation of the GALT and microbial translocation. Alternatively, early events during the infection may have significant structural effects on GALT, which do not reverse with ART, resulting in chronic microbial translocation and immune activation with subsequent virus production. As to circulating CD4 counts, they were significantly lower in patients with LLV, but adjusting for co-founding factors such as duration of ART and CDC stage made their difference insignificant (Martin-Blondel et al., 2012).

We report that current literature varies remarkably as to the definition of LLV and study design. We applied strict eligibility criteria in an attempt to collect comparable data. Despite the small number of relevant studies (n=15) included, methodology strategies adopted by

authors differed significantly making impossible to conduct of a collective approach and comparison, such as a meta-analysis. For instance, two studies with similar design utilised different definitions for LLV with one adapting a VL between 20 and 200 copies/mL as determined from a single measurement and the other using a VL range between 20 and 50 copies/mL as measured on one or two occasions (Leierer et al., 2015, Martin-Blondel et al., 2012). Moreover, the control group in the former probably (but not clearly stated) included patients with detected VL below the LLoQ, whereas patients with detected but not quantified (VLLV) VL shaped a separate category in the latter. The same model was applied to the VLLV group and the same associations as the LLV group were identified with the only exception of treatment combination that was not different. Yet, both studies identified higher pre-ART VL, shorter ART duration and PI-based regimens as factors associated with LLV.

The same factors were also demonstrated in a work by *Vancoillie et al.*, but isolated blips were allowed in the "aviraemic" control group (Vancoillie et al., 2014). In literature isolated viral blips are generally accepted as insignificant clinical events reflecting random biological or statistical variation (Nettles et al., 2005). Blips were reported in 10% of chronically suppressed patients during routine follow-ups with a median VL of 78 and 116 copies/mL with the TaqMan v1.0 and TaqMan v2.0, respectively (Garrett et al., 2012). Also, 38% of LLV may also evolve in future blips and vice versa, more frequent blips may predict future LLV (Charpentier et al., 2012, Wojewoda et al., 2013). Hence, viral blips are different from LLV and as a result we decided to exclude them from this work. However, we included studies that defined LLV based on a single measurement and in this scenario a proportion would represent isolated blips and not persistent LLV. Only 2 studies used strict criteria in their definition of persistent LLV including at least two consecutive measurements of VL. As a result, the size of the case group is smaller compared to the control group. Of note, we report that the proportion of patients with LLV ranges in selected studies from 6% to 77%.

We utilised a time frame beginning in 2008, which is the transition year from a decade of use of the Amplicor method in clinical settings to third generation assays. The rationale was to reduce under-reporting of LLV <200 copies/mL as demonstrated in five selected studies (Do et al., 2011, Karasi et al., 2011, Lima et al., 2009, Swenson et al., 2014, Yan et al., 2010). Third generation real-time assays detect nucleic acids during the exponential phase of each amplification cycle producing faster results and reducing the risk of contamination. The main representatives of real-time platforms, the TaqMan v2.0 and RealTime, show good correlation of results within their entire range of reporting values (Do et al., 2011, Paba et

al., 2011, Swenson et al., 2014, Yan et al., 2010). However, concordance is getting poorer close to their LLoQ. Percent agreement at the 50 copies/mL cut-off was reported around 90% (Karasi et al., 2011, Swenson et al., 2014). Intra-assay variability of the RealTime and TaqMan v2.0 was significant with over half of clinical samples being discordant as to their detection status at the 50 copies/mL cut-off for at least one out of three replicates (Ruelle et al., 2009). Discordant results around the assay LLoQ differed between the two methods and were more pronounced for the TaqMan v2.0 compared to the RealTime. *Leierer et al.* observed that patient samples tested with the TaqMan v2.0 had higher frequency of LLV between 20 and 200 copies/mL compared to the RealTime (Leierer et al., 2015). However, more than half of them (57%) had a VL between 20 and 50 copies/mL for which both inter- and intra- assay repeatability is poor. Non-B HIV subtypes and sample handling may have affected reported results in the past, but they are less of an issue with new fully automated assays (Ndiaye et al., 2015, Parekh et al., 2018).

A main drawback of this systematic review is that it is based on a qualitative synthesis. We tried to present the data accurately within the context of the selected studies. Yet, there is an element of subjectivity. In addition, only one reviewer conducted the search and selection of studies as part of the original work presented by the student in this thesis. Moreover, all selected studies have a cross-sectional design and causality is not proven but assumed for factors preceding LLV.

Current literature addressing the issue of LLV is subject to variability due to lack of a broadly accepted definition which would specify range of HIV-1 RNA levels and duration. We propose that the factors identified in literature to be associated with LLV between 10 and 200 copies/mL reflected a larger size of cellular reservoirs, adherence issues and enhanced microbial translocation. Due to assay limitations, we need to treat with caution any findings regarding viraemia between 20 and 50 copies/mL. Further work is required to confirm predictors of LLV.

CHAPTER 3

Virological and immunological factors associated with residual viraemia in optimally treated patients

3.1 Introduction

Combination ART effectively suppresses viral replication, which is reflected in a steep decline in VL, but it cannot eradicate HIV as indicated by persistence of a latent reservoir and residual viraemia (Dornadula et al., 1999, Havlir et al., 2005, Maggiolo et al., 2012, Maldarelli et al., 2007, Palmer et al., 2008, Palmisano et al., 2005). Moreover, treatment cessation results in viral rebound with new cycles of viral production and infection (Wen et al., 2018). Replication-competent virus resides as integrated provirus mainly in resting CD4 T cells, a latent reservoir which is established early after infection and persists at stable levels in spite of effective ART (Ho et al., 2013, Hosmane et al., 2017, Ruggiero et al., 2015, Shen and Siliciano, 2008b, Siliciano et al., 2003, Siliciano and Siliciano, 2004). Ability to propagate via cell proliferation without requiring virus production and potential for generating intermittent bursts of virus production upon cell activation are key features of the HIV reservoir (Ananworanich et al., 2016, Bachmann et al., 2019, McManus WR et al., 2018, Pitman et al., 2018, Wang et al., 2018).

Residual viraemia is the presence of HIV-1 virus in the plasma at levels below the LoD of commercial assays. Residual viraemia is stable with a mean of 3 copies/mL and it is detected with ultrasensitive methods in the majority of ART-suppressed patients (Amendola et al.,

2011, Hatano et al., 2009, Margot et al., 2018, Palmer et al., 2008, Yukl et al., 2011). Palmer and colleagues found that all of their suppressed patients who were sampled longitudinally had at least one specimen with residual viraemia quantified with a SCA. Residual viraemia confirms the existence of an active, or intermittently active, HIV-1 reservoir. Yet, it remains to be elucidated which are the particular cell types or the circumstances that drive virus release. It has been proposed that direct or bystander activation of resting memory CD4 T cells harboring HIV-1 may result in intermittent virus shedding identified by SCA (Anderson et al., 2011, Hermankova et al., 2001, Kieffer et al., 2004). The clonal character of sequences found in residual viraemia indicate production of virus by a single type of cell with proliferative capacity (Sahu, 2015). The lack of occurrence of new drug resistance mutations following ART initiation and the inability of intensification strategies to clear residual virus in plasma are substantial arguments against ongoing cycles of viral replication and infection (Bailey et al., 2006, Brennan et al., 2009, Buzon et al., 2010, Hatano et al., 2011, McMahon et al., 2010).

Eradication of this cellular reservoir by the immune system appears unattainable. HIV-specific cytotoxic lymphocytes, although abundant during chronic infection, are unable to clear cellular reservoirs harboring integrated viral DNA (Lieberman et al., 2001). Antigen presentation to naïve T cells (CD45RA+) drives their differentiation to antigen-specific effector cells (CD45RA-) which reverse to a memory state, either as central (CCR7+) or effector (CCR7-), upon pathogen clearance (Ahmed and Gray, 1996, Bevan and Goldrath, 2000, Parish and Kaech, 2009, Seder and Ahmed, 2003, Wherry and Ahmed, 2004). Antigen persistence during chronic infection, on the other hand, results in functional impairment characterised by reduced cytokine production, proliferation and cytotoxicity (Wherry et al., 2003). Functional impairment appears to be associated with increased expression of PD1 (Barber et al., 2006, Freeman et al., 2006), which is also a marker of latency in HIV infection (Ghosn et al., 2018). PD1 binds to its ligands (PD-L1 and PD-L2) and approximates TCR while TCR interacts with antigens. The complex is a negative regulator of lymphocyte activation by downsizing intracellular signals (Freeman et al., 2000, Greenwald et al., 2005, Latchman et al., 2001, Okazaki and Honjo, 2007). PD1 overexpression on HIV-specific memory T cells has been observed and associated with cell exhaustion and apoptosis (Day et al., 2006, Petrovas et al., 2006).

We conducted a study in a strictly defined population of HIV-1 positive patients on first line ART experiencing consistent VL suppression since treatment commencement. Our aim was to identify virological and immunological factors associated with residual viraemia. We

focused on HIV-1 DNA load in PBMC, which is a good indicator of HIV-1 reservoirs and PD1 expression on CD4 and CD8 subsets, which appears to play an important role in chronic infection.

3.2 Methods

3.2.1 Study population

To circumvent heterogeneity and identify a highly stable on treatment population we applied strict eligibility criteria (Table 3.1). We screened the records of all patients attending the HIV clinic at the Royal Liverpool University Hospital between October 2013 and August 2014. Eligible patients were on first line combination ART with two NRTIs and either a NNRTI or a PI. We allowed a single switch of PI to another PI and modifications of the NRTI backbone for tolerability reasons. ART was commenced at least 6 months prior to recruitment and VL was regularly monitored with commercial assays at a minimum of two routine visits per year. VL was suppressed below 50 copies/mL within 6 months of ART commencement and remained persistently suppressed since and until all samples were collected. Patients on PIs were allowed single blips up to 200 copies/mL which were confirmed with suppressed VL preceding and following the isolated events of viraemia. Eligible patients had to report good adherence with no missed doses and no recorded treatment interruptions.

The study complied with the Standard Operating Procedures for Research Ethics Committees (REC) in the UK and received approval by the National Research Ethics Service (NRES) committee and local Research Development and Innovation (RD&I) department (REC: 10/H0808/75). All patients were provided with an information leaflet and signed a consent form. This project is funded by the Engineering and Physical Sciences Research Council (EPSRC) and it is included in the National Institute for Health Research Clinical Research Network (NIHR CRN) portfolio.

3.2.2 Sampling

Up to 36mL of whole blood was collected in EDTA tubes and processed within 1.5 hours of venipuncture to maximise yield and cell viability. Samples underwent centrifugation at 2,500 rpm for 10 min at 18^0C and plasma was immediately stored at $- 80^0$C. The remaining fractions were mixed with equal volume of Roswell Park Memorial Institute (RPMI) medium (80% RPMI 1640 and 20% fetal bovine serum [FBC]) and used to isolate PBMC by density gradient centrifugation. Briefly, 20mL of diluted sample was layered on 15mL of Ficoll-Paque

medium and centrifuged at 500 xg for 30 min with no break at 18^0C. The PBMC layer was harvested and washed twice to remove Ficoll-Paque and platelet contaminants. The washing steps included resuspension in RPMI medium up to a total volume of 50 mL and centrifugation at 300 xg for 10 minutes (18^0C).

Cells were resuspended in 10 mL of RPMI medium and the number of viable cells was counted with the haematocytometer. Tryptan Blue stain 0.4% was mixed with the cells at a dilution 1:10 (10 µL of specimen and 90 µL of stain). Using a hand tally counter the number of live cells (unstained by Tryptan Blue) were counted in all four corner squares (a). The total number of cells was calculated from the formula: (a/4)*10*(10^4)*10. The viability requirement was set at >90%. If less than 90% of cells were viable, another aliquot was tested. In cases of viability <90% in the repeated aliquot, the sample was discarded and a new sample from the patient was sought. However, cell viability was above 97% in all samples and none of the above was required. Cells for freezing were centrifuged and resuspended in cold sterile freezing medium made up of 80% FBS and 20% dimethylsulphoxide (DMSO). Aliquots were then placed in Mr Frosty, a container with isopropyl alcohol, which was kept for at least 2.5 hours at -80^0C. This way the temperature was gradually decreased to -80^0C at cooling rate of about 1^0C/min to avoid osmotic intracellular dehydration. Aliquots were transferred to liquid nitrogen the following day. Fresh PBMC aliquots (10×10^6) were used immediately for flow cytometry. Aliquots of 5×10^6 were stored in 1mL freezing medium at -80^0C or liquid nitrogen. This process was carried out by three colleagues, Yufei Wang (post-doctoral researcher), Alessandra Ruggiero (doctoral student) and me.

3.2.3 Ultrasensitive quantification of plasma HIV-1 RNA

HIV-1 RNA levels were measured with a validated in-house ultrasensitive assay that applied a modified version of the RealTime assay on the Abbott m2000 (sp/rt) platform (Maidenhead, UK) (Kiselinova et al., 2015, Ruggiero et al., 2015). A total of 8 mL of plasma was ultra-centrifuged at 35,000 rpm (209,490.6 xg) for 15 min at 4^0C with a centrifuge rotor Beckman SW41. The pellet was resuspended in 1 ml of basematrix (SeraCare, USA) and processed on the Abbott m2000 platform as per the manufacturer's instructions. The assay employs automated RNA extraction, followed by cDNA transcription and amplification using internally controlled real-time PCR. Three types of results were reported: (i) HIV-1 RNA copies/mL if the level was above the LLoQ, (ii) target detected if below the LLoQ but above

the LoD and (iii) target not detected if less than the LoD. For samples reported in (i) and (ii) the output Ct value was used in a formula with the run intercept and slope values to estimate the number of copies per mL of plasma (VL = $10^{[(Ct-Intercept)/Slope]}/8$). Results were reported in copies/mL.

The assay was previously validated by spiking HIV-negative plasma with four serial dilutions of the WHO 3rd International HIV-1 RNA Standard in quadruplicates (Palmer et al., 2003). The assay detection rates for 5, 3 and 1 HIV-1 RNA copies/mL were 100%, 80% and 60%, respectively (Ruggiero et al., 2015).

3.2.4 Quantitation of cellular HIV-1 DNA

3.2.4.1 DNA extraction

HIV-1 DNA load was measured using cryopreserved PBMC. For each patient, an aliquot of 5 x 10^6 PBMC stored at -80^{0}C was thawed to room temperature. DMSO-containing freezing solution was immediately removed with two washing steps by adding phosphate-buffered saline (PBS) and centrifugation at 800 xg for 10 min on a bench microcentrifuge. Cell death and release of viscous DNA causing cell clumping was expected to be minimal only from thawing, as cells were frozen gradually with Mr Frosty. Cells were resuspended in 200 µL of PBS. The QIAamp® DNA Mini and Blood Mini kit (Thermo Fisher Scientific) was used to isolate DNA as described by Geretti *et al.* (Geretti et al., 2013a). In brief, cell lysis was performed with addition of Proteinase K (20 µL) and lysis buffer (200 µL) and incubation at 56^{0}C for 30 min and 100^{0}C for 30 min. DNA was then purified with QIAamp Mini spin columns following the manufacturer's instructions to yield a 100 µL DNA eluate which was stored at -20^{0}C until DNA quantification.

3.2.4.2 DNA quantitation

Total DNA concentration in extracts was determined by a spectrophotometer (Thermo Scientific NanoDropTM 1000) at an optical density of 260 nm. DNA concentration was adjusted to 50 ng/µL in a volume of 50 µL, which corresponds to 1 µg of double stranded DNA per PCR input volume (20 µL) and 150,000 cells (Dib et al., 1996). The same buffer as the eluate (Buffer AE, Qiagen) was used as blank before measurements and to dilute DNA. DNA purity was assessed with the 260/280 and 260/230 ratios. Any ratio lower than 1.8

indicated contamination (e.g. protein) and extraction was repeated. Diluted samples were checked for concentration and readings were recorded, or further dilutions were performed if required.

Total HIV-1 DNA load in PBMC was quantified by a quantitative real-time PCR using the Applied Biosystems 7500 Real-Time PCR platform. A standard curve incorporating four quantities of HIV-1 DNA (6,000, 600, 60 and 6 copies/reaction) was produced for every run using the Generic HIV DNA CELL kit (Biocentric Laboratories, France) (Avettand-Fenoel et al., 2009). A 50 µL volume per reaction contained 1 µg of DNA extract. The target was a conserved LTR region. Thermal cycles included 2 minutes at 50^0C (uracil-DNA glycosylases incubation), 10 minutes at 95^0C (polymerase activation) and 50 cycles of 15 seconds at 95^0C (denaturation/melting step) and 1 minute at 60^0C (hybridisation/annealing step). The software reported HIV-1 DNA copies/µg (b) which was converted into HIV-1 DNA copies/10^6 PBMC using the formula: b * 10^6 / 150,000.

3.2.5 PD1 expression on CD4 and CD8 T cell subsets

A post-doctoral researcher in the group (Yufei Wang) performed all immunophenotyping on fresh PBMC isolated from whole blood within 2 hours of collection[1]. Immunophenotyping was part of a separate project and I was given access to the final data only. An aliquot of 10 million PBMC was used. PBMCs were stained for markers of differentiation (CD3, CD4, CD8, CD45RA, CCR7) and exhaustion (PD1) with directly conjugated antibodies purchased from Biolegend (CD3/4/8-PE, CD45RA-perCP, CCR7-FITC, PD1-APC). Markers co-expression was measured on the BD FACSCalibur platform as per manufacturer instructions and raw data were processed with the accompanying software.

3.2.6 Statistical analysis

Statistics were conducted with SPSS version 25 (IBM). Continuous variables that were normally distributed are presented as means with SD whereas continuous variables that did not have normal distribution are presented as medians and IQR. Normality of continuous variables was assessed through Shapiro-Wilk test (Shapiro and Wilk, 1965). Categorical

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1: All experiments were performed by myself, unless otherwise stated.

variables are presented with frequencies and percentages. We assigned the value of 0.5 copies/mL to undetectable HIV-1 RNA with the SCA to use in descriptive statistics and further analysis. A comparative analysis between patients with detectable and undetectable HIV-1 RNA below 50 copies/mL was conducted using the chi-square or Fisher's exact test for categorical variables. Student's t-test and Mann-Whitney test were applied to continuous variables that were normally and non-normally distributed, respectively. Levene test was applied to confirm the assumption of equal variance between groups before proceeding with comparison of means. Univariate and multivariate logistic regression models were used to explore factors associated with residual viraemia. The outcome (dependent) variable was detectable HIV-1 RNA with the SCA. A stepwise model selection process was used for the adjusted analysis; initially every variable from the univariate analysis with a p value of less than 0.20 was added in the model and then the non-significant ones were removed sequentially with the "Backward: LR" method in SPSS. Predictor variables were tested for multicollinearity before included in the model with the variance inflation factor (VIF) being less than 5. The analysis was performed under the kind guidance of Eftychia Psarelli, a senior statistician in Molecular and Clinical Cancer Medicine, at the University of Liverpool.

Table 3.1: Eligibility criteria for inclusion in the study.

Eligibility criteria
Adult (>16 years old) HIV-1 positive patients
First line ART with two NRTIs and either an NNRTI or a PI
No changes of the NNRTI
Changes from one PI to another PI allowed
Changes of the NRTI arm allowed within the same drug class
ART commenced at least 6 months prior to recruitment
Viral load suppression <50 copies/mL documented within 6 months of starting ART
No recorded viral load rebound[1]   No treatment interruption   At least two viral load measurements documented per year

1: Episodes of viral rebound were defined as VL >50 copies/mL for the NNRTI-based group and VL >200 copies/mL for the PI group. Isolated blip events between 50 and 200 copies/mL were permitted in patients on PIs.

ART: anti-retroviral therapy; NRTI: nucleoside reverse transcriptase inhibitor; NNRTI: non-nucleoside reverse transcriptase inhibitor; PI: protease inhibitor; VL: viral load

3.3 Results

3.3.1 Study population

A total of 59 HIV-1 positive patients were recruited. Three moved to another region before sampling and we had inadequate blood volume for both virological and immunological experiments for three, yielding 53 patients for analysis. Their characteristics are shown in Table 3.2. In brief, all subjects were receiving ART with either an NNRTI (n=42, 79%) or a ritonavir-boosted PI (n=11, 21%). At sampling patients were on ART for a median duration of 5.1 years (IQR: 1.7-7.2) with VL suppression (<50 copies/mL) for a median of 4.8 years (IQR: 1.5-7.0). The shortest duration of ART was almost a year (34 weeks) and the longest 16.5 years. The most commonly used ART combination was TDF/emtricitabine (FTC) plus efavirenz (EFV) or darunavir/ritonavir (DRV/r) (Table 3.2). In the PI group, only 2 subjects changed from the initial PI (lopinavir/ritonavir and saquinavir/ritonavir were switched to DRV/r and atazanavir/ritonavir due to diarrhoea and hypercholesterolaemia, respectively). There were 15 cases of NRTI changes in 12 patients (Appendix, table A.2).

3.3.2 Virological parameters

Residual HIV-1 RNA in plasma was detected in 19 patients. Almost two thirds (n=34, 64.2%) of all patients had undetectable HIV-1 RNA in the plasma. Although almost one third (n=13, 31%) experienced detectable HIV-1 RNA in the group of patients receiving NNRTI-based regimens, more than half (n=6, 54.5%) of patients on a PI-based regimen had residual viraemia quantified with our SCA. Reflecting these findings, the median was an undetectable HIV-1 RNA (assigned an arbitrary level of 0.5 copies/mL, IQR: 0.5-2) overall, and 2 copies/mL (IQR: 0.5-3) in patients on PI-based regimens (Table 3.2). Residual HIV-1 RNA which was quantified with our SCA had a median of 3 copies/mL (IQR: 2-7). Only three patients had viraemia quantified above 10 copies/mL. The highest value of 38 copies/mL was measured in a subject who at the time of sampling had been receiving TDF/FTC/DRV/r for 45 weeks and had shown a suppressed VL (<50 copies/mL) for 23 weeks, which was the shortest duration of suppression in our cohort.

Total HIV-1 DNA had a median of 2.6 $\log_{10}$ copies/10^6 PBMC (IQR: 2.4-3.1) and a range between 1.74 and 3.59 $\log_{10}$ copies/10^6 PBMC.

We first explored differences between groups with undetectable (n=34) and detectable HIV-1 RNA in plasma (n=19) combining the NNRTI and PI group together (Table 3.3). HIV-1 DNA load in PBMC, PD1 expression on effector memory CD4 T cells and on central and effector memory CD8 T cells differed significantly among the two groups (Figure 3.1). We noted that duration of suppression below 50 copies/mL and ART duration were shorter in the group with detected HIV-1 RNA in plasma, although that was not statistically significant.

3.3.3 Immunological parameters

CD4 and CD8 were balanced with a ratio of 1. The highest PD1 expression was observed on effector memory CD4 T cells (CD4+/CD45RA-/CCR7-) and the lowest on naive CD4 T cells (CD4+/CD45RA+) as shown in Table 3.2. CD4+ and CD8+ cells had similar expression levels of PD1. However, PD1 expression was higher on the effector memory CD4 subset compared to CD8 and naïve CD8 subset. PD1 expression on all CD4 and CD8 subsets was higher in patients receiving NNRTI-based ART compared to PI-based ART with the differences being more prominent for CD8 than CD4 cells.

PD1 expression on total and memory CD4 and CD8 subsets was consistently higher in patients with undetectable HIV-1 RNA compared to the group with detectable HIV-1 RNA (Figure 3.2). The highest PD1 expression was observed on effector memory CD4 cells in both undetected and detected groups. There was a consistent 5% difference between the two groups for total and central memory CD4 cells and this difference more than tripled for effector memory CD4 cells (17%). A similar trend was demonstrated among CD8 subsets with PD1 expression on effector memory CD8 cells being also the highest, but the difference between the two groups of patients remained stable around 10% for all memory subsets (Figure 3.2).

3.3.4 Factors associated with residual viraemia

Factors independently associated with the detection of residual HIV-1 RNA were first explored by univariate logistic regression analysis and the results are summarized in Table 3.4. There was an association between the detection of HIV-1 RNA and total HIV-1 DNA in PBMC, and PD1 expression on effector memory CD4 cells and central and effector memory CD8 cells. More specifically, for every unit increase in the $\log_{10}$ DNA copies, the odds of

detectable HIV-1 RNA were increasing 9 times, although with 95% assurance this estimate could have been between 1.8 to 45 times greater (p = 0.007). Similarly, for every unit increase in the % of PD1 expression on CD4+/CD45RA-/CCR7-, the odds of residual viraemia as quantified with our SCA were decreased by 4%, although with 95% assurance this estimate could have been between 1% and 8% (p=0.034).

Age, third agent, total HIV-1 DNA in PBMC, time from suppression to recruitment, CD8%, PD1 on CD8+ (%), PD1% on CD8+/CD45RA-/CCR7+ (%), PD1 on CD8+/CD45RA-/CCR7- (%) and PD1 on CD4+/CD45RA-/CCR7- (%) had a p-value less than 0.2 in the univariate analyses and passed the multicollinearity check allowing for inclusion as predictor variables in the logistic regression (VIF for PD1 on CD8+/CD45RA- was 7.38 and the variable was excluded). The relation between total HIV-1 DNA and residual viraemia remained significant adjusting for PD1 expression on effector memory CD4 T cells and age in the final model (Table 3.5). The steps to the final model are shown in Appendix, Table A.3.

Figure 3.1: Factors associated with residual viraemia in the univariate analysis.
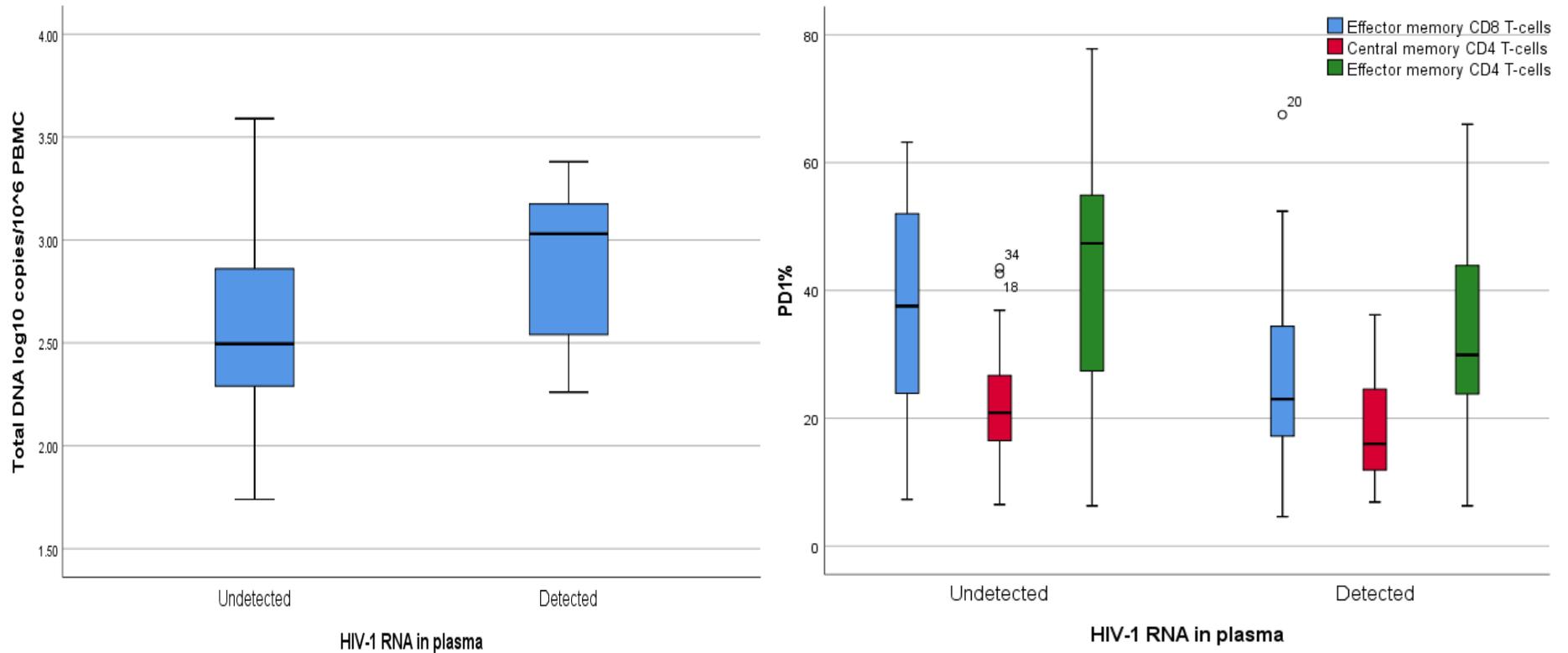
(a) median total HIV-1 DNA in the group with undetectable and detectable HIV-1 RNA in plasma and (b) median PD1 expression on CD4 and CD8 subsets in the group with undetectable and detectable HIV-1 RNA in plasma. P-value <0.05

Figure 3.2: *PD1 expression (%) on CD4 and CD8 subsets in patients with undetected and detected HIV-1 RNA in plasma.*
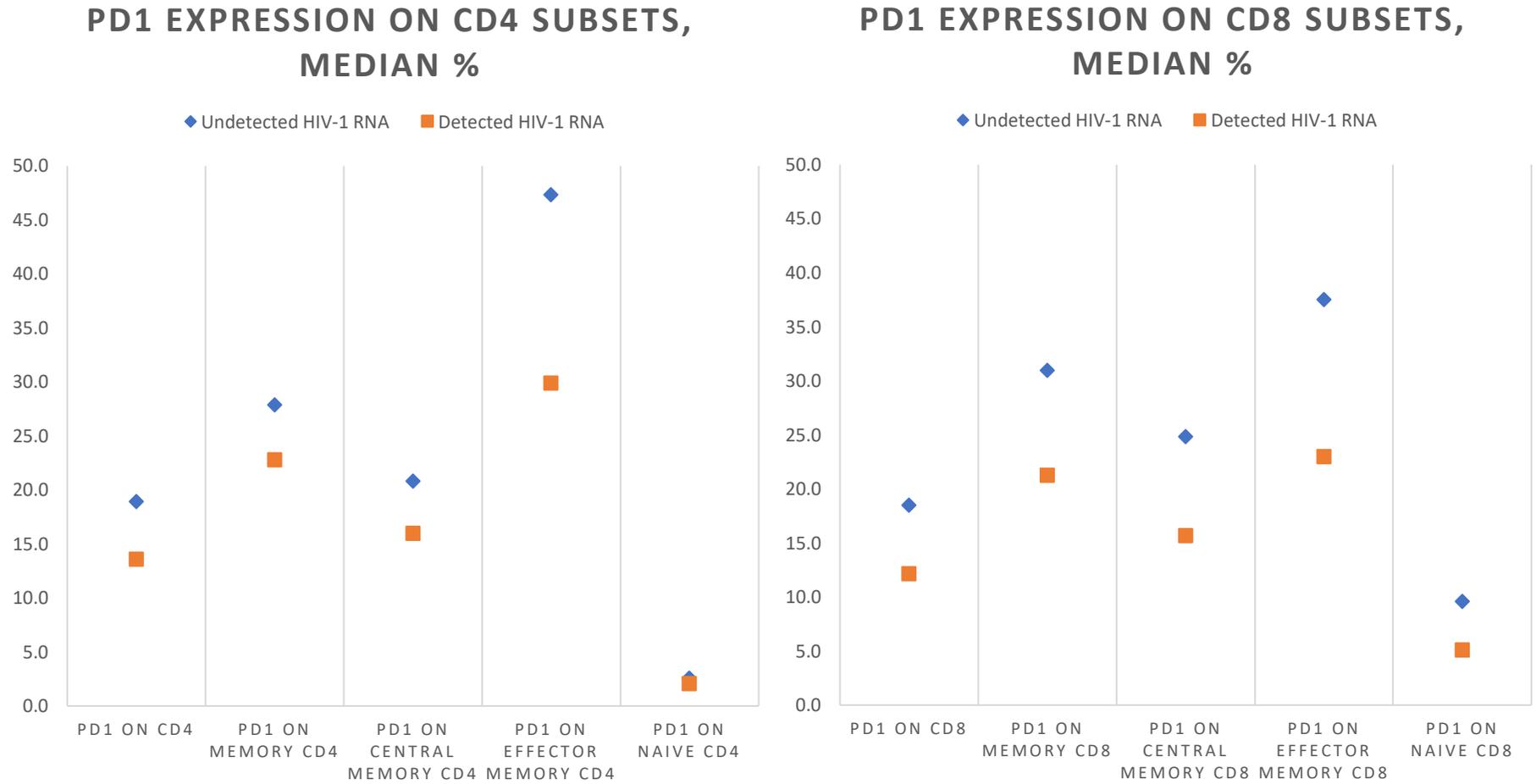
Table 3.2: Baseline characteristics of the study population overall and stratified by therapy as non-nucleoside reverse transcriptase inhibitor and protease inhibitor -based regimens.

Characteristic	Total (n=53)	NNRTI (n=42)	PI (n=11)
Age (years), mean (SD)	43.5 (9.9)	44.4 (10.1)	40.2 (8.5)
Gender, n (%) Male	28 (53)	25 (59)	3 (27)
Female	25 (47)	17 (41)	8 (73)
Ethnicity, n (%) Black	20 (38)	14 (33)	6 (55)
White	33 (62)	28 (67)	5 (45)
Risk group, n (%) Heterosexual	32 (60)	24 (57)	8 (73)
MSM	18 (34)	16 (38)	2 (18)
Other	3 (6)	2 (5)	1 (9)
Time from diagnosis to start of ART), median months (IQR)	9.9 (1.9 – 44.2)	10.0 (2.0 – 42)	4.9 (3.3 – 5.9)
HIV-1 clade, n (%) B	23 (43)	19 (45)	4 (36)
C	9 (17)	5 (12)	4 (36)
A	1 (2)	0	1 (9)
Unknown	20 (38)	18 (43)	2 (18)
TDR, n (%) Yes	12 (23)	5 (12)	7 (64)
No	34 (64)	31 (74)	3 (27)
Missing	7 (13)	6 (14)	1 (9)
Nadir CD4 count (cells/mm^3), mean (SD)*	198 (108)	191 (109)	222 (109)
Nadir CD4 count (cells/mm^3), median (IQR)*	183 (124-276)	179 (125-239)	233 (139 – 313)
Pre-ART VL (log$_{10}$ copies/mL), median (IQR)	4.8 (4.5 – 5.2)	4.8 (5 – 5.2)	4.9 (0.8) [†]
Calendar year for ART commencement, median (IQR)	2009 (2006 – 2012)	2008 (2006-2011)	2011 (2009-2012)
ART composition, n (%) EFV	38 (72)	38 (90)	N/A
NVP	2 (4)	2 (5)	N/A
RPV	2 (4)	2 (5)	N/A
DRV/r	8 (15)	N/A	8 (73)

Characteristic	Total (n=53)	NNRTI (n=42)	PI (n=11)
LPV/r	2 (4)	N/A	2 (18)
ATV/r	1(2)	N/A	1 (9)
TDF/FTC	39 (74)	31 (74)	8 (73)
ABC/3TC	12 (22)	11 (26)	1 (9)
ABC/AZT	2 (4)	0	2 (18)
No of NRTI changes, n 1	11	8	3
2-3	2	2	0
No of PI changes, n 1	2	N/A	2
Duration of ART (years), median (IQR)	5.1 (1.7 – 7.2)	5.9 (2.5 – 7.8)	2.8 (1.3 – 5.2)
Duration of viral load suppression (years), median (IQR)	4.8 (1.5 – 7.0)	5.2 (3.3) [†]	1.9 (0.8 – 4.8)
Residual HIV-1 RNA, median copies/mL (IQR)	0.5 (0.5-2)	0.5 (0.5-1.8)	2 (0.5-3)
Total HIV DNA, median $\log_{10}$ copies/10^6 PBMC (IQR)	2.6 (2.4 – 3.1)	2.6 (0.4) [†]	3.1 (0.4) [†]
CD4 count mean cells/mm^3, (SD)	650 (223)	637 (220)	695 (241)
CD4 (%), mean (SD)	37.5 (8.2)	37.6 (8.3)	37 (8.5)
CD8 (%), mean (SD)	38.5 (11.3)	38.4 (10.6)	39 (14.0)
CD4/CD8 ratio, median (IQR)	1.0 (0.7 – 1.4)	1.0 (0.7 – 1.4)	1.1 (0.5) [†]
PD1 on CD8+ (%), median (IQR)	17.1 (10.2 – 24.0)	19.1 (9.0) [†]	12.1 (6.1) [†]
PD1 on CD8+/CD45RA- (%), median (IQR)	28.0 (18.9 – 39.0)	32.3 (14.9) [†]	22.5 (14.6) [†]
PD1 on CD8+/CD45RA-/CCR7+ (%), median (IQR)	22.6 (14.3 – 35.8)	25.7 (13.9) [†]	19.5 (13.2) [†]
PD1 on CD8+/CD45RA-/CCR7- (%), median (IQR)	32.3 (20.7 – 48.0)	36.7 (16.9) [†]	20.8 (15.1) [†]

Characteristic	Total (n=53)	NNRTI (n=42)	PI (n=11)
PD1 on CD8+/CD45RA+ (%), median (IQR)	6.4 (3.9 – 11.9)	7.2 (4.0 – 13.6)	5.7 (3.1) [†]
PD1 on CD4+ (%), median (IQR)	17.6 (11.5 - 25.1)	18.5 (11.8 – 25.2)	16.6 (7.4) [†]
PD1 on CD4+/CD45RA- (%), median (IQR)	25 (18.9 – 31.9)	26.8 (21.9 – 33)	23.4 (10.7) [†]
PD1 on CD4+/CD45RA-/CCR7+ (%), mean (SD)	21.3 (9.0)	22.1 (8.8)	18.0 (9.3)
PD1 on CD4+/CD45RA-/CCR7- (%), mean (SD)	40.2 (16.3)	42.5 (16.2)	31.3 (13.9)
PD1 on CD4+/CD45RA+ (%), median (IQR)	2.4 (1.0 – 4.7)	2.5 (1.0 – 5.9)	2.4 (1.7 – 2.7)

*Shapiro Wilcoxon test indicated a normal distribution for nadir CD4 count (P >0.05). Here we present both mean and median as per Shapiro Wilcoxon test and as frequently encountered in literature, †: mean (SD)

NNRTI: non-nucleoside reverse transcriptase inhibitor; PI: protease inhibitor; VL: viral load; SD: standard deviation; MSM: men who have sex with men; IQR: interquartile; TDT: transmitted drug resistance; ART: anti-retroviral therapy; VL: viral load; EFV: efavirenz; NVP: nevirapine; RPV: rilpivirine; DRV/r: darunavir/ritonavir; LPV/r: lopinavir/ritonavir; ATV/r: atazanavir/ritonavir; TDF/FTC: tenofovir/emtricitabine; ABC/3TC: abacavir/lamivudine; ABC/AZT: abacavir/zidovudine

Table 3.3: Comparative analysis of the group with undetected HIV-1 RNA in plasma vs. detected HIV-1 RNA in plasma.

Characteristic	Undetected HIV-1 RNA (n=34)	Detected HIV-1 RNA (n=19)	p-value
Age (years), mean (SD)	44.9 (9.9)	40.9 (9.7)	0.162*
Gender, n (%) Male	17 (61)	11 (39)	0.581†
Female	17 (68)	8 (32)	
Ethnicity, n (%) Black	13 (65)	7 (35)	0.920†
White	21 (64)	12 (36)	
Risk group, n (%) Heterosexual	22 (69)	10 (31)	0.716‡
MSM	10 (56)	8 (44)	
Time from diagnosis to start of ART), median months (IQR)	9.5 (1.8 – 37.1)	16.1 (1.9 – 54.3)	0.711**
TDR, n (%) Yes	6 (50)	6 (50)	0.308‡
No	22 (65)	12 (35)	
Nadir CD4 count (cells/mm^3), mean (SD)¥	195 (119)	202 (89)	0.818*
Nadir CD4 count (cells/mm^3), median (IQR)¥	172 (114-242)	203 (147-288)	0.565**
Pre-ART VL (log$_{10}$ copies/mL), median (IQR)	4.7 (4.6 – 5.2)	4.9 (4.5 – 5.0)	0.857**
Third agent, n (%) NNRTI	29 (69)	13 (31)	0.136‡
PI	5 (45)	6 (55)	
Duration of ART (years), median (IQR)	5.9 (2.4-8.0)	3.9 (1.6-6.0)	0.111**
Duration of viral load suppression (years), median (IQR)	5.6 (1.5-7.8)	3.5 (1.4-5.9)	0.097**
Total HIV DNA, median log$_{10}$ copies/10^6 PBMC (IQR)	2.5 (2.3 – 2.9)	3.0 (2.5 – 3.2)	*0.004**

Characteristic	Undetected HIV-1 RNA (n=34)	Detected HIV-1 RNA (n=19)	p-value
CD4 count mean cells/mm^3, (SD)	627 (223)	691 (224)	0.359*
CD4 (%), mean (SD)	38.4 (7.7)	35.9 (9.0)	0.297*
CD8 (%), mean (SD)	36.7 (10.5)	41.7 (12.2)	0.124*
CD4/CD8 ratio, median (IQR)	1.0 (0.8 – 1.5)	1.0 (0.6 – 1.4)	0.250**
PD1 on CD8+ (%), median (IQR)	18.5 (12.7 – 27.6)	12.1 (9.2 – 18.2)	0.064**
PD1 on CD8+/CD45RA- (%), median (IQR)	31.0 (2.07 – 41.9)	21.3 (15.3 – 32.4)	0.097**
PD1 on CD8+/CD45RA-/CCR7+ (%), median (IQR)	24.9 (17.8 – 37.3)	15.7 (10.3 – 25.2)	*0.034**
PD1 on CD8+/CD45RA-/CCR7- (%), median (IQR)	37.5 (23.9 – 52.0)	23.0 (16.5 – 34.8)	*0.022**
PD1 on CD8+/CD45RA+ (%), median (IQR)	9.6 (3.9 – 13.8)	5.1 (3.9 – 9.0)	0.179**
PD1 on CD4+ (%), median (IQR)	19.0 (2.6 – 27.9)	13.6 (2.1 – 22.8)	0.383**
PD1 on CD4+/CD45RA- (%), median (IQR)	25.2 (3.7 – 33.9)	22.6 (6.7 – 27.8)	0.105**
PD1 on CD4+/CD45RA-/CCR7+ (%), mean (SD)	22.4 (8.8)	19.2 (9.1)	0.219*
PD1 on CD4+/CD45RA-/CCR7- (%), mean (SD)	43.9 (16.0)	33.7 (15.2)	*0.028*
PD1 on CD4+/CD45RA+ (%), median (IQR)	12.0 (1.0 – 22.0)	11.6 (1.4 – 17.5)	0.809**

*Student's t-test (normally distributed continuous variables), †: Chi-squared test (categorical variables), **: Mann-Whitney test (non-normal continuous variables), ‡: Fisher's Exact test (categorical variables with at least one expected frequency cell ≤5), ¥: Shapiro Wilk test

indicated a normal distribution for nadir CD4 count (P >0.05). Here we present both mean and median as per Shapiro Wilcoxon test and as frequently encountered in literature

VL: viral load; SD: standard deviation; MSM: men who have sex with men; IQR: interquartile; TDT: transmitted drug resistance; ART: anti-retroviral therapy; VL: viral load; TDF: tenofovir; FTC: emtricitabine; EFV: efavirenz; ABC: abacavir; 3TC: lamivudine; RPV: rilpivirine; NVP: nevirapine; DRV: darunavir; r: ritonavir; AZT: zidovudine; LPV: lopinavir; ATV: atazanavir

Table 3.4: Univariate logistic regression of clinical, virological and immunological factors on the outcome of residual viraemia detection.

Factor	Odds Ratio	95% CI	*p*-value
Age (years)	0.96	[0.90 – 1.02]	0.163
Gender (F vs. M)	0.73	[0.23 – 2.25]	0.581
Ethnicity (Black vs. White)	0.94	[0.29 – 3.00]	0.920
Risk group (MSM/Other vs. Hetero)	1.65	[0.53 – 5.17]	0.390
Time from diagnosis to start of ART (months)	1.01	[0.99 – 1.02]	0.533
HIV-1 clade (B vs. A/C)	0.80	[0.17 – 3.69]	0.775
TDR (Yes vs. No)	1.83	[0.48 – 6.95]	0.373
Nadir CD4 count (ln cells/mm^3)	1.26	[0.59 – 2.72]	0.547
Pre-ART VL (log$_{10}$ copies/mL)	1.02	[0.45 – 2.32]	0.957
Third agent (PI vs. NNRTI)	2.68	[0.69 – 10.38]	0.154
Time from suppression to recruitment (years)	0.84	[0.70 – 1.01]	0.068
Total HIV-1 DNA (log$_{10}$ copies/10^6 PBMC)	9.15	[1.85 – 45.4]	*0.007*
CD4 count (ln cells/mm^3)	2.45	[0.42 – 14.4]	0.319
CD4 (%)	0.96	[0.90 – 1.03]	0.293
CD8 (%)	1.04	[0.99 – 1.10]	0.126
CD4/CD8 ratio	0.50	[0.15 – 1.60]	0.243
PD1 on CD8+ (%)	0.94	[0.87 – 1.01]	0.071
PD1 on CD8+/CD45RA- (%)	0.97	[0.93 – 1.01]	0.119
PD1 on CD8+/CD45RA-/CCR7+ (%)	0.95	[0.91 – 0.99]	*0.049*
PD1 on CD8+/CD45RA-/CCR7- (%)	0.96	[0.92 – 0.99]	*0.029*
PD1 on CD8+/CD45RA+ (%)	0.92	[0.83 – 1.02]	0.117
PD1 on CD4+ (%)	0.97	[0.91 - 1.4]	0.374
PD1 on CD4+/CD45RA- (%)	0.96	[0.90 – 1.01]	0.142
PD1 on CD4+/CD45RA-/CCR7+ (%)	0.96	[0.89 – 1.03]	0.218
PD1 on CD4+/CD45RA-/CCR7- (%)	0.96	[0.92 - 0.99]	*0.034*

Factor	Odds Ratio	95% CI	*p*-value
PD1 on CD4+/CD45RA+ (%)	1.02	[0.94 – 1.12]	0.575

CI: confidence interval; F: female; M male; MSM: men who have sex with men; Hetero: heterosexual; TDT: transmitted drug resistance; ART: anti-retroviral therapy; VL: viral load; NNRTI: non-nucleoside reverse transcriptase inhibitor; PI: protease inhibitor; PBMC: peripheral mononuclear cells

Table 3.5: Multivariate logistic regression model of factors associated with detection of residual viraemia in plasma.

Factor	Odds Ratio	95% CI	*p*-value
Total HIV-1 DNA, $\log_{10}$copies/10^6 PBMC	3.42	[1.32 – 8.83]	*0.011*
PD1 on CD4+/CD45RA-/CCR7-	0.97	[0.93 – 1.00]	0.05
Age	0.95	[0.89-1.01]	0.072

CI: confidence interval

3.4 Discussion

This study investigated a cohort of successfully treated HIV-1 patients on first line ART and no evidence of virological failure for a duration of around 5 years. HIV-1 RNA in plasma was detected and quantified with a SCA in almost one third of patients and the median was 3 copies/mL. Previous studies have shown that residual viraemia of this level has identical HIV sequences implying that the source is a clonally expanded infected cell (Bailey et al., 2006, Bozzi et al., 2019, Jacobs et al., 2019, Rosenbloom et al., 2017). Hence, ongoing virus replication is probably rare, if not absent at all, in our cohort of patients. Antigen or cytokine triggered activation of cells harboring HIV-1 DNA is responsible for production of virus (McManus WR et al., 2018, Wang et al., 2018). Reservoirs are maintained by proliferation and escape clearance by cytotoxic cells (Shen and Siliciano, 2008b, Zheng et al., 2014). Immune checkpoints such as the PD1 play a critical role in responses which are pivotal in controlling HIV-1 replication and viraemia (Schmitz et al., 1999, Zheng et al., 2014) and appear to be a mechanism of short-term latency (Boyer and Palmer, 2018).

Patients on a PI-based regimen demonstrated some trends different from the group of patients on NNRTI-based ART. Subjects on PIs were allowed to have changes of the PI arm and isolated VL measurements up to 200 copies/mL post suppression. The rationale was that PI-based regimens exhibit increased toxicity and pharmacokinetics which make drug switches and isolated episodes of viraemia (viral blips), respectively, more frequent than NNRTIs. Moreover, blips of this magnitude have not been associated with virological failure (Grennan et al., 2012, Raffi et al., 2017). Regardless, the number of patients on PIs was very small and only one fifth of our population. Although our study lacked sufficient statistical power to find differences by regimen, we observed some trends. Subjects receiving PI-based ART compared to NNRTI-based ART appeared to have a distinct virological profile with a higher frequency of transmitted drug resistance mutations, detectable HIV-1 RNA in plasma and total HIV-1 RNA in PBMC, shorter duration of ART and lower PD1 expression on CD4 and CD8 subsets compared to the NNRTI group. These observations agree with previous studies suggesting that PI-based ART is less suppressive than NNRTI and InSTI based regimens (Bonora et al., 2009, Darcis et al., 2020, Geretti et al., 2019, Gianotti et al., 2018, Gianotti et al., 2012, Maggiolo et al., 2012, McKinnon et al., 2016, Sarmati et al., 2012). PIs have insufficient tissue penetration and even small decreases in their plasma concentration can cause significant reduction in their activity (Shen et al., 2008, Yeh et al., 2009). These

pharmacological features coupled with shorter ART duration in our cohort may imply that ongoing viral replication may occur more frequently in the PI group. Yet, the level of residual viraemia was the same as in the NNRTI group and except for one sample, which also had the shortest duration of suppression in the study (23 weeks), all the rest were below 5 copies/mL.

This study found that successfully treated patients had their total HIV-1 DNA in PBMC strongly associated with residual viraemia. HIV-1 DNA load in blood was a strong predictor of detectable HIV-1 RNA in plasma when adjusting for PD1 expression on effector memory CD4 T cells (CD4+/CD45RA-/CCR7-) and age. This should be interpreted with caution due to the wide 95% confidence interval (1.2 – 31). Two previous studies reported a positive correlation between residual viraemia (<10 copies/mL) and HIV-1 DNA in circulating CD4 T cells or total HIV-1 DNA in PBMC (Chun et al., 2011, Hong et al., 2018). Indeed, we may speculate that a larger reservoir may increase the probability of bursts of virus production upon cell activation. The argument is that only a small proportion of the reservoir (~5%) can produce intact viruses without defects and this subset appears to lack the capacity to clonally expand in response to TCR stimulation *ex vivo* (Bruner et al., 2019). On the contrary, cells harboring defective viruses proliferate with the potential to produce residual viraemia upon activation (Imamichi et al., 2016). Our group has previously reported no association between residual viraemia and integrated or total HIV-1 DNA (Ruggiero et al., 2018, Ruggiero et al., 2015). This finding accrued from a very similar population, but on NNRTI-based ART exclusively, while in this cohort we have included patients on PI-based regimens. The characteristics of the PI group may reflect a more active and bigger cellular reservoir which could have strengthened the association between HIV-1 DNA in PBMC and HIV-1 RNA in plasma. From a technical perspective, it has been demonstrated that co-amplification of cellular HIV-1 DNA along with HIV-1 RNA results in increased HIV-1 RNA values (Wan et al., 2010). However, the Abbott m2000sp platform employs an RNA-centric extraction method with the use of purification reagents specific for RNA. It has been shown that co-amplification of HIV-1 DNA is minimal with the RealTime assay compared to the TaqMan v2.0 (Fernandes et al., 2010).

Surprisingly, residual viraemia did not associate with increased PD1 expression on CD4 and CD8 subsets. On the contrary, PD1 expression on effector memory CD4 and CD8 T cells and central memory CD4 T cells was significantly increased in patients with undetectable HIV-1 RNA in a univariate model. Moreover, PD1 expression was consistently higher on all CD4 and CD8 subsets in the group with undetectable HIV-1 RNA compared to the group with

residual viraemia with the biggest difference seen on effector memory CD4 T cells. PD1 is expressed on activated CD4 and CD8 T cells to orchestrate the balance between immune stimulation and inhibition (Sharpe et al., 2007). PD1 regulatory and inhibitory roles are evident in two models of acute and chronic LCMV infection. Transient expression of PD1 on early effector CD8 T cells precedes acute infection clearance contrary to persistent PD1 up-regulation during chronic infection which attenuates a constant TCR signaling from antigen presence (Barber et al., 2006). PD1 has been associated with immune exhaustion and T cell dysfunction in HIV-1 infection (Day et al., 2006, D'Souza et al., 2007, Trautmann et al., 2006). High PD1 expression on T cells during chronic HIV infection is epitope-dependent driving functional inactivation and facilitating HIV persistence (Blattman et al., 2009, Day et al., 2006, Gilboa, 1999, Oldstone, 1997, Petrovas et al., 2006, Trautmann et al., 2006). However, the interaction of HIV-specific CD8 cells with mutated epitopes does not result in increased PD1 expression (Blattman et al., 2009, Conrad et al., 2011). Indeed, RNA viruses escape cytotoxicity and establish chronic infection with the acquisition of mutations at the sites of interaction with TCR receptors or by impairing important helper CD4 functions disabling their communication with CD8 cells (Blattman et al., 2009, Oldstone, 1997). Assuming that residual viraemia is clonal and bears mutations at the antigenic regions, it would not trigger PD1 expression. This would not explain the increased expression in patients with undetectable HIV-1 RNA in plasma, though.

Studies blocking the PD1-PD-L1/2 pathway in vitro and in vivo during SIV infection demonstrated immunological recovery with potential clinical benefit (Finnefrock et al., 2009, Freeman et al., 2006, Velu et al., 2009). Contrary to these promising findings, a model of rhesus macaques failed to show any VL suppression with PD1-PD-L1 inhibition in vivo (Amancha et al., 2013). These reports underscore the complex interplay of immune-regulatory mechanisms. Our group and others have provided evidence of immune activation during long-term suppressive ART (Ruggiero et al., 2018, Ruggiero et al., 2015, Zaidan et al., 2019). As a result, PD1 may increase to control an immune response that effectively clears residual virus production in patients with undetectable HIV-1 RNA in plasma. For instance, the interaction of PD1 with PD-L2 on APC exhibits a costimulatory role for T cell activation and prevents PD-L1 induced exhaustion in murine models (Dyck and Mills, 2017b, Karunarathne et al., 2016). PD1 is also normally expressed on Tfh which comprise around 20% of circulating CD4 T cells (CD4+/CD45RA-/CXCR5+/PD1+) and trigger B cells to produce antibody responses in secondary lymphoid tissues (Eivazi et al., 2016, Kawamoto et al., 2012, Tangye et al., 2013). Frequency of Tfh cells in HIV positive subjects correlates positively with

the capacity to develop bnAbs and stronger immune responses (Locci et al., 2013). Hence, PD1 expression in patients with undetectable HIV-1 RNA may reflect the activation of protective immune mechanisms.

Our study has limitations. The main disadvantage is our small sample size due to the rarity of the population we targeted to recruit. For this reason, we combined data from patients on NNRTI and PI based regimens and we allowed single episodes of viral rebound up to 200 copies/mL in the latter. Moreover, we included the three patients with LLV in our group with detectable HIV-1 RNA. Multi-centre studies are required to achieve larger numbers of participants on each type of therapy. We also focused on the PD1 pathway to address immune exhaustion and impairment of the cytotoxic machinery. A future approach would be to measure additional markers on CD4 and CD8 T cells, such as the exhaustion markers Tim-3 and CD160 (Kuchroo et al., 2014) and Tfh cell markers with mass cytometry. It would also be useful to have longitudinal immunological data to demonstrate the dynamics of PD1 expression.

Our findings imply that residual viraemia in successfully treated patients can be predicted by the size of circulating reservoirs when adjusting for PD1 expression on effector memory CD4 T cells and age. To the best of our knowledge there is no previous work on PD1 expression on CD4 and CD8 T cells in patients with residual viraemia. In this work it was demonstrated that patients with undetectable HIV-1 RNA express PD1 at increased levels compared to those with residual viraemia. Further work is required to investigate this finding and delineate additional immune factors associated with residual viraemia.

CHAPTER 4

Residual viraemia persists despite optimal drug levels

4.1 Introduction

ART initiation causes decay of HIV-1 RNA in plasma in four phases (Palmer et al., 2008). A very rapid decrease in VL during the first and second phase ($t_{1/2}$ = 1.5 and $t_{1/2}$ = 28 days, respectively) is followed by a slower decay in the third phase ($t_{1/2}$ = 39 weeks). Eventually, the fourth phase is established during which HIV-1 RNA in plasma persists indefinitely in most patients on successful ART (Hatano et al., 2009, Havlir et al., 2005, Palmer et al., 2008, Ruggiero et al., 2018). Accumulating evidence suggests that residual viraemia at the level of around 3 copies/mL has an archival character and derives from clonally expanded cells harbouring HIV-1 DNA (Bailey et al., 2006, Hosmane et al., 2017, Lorenzi et al., 2016, Simonetti et al., 2016, Wang et al., 2018). Some of these clones persist longitudinally while others disappear with residual viraemia remaining stable (Wang et al., 2018).

However, sub-optimal drug levels at sanctuary sites may allow ongoing cycles of viral replication and virus detection in plasma (Fletcher et al., 2014, Nightingale et al., 2016). At the same time, modest lapses in adherence may result in low level HIV-1 replication which may not be detected with commercial assays (Konstantopoulos et al., 2015, Pasternak et al., 2012); consistent with this view, adherence levels have been associated with persistent HIV-1 RNA detection below 50 copies/mL as measured with ultra-sensitive assays (Li et al., 2014). Yet, patients with optimal adherence can also have detectable HIV-1 RNA in plasma (Li et al., 2014, Maggiolo et al., 2017).

Here, we investigated whether detection of residual plasma HIV-1 RNA at levels below 10 copies/mL during suppressive first-line ART remains stable in subsequent measurements and explored its association with plasma drug concentrations.

4.2 Methods

4.2.1 Study population

Methods have been described in Chapter 3. In brief, we recruited HIV-1 infected individuals on first-line ART comprising of two NRTI and either a NNRTI or a PI. ART regimen modifications were allowed for the NRTI and PI arm and within the same drug class. Eligible patients were on therapy for at least 6 months. Their VL was consistently suppressed below 50 copies/mL. Patients on PI based regimens were allowed blips up to 200 copies/mL.

4.2.2 HIV-1 RNA and DNA quantification

Whole blood was collected at two time points (baseline, S0, and second sampling, S1). On the day of collection PBMC and plasma were separated, as described in Chapter 3, and stored at -80^0C. We measured HIV-1 RNA in plasma with our in-house SCA. In brief, 8mL of plasma were concentrated with ultra-centrifugation. HIV-1 RNA was measured in the concentrate with the Abbott m2000 platform and HIV-1 copies/mL were calculated from the formula: 10^[(Ct-Intercept)/Slope]/8. Samples with undetectable HIV-1 RNA were assigned the value of 0.5 copies/mL (Mens et al., 2011). The assay detection rates for 5, 3 and 1 HIV-1 RNA copies/mL were 100%, 80% and 60%, respectively.

Total HIV-1 DNA in PBMC was extracted with the QIAamp® DNA Mini and Blood Mini kit. DNA extract concentration was quantified by spectrophotometry (NanoDrop 1000, Thermo Scientific) and adjusted to 50 ng/μL corresponding to yield 1 μg DNA per PCR reaction. A real-time qRT-PCR targeting an LTR region was applied to measure the copies of HIV-1 DNA per 10^6 PBMC (Avettand-Fenoel et al., 2009).

4.2.3 Plasma drug levels

Plasma concentrations of EFV, DRV and lopinavir (LPV) were measured by validated high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS) at the Department of Pharmacology, University of Liverpool, as previously described (Dickinson et al., 2005). Time of sample relative to dose intake was recorded. We used untimed drug levels and categorised them as optimal or suboptimal based on target trough concentrations as

per drug manufacturer's prescribing information for drug-susceptible virus: 1,000 ng/mL for EFV, 1,000 ng/mL for LPV and 550 ng/mL for DRV (Acosta et al., 2012, Gonzalez-Serna et al., 2016). To investigate HIV-1 RNA levels in relation to drug levels we stratified samples as <1 copy/mL or undetectable (UD), 1 to 10 copies/mL or residual viraemia (RV) and >10 copies/mL or LLV.

4.2.4 Statistical analysis

Data were analysed with SPSS version 25 (IBM) as in Chapter 3. We utilised the McNemar test for repeated measurements of categorical data. The paired t-test (parametric) and Wilcoxon rank sum test (non-parametric) were used for repeated (correlated) variables. A sensitivity sub-analysis was performed for patents on EFV.

4.3 Results

4.3.1 Individual characteristics

Almost two third (60%, n=32) of subjects from our original cohort of patients were sampled twice (n=64 samples). The majority (81%, n= 26) were on first line NNRTI-based regimens with EFV (n=25) and rilpivirine (RLP, n=1). Ritonavir boosted PI-based ART included DRV (n=5) and LPV (n=1). Baseline characteristics for our longitudinal cohort and for patients on EFV are shown in Table 4.1. The median duration of first-line ART at the time of sampling was 205 weeks (IQR: 80-356) with a range between 37 and 672 weeks and the median duration of suppression was 193 weeks (IQR: 63-351). CD4 count, HIV-1 RNA in plasma, total HIV-1 DNA in PBMC and drug levels for EFV, DRV and LPV were measured at two time points separated by a median 4 months (IQR: 3-6, range: 2-7) interval (Table 4.2). CD4% was around 33% and mean total HIV-1 DNA in PBMC was 2.6 $\log_{10}$ copies/10^6 cells at each sampling point (Table 4.2). No treatment changes or interruptions were recorded during this period and VL remained suppressed (50 copies/mL) at routine clinic visits.

4.3.2 Residual viraemia

Of 32 patients, 17 (53%) had undetectable HIV-1 RNA in plasma at both time points and 8 (25%) had consistently detectable HIV-1 RNA. Undetectable HIV-1 RNA was preceded (n=4) or followed (n=3) by detected viraemia in seven subjects (Figure 4.1 & Appendix Figure A.1). The median plasma HIV-1 RNA level was 0.5 copies/mL (IQR: 0.5-2) at each time point (Table 4.2).

Of 23 samples with detectable HIV-1 RNA, 18 (78%) samples from 13 patients had residual viraemia between 1 and 10 copies/mL and 5 samples from 3 subjects had LLV between 10 and 40 copies/mL. Samples with quantifiable HIV-1 RNA had median 3 copies/mL (IQR: 2-9) and 4 copies/mL (IQR: 2-7) at S0 and S1, respectively. Patients with residual viraemia at one or both time points (n=12) fluctuated within a very confined range of 0 to 6.5 copies/mL (median 1.5; IQR: 1.5-3.3) (Figure 4.1 and Appendix Figure A.1). Only one patient crossed the adapted threshold of 10 copies/mL and had LLV (20 copies/mL) at S0 and residual viraemia (2 copies/mL) at S1.

4.3.3 Drug levels

Drug levels were measured in 59 of 64 samples for which the volume was adequate. Plasma levels of EFV relative to time post dosing were measured in 48 samples (23 at S0 and 25 at S2) and they are shown in Figure 4.2. The concentration of EFV was above the recommended threshold of 1,000 ng/mL for the majority of samples (n=42, 87.5%). Sub-optimal levels timed between 12 and 16 hours after dosing were identified in six samples from five patients (one patient, P5-Figure 4.2, had EFV <1,000 ng/mL at S0 and S2). The highest EFV levels were above the upper therapeutic target of 4,000 ng/mL in six samples from three female patients at both time points (Figure 4.2).

Concentration of DRV and LPV in the plasma was above the recommended threshold for all 11 samples (inadequate volume for one DRV specimen at S0). The median time of dosing was 10.5 hours (IQR: 4.3-15.3) prior to S0 and 13 hours (IQR: 6.6-16.8) before S1.

4.3.4 HIV-1 RNA level and drug levels

Of the 59 samples, 37 (62.7%) were UD and 17 (28.8%) and 5 (8.5%) were categorised as RV and LLV, respectively. Drug levels were optimal in 92% (n=34) of the UD samples. This proportion lowered to 88% (n=15) and further to 80% (n=4) in samples with RV and LLV, respectively. Of the 53 samples with optimal drug concentration, 36% (n=19) had detectable HIV-1 RNA in plasma between 1 and 40 copies/mL of which 28% (n=15) had residual viraemia (1-10 copies/mL).

A sensitivity sub-analysis was performed for samples taken from patients on EFV (25 patients, 48 samples). Baseline characteristics are shown in Table 4.1. There were no differences in HIV-1 RNA in plasma and total HIV-1 DNA in PBMC between the two time points (Table 4.3). The proportions of UD, RV and LLV were 65% (n=31), 29% (n=14) and 6% (n=3), respectively. Of the 48 samples with EFV levels, six (13%) had sub-optimal concentration. Of those six, three were UD, two RV and one LLV. The proportion of samples with EFV levels less than 1,000 ng/mL and detectable HIV-1 RNA (RV and LLV combined) was almost twice the proportion of those with UD [17.6% (3/17) vs. 9.7% (3/31)]. One patient had RV (7 copies/mL) with sub-optimal EFV levels (417 ng/mL) at S0 and UD with optimal drug concentration (1,263ng/mL) at S1. However, another patient with sub-optimal EFV levels at S0 and S1 had detectable HIV-1 RNA only at S1.

Figure 4.1: Reproducibility of HIV-1 RNA in plasma.

a.		SO		
		Undetected	Detected	
S1	Undetected	17 (53%)	4 (13%)	21 (66%)
	Detected	3 (9%)	8 (25%)	11 (34%)
		20 (62%)	12 (38%)	

b.
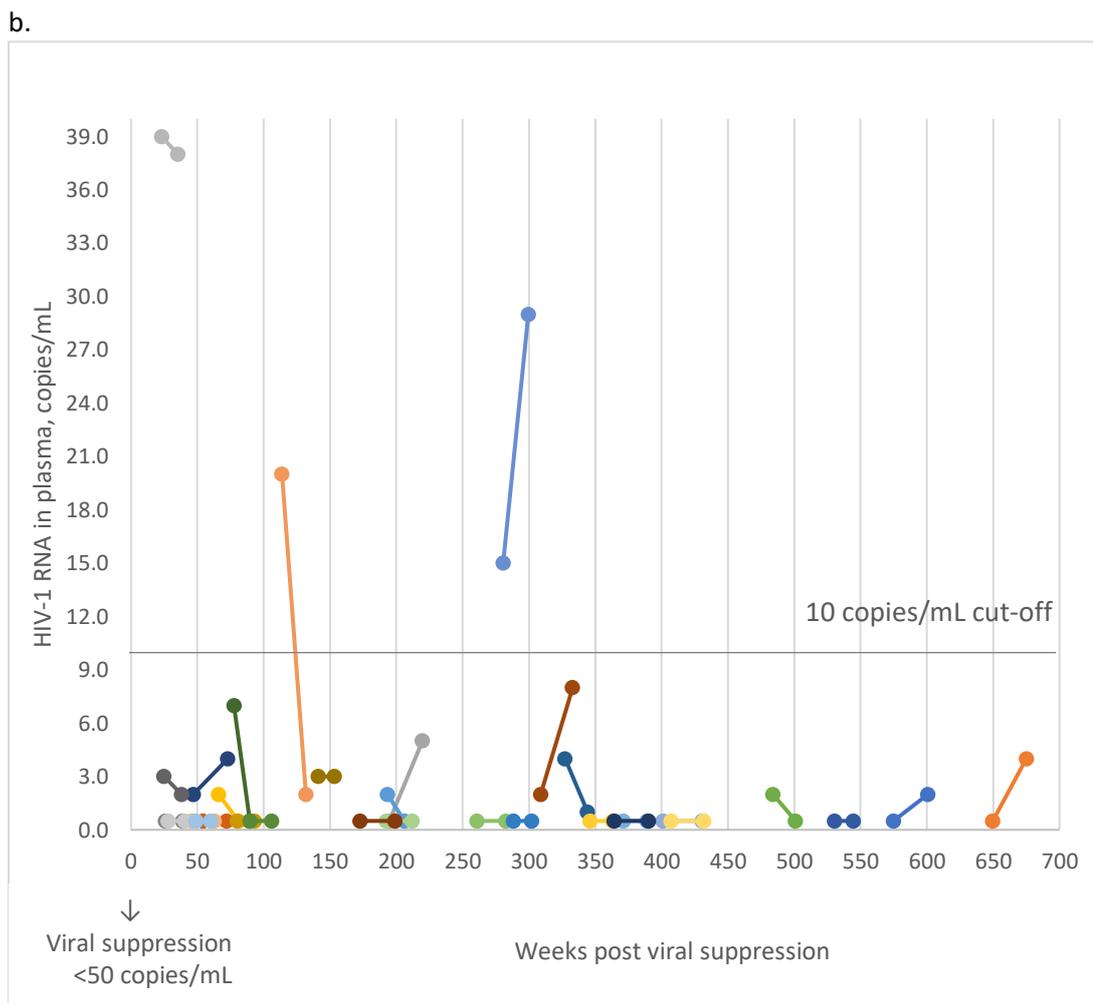
(a) frequency of patients with detected and undetected HIV-1 RNA at baseline (S0) and second sampling (S1) and (b) level of HIV-1 RNA in plasma measured with a single copy assay at least 24 weeks after starting anti-retroviral therapy (ART). Each line represents a patient and the dots represent baseline sampling (S0) and second sampling (S1). The 10 copies/mL cut-off was used to define residual viraemia.

Figure 4.2: Efavirenz levels in plasma at baseline (S0) and second sampling (S1) in patients with detected and undetected HIV-1 RNA in plasma.
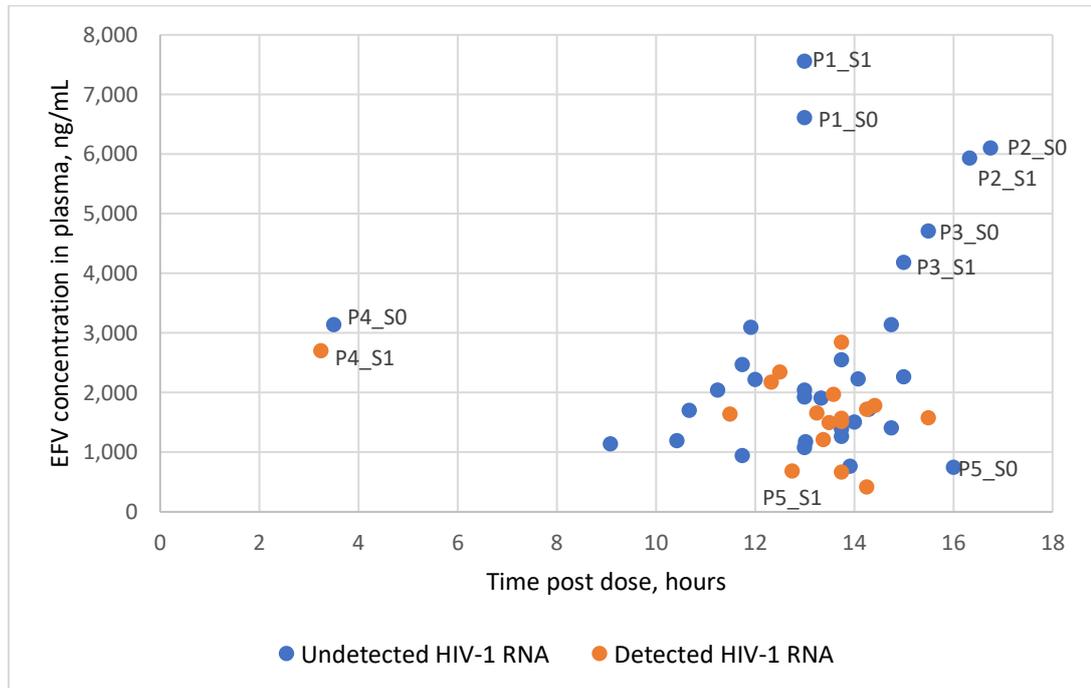
Outliers have been labelled with patient number (P1-5) and sampling point (S0 or S1). P5 is the single patient with efavirenz (EFV) levels below the target of 1,000 ng/mL at both sampling points.

Table 4.1: Baseline characteristics of the whole cohort and of patients on efavirenz based regimens at recruitment.

Characteristic	Total (n=32)	EFV (n=25)
Age (years), mean (SD)	42 (10.3)	42 (10.7)
Gender, n (%) Male	17 (53)	14 (56)
Female	15 (47)	11 (44)
Ethnicity, n (%) Black	10 (31)	8 (32)
White	22 (69)	17 (68)
Risk group, n (%) Heterosexual	19 (59)	15 (60)
MSM	12 (38)	9 (36)
Other	1 (3)	1 (4)
Time from diagnosis to start of ART, median months (IQR)	10 (2 – 36)	5 (2-17)
HIV-1 clade, n (%) B	15 (47)	11 (44)
C	5 (16)	3 (12)
A	1 (3)	0
Unknown	11 (34)	11 (44)
TDR, n (%) Yes	7 (22)	2 (8)
No	23 (72)	21 (84)
Missing	2 (6)	2 (8)
Nadir CD4 count, mean cells/mm^3 (SD)	229 (102)	199 (77.5)
Pre-ART VL, mean log$_{10}$ copies/mL (SD)	4.6 (0.7)	4.6 (0.7)
ART composition, n (%) TDF/FTC/EFV	20 (63)	20 (80)
ABC/3TC/EFV	5 (16)	5 (20)
TDF/FTC/RLP	1 (3)	0
TDF/FTC/DRV/r	4 (13)	0
ABC/3TC/DRV/r	1 (3)	0
ABC/3TC/LPV/r	1 (3)	0
Subjects with NRTI backbone changes[¥], n (%)	6 (19)	6 (24)
Calendar year for start of ART, median (IQR)	2010 (2007-2012)	2009 (2007-2011)

Characteristic	Total (n=32)	EFV (n=25)
Duration of ART median weeks (IQR)	205 (80-356)	266 (127-378)
Duration of VL suppression median weeks (IQR)	193 (63-351)	261 (114-371)

¥: number of changes was one with the exception of one subject who had two changes

EFV: efavirenz; VL: viral load; SD: standard deviation; MSM: men who have sex with men; IQR: interquartile; TDT: transmitted drug resistance; ART: anti-retroviral therapy; TDF: tenofovir; FTC: emtricitabine; ABC: abacavir; 3TC: lamivudine; RPV: rilpivirine; NVP: nevirapine; DRV: darunavir; r: ritonavir; AZT: zidovudine; LPV: lopinavir; ATV: atazanavir

Table 4.2: Comparative analysis of virological parameters, CD4 and drug levels between the two time points (S0 and S1).

Characteristic	S0 (n=32)	S1 (n=32)	p-value
Residual HIV-1 RNA, median copies/mL (IQR)	0.5 (0.5-2)	0.5 (0.5-2)	0.900**
HIV-1 RNA, n (%) Undetected	20 (63)	21 (66)	1.00†
Detected	12 (37)	11 (34)	
Total HIV DNA mean $\log_{10}$ copies/10^6 PBMC (SD)	2.65 (0.41)	2.64 (0.38)	0.938*
CD4 count (cells/mm^3), mean (SD)	572 (202)	662 (226)	*0.002**
CD4 (%), mean (SD)	32.5 (9.7)	33.3 (8.4)	0.282*
EFV levels in plasma (ng/mL), median (IQR)	1,699 (1,439-2,301) [a]	1,777 (1,263-2,703) [a]	0.465**
DRV levels in plasma (ng/mL), median (IQR)	3,781.7 (3,144.8-9,860.2) [b]	3,058.6 (2,568.3-3,983.7) [b]	0.273**
LPV levels in plasma (ng/mL), n=1	9,050.4	7,857.2	N/A

*Paired t-test (normally distributed continuous variables), **: Wilcoxon rank sum test (non-normal continuous variables), †: McNemar's test (categorical variables), a: n=23 at S0 (x2 missing values) and n=25 at S1, b: n=4 at S0 (x1 missing value) and n=5 at S1,

S0: baseline sampling; S1: second sampling; IQR: interquartile range; SD: standard deviation; PBMC: peripheral mononuclear cells; EFV: efavirenz; DRV: darunavir; LPV: lopinavir; N/A: not applicable

Table 4.3: Comparative analysis of virological parameters, CD4 and drug levels between the two sampling points (S0 and S1) for patients receiving efavirenz.

Characteristic	S0 (n=25)	S1 (n=25)	p-value
Time post dose, median hours (IQR)	13.6 (13-14.3)	13.7 (12.5-14.1)	0.654*
Residual HIV-1 RNA, median copies/mL (IQR)	0.5 (0.5-2)	0.5 (0.5-2)	0.964*
HIV-1 RNA, n (%) Undetectable	15 (60)	16 (64)	1.00†
Detected	10 (40)	9 (36)	
Total HIV DNA mean $\log_{10}$ copies/10^6 PBMC (SD)	2.65 (0.38)	2.59 (0.38)	0.484**
CD4 count (cells/mm^3), mean (SD)	558 (198)	650 (215)	0.018**
CD4 (%), mean (SD)	32 (10)	33 (9)	0.423**

*Paired t-test (normally distributed continuous variables), **: Wilcoxon rank sum test (non-normal continuous variables), †: McNemar's test (categorical variables)*

S0: baseline sampling; S1: second sampling; IQR: interquartile range; SD: standard deviation; PBMC: peripheral mononuclear cells

4.4 Discussion

We recruited a unique cohort of successfully treated patients on long term ART and we sampled more than half of them (32/53) twice within a median interval of four months (S0 and S1). Most patients (n=25, 78%) were on suppressive first line ART for more than a year and viral reservoirs represented by total HIV-1 DNA in PBMC were stable between S0 and S1. The undetected and detected status of HIV-1 RNA in plasma was reproducible at second sampling in 78% of cases (n=17 and n=8, respectively). The level of residual viraemia also remained stable between time points. This remarkable reproducibility in our cohort reflects the stability of HIV-1 RNA levels in plasma during chronic effective treatment (Maldarelli et al., 2007, Palmer et al., 2008).

We investigated if sub-optimal drug concentrations were associated with residual HIV-1 RNA in plasma. Over one third of samples had plasma HIV-1 RNA levels ranging from 1 to 40 copies/mL despite optimal drug levels, of which the majority had residual viraemia (<10 copies/mL). If we infer adherence from drug concentrations, our findings are consistent with previous studies demonstrating that one third to half of patients with persistent viraemia below 50 copies/mL had optimal adherence (Li et al., 2014, Maggiolo et al., 2017). These findings also support the hypothesis that residual viraemia derives from bursts of virus production from latently infected reservoirs which is quickly suppressed by ART. However, a small subset of samples with residual viraemia had sub-optimal drug levels reflecting the possible presence of viral replication replenishing HIV-1 RNA in plasma (Li et al., 2014, Maggiolo et al., 2017). Our study differs from previous work because we treated residual viraemia <10 copies/mL as a separate entity from LLV between 10 and 50 copies/mL. This was justified by the observation that there is a gradual increase in the risk of viral rebound with increasing HIV-1 RNA above 10 copies/mL (Doyle et al., 2012).

We identified three outliers in our cohort having LLV between 10 and 40 copies/mL. One subject had viral suppression for less than a year (23 and 35 weeks at S0 and S1, respectively), during which viraemia may be expected to continue to decline (Hatano et al., 2010) and one had sub-optimal drug levels which may reflect poor adherence (Li et al., 2014). The third subject went from LLV (S0) to residual viraemia (S1) but none of the above explanations could be applied.

Contrary to previous studies using drug refill and pill count, we utilised drug levels to measure adherence. Although it is indicated in Chapter 3 that all our patients reported optimal adherence, recall bias and social acceptance may result in an overestimate (Goldman et al., 2008). Liechty and colleagues showed that subtherapeutic untimed drug levels were an acceptable indicator of non-adherence, but therapeutic drug concentrations, particularly for EFV which has a long half-life, were not a good marker of adherence in clinical practice (Liechty et al., 2004). Therapeutic EFV levels have been associated with self-reported adherence at 85% and above in a study investigating the aetiology of blips (Farmer et al., 2016). Sub-optimal untimed drug levels have not been associated with viral blips, but they have been proposed as an independent predictor of virologic failure in patients with episodic or recurrent viraemia between 50 and 1,000 copies/mL (Gonzalez-Serna et al., 2016). Taken together, we linked sub-optimal levels with decreased adherence, but we note that our untimed analysis cannot adjust for pharmacokinetic variability causing reduced levels. We also acknowledge that a small proportion of our patients with optimal drug levels in plasma may not have been 100% adherent to ART.

Our effort to reduce variability by sampling a strictly pre-defined population stable on treatment came at the cost of a small sample size. As a result, we did not model repeated measures and treated each sample as independent in a descriptive analysis. The small number of patients and time points is the main drawback, which does not allow for robust findings. The study also lacked a longitudinal assessment of immunological parameters. Lastly, at the time of study conduct InSTI were not part of first line recommendations in guidelines, as they are now, and our subjects were on rather old-fashioned regimens.

Nevertheless, we noted some interesting trends. We report that HIV-1 RNA in successfully suppressed patients on ART fluctuated in less than half of patients and within a very stringent range between routine visits. Residual virus still persists at a stable level despite optimal drug levels reflecting the presence of an active reservoir as the source of HIV-1 RNA in plasma. In a small proportion of patients, though, inadequate drug levels may contribute to new rounds of virus replication and residual viraemia.

CHAPTER 5

General discussion

HIV-1 RNA in plasma persists in patients under suppressive ART and factors associated with residual viraemia remain elusive. Treatment initiation causes a rapid decline in VL which continues at a slower rate until HIV-1 RNA reaches a level around 3 copies/mL (Maldarelli et al., 2007, Palmer et al., 2008). In line with the kinetics of viral decay, our systematic review showed that patients who had been on suppressive ART for less than two years experienced LLV more frequently than those on longer duration of treatment (Leierer et al., 2015, Martin-Blondel et al., 2012, Pascual-Pareja et al., 2010). Hints of this notion were also demonstrated in isolated cases of our observational cohort with residual viraemia.

The terms of low level and residual viraemia have been widely used to stratify HIV-1 RNA levels, but their definitions vary significantly in literature. Our systematic review included 15 studies and the proportion of patients with LLV was between 6% and 77%. We adapted a threshold of 10 copies/mL to distinguish between the two entities, because the risk of viral rebound starts to increase gradually around this cut-off in proportion with the level of viraemia (Doyle et al., 2012, Maggiolo et al., 2012, Pugliese et al., 2013, Ryscavage et al., 2014). Residual viraemia around 3 copies/mL is clonal and derives from reservoirs established during primary infection (Anderson et al., 2011, Bailey et al., 2006, Buzon et al., 2010, Gianotti et al., 2012, Palmer et al., 2008, Wang et al., 2018). However, higher levels of HIV-1 RNA may result from additional mechanisms such as ongoing viral replication due to sub-optimal ART penetration at sanctuaries or moderate adherence (Fletcher et al., 2014, Konstantopoulos et al., 2015, Li et al., 2014, Nightingale et al., 2016), and drug resistant mutations (Delaugerre et al., 2012, Mackie et al., 2010, Taiwo et al., 2011).

Consistent with the hypothesis of activation of cellular reservoirs as the source of residual HIV-1 RNA during suppressive ART, our systematic review and our observational study showed that LLV and residual viraemia, respectively, were associated with a larger cellular reservoir as measured by levels of total HIV-1 DNA (Charpentier et al., 2012, Falasca et al., 2017, Leierer et al., 2015, Martin-Blondel et al., 2012, Pascual-Pareja et al., 2010, Reus et

al., 2013). Our review identified that higher pre-ART VL, longer time since HIV-1 diagnosis and advanced clinical stage were more frequently encountered in patients with LLV. These factors reflect a prolonged course of infection prior to treatment initiation resulting in an increased number of cells being infected (Hong and Mellors, 2015, Jain et al., 2013, Maldarelli et al., 2007). In this context, a larger latent reservoir in blood, as indicated with increased total HIV-1 DNA in PBMC, was associated with LLV in one of the included studies (Falasca et al., 2017). Among the population of our observational cohort, one third had residual viraemia around 3 copies/mL and HIV-1 RNA levels in plasma were reproducible at re-sampling. These findings are consistent with previous reports describing clonal expansion of cells harbouring HIV-1 DNA as a probable source of residual virus before being effectively suppressed by ART (Josefsson et al., 2013, Shen and Siliciano, 2008b). These cells may be circulating in peripheral blood as illustrated in our data by the observed association between total HIV-1 DNA in PBMC and residual HIV-1 RNA in plasma (Mullins and Frenkel, 2017).

Virus replication, on the other hand, can be driven by inefficient ART penetration in tissues and decreased adherence. Patients on PI-based regimens or during ART holidays experienced LLV with increased frequency in our systematic review (Falasca et al., 2017, Leierer et al., 2015, Saison et al., 2013). A small proportion of our study patients were on PIs (n=11) and we performed drug levels in plasma on two different occasions in almost half of them which were found optimal. However, PI levels in lymphoid tissues may have been sub-optimal and this may have caused some trends (e.g. higher frequency of patients with residual viraemia) seen in the PI group compared to the NNRTI group. A small fraction of EFV samples with detectable HIV-1 RNA in plasma had sub-optimal drug concentration. This observation implies that virus replication may generate residual viraemia in a limited number of patients with sub-optimal treatment and this warrants further investigation.

Sometimes, both mechanisms may co-exist. In our review, we identified an association between enhanced microbial translocation and LLV (Falasca et al., 2017, Reus et al., 2013, Torres et al., 2014). Ongoing residual replication at the sanctuary of GALT causes structural changes and microbial translocation which may activate cells harbouring HIV-1 DNA and trigger immune responses with further local damage in a self-perpetuating manner (Chun et al., 2008, Cory et al., 2013). The interplay of immune activation is much more complex during residual viraemia (Hatano, 2013, Ruggiero et al., 2015, Younas et al., 2016). Increased PD1 expression on memory CD4 and CD8 subsets in our patients with undetectable HIV-1 RNA entailed the existence of a pathway with immunoregulatory properties. At first this seemed contradictory to current evidence presenting PD1 as an indicator of immune

exhaustion and viral latency (Blattman et al., 2009, Filaci et al., 2018). However, PD1 expression has also been associated with protective immune mechanisms via different pathways (e.g. interaction with PD-L2) in HIV-1 infection (Dyck and Mills, 2017b, Karunarathne et al., 2016, Locci et al., 2013). Moreover, PD1 expression on CD4 and CD8 T cells may exert opposite effects. It has been suggested that PD1 expression on regulatory CD8 T cells may hamper cytotoxic anti-viral responses against cellular reservoirs (Filaci et al., 2018), whereas on memory CD4 T cells it may reflect increased frequency of Tfh which correlates with broadly neutralizing antibodies to residual virus (Locci et al., 2013). Further work is required to shed light on the role of PD1 as to activation of cellular reservoirs and/or viral replication.

Our systematic review underscored poor inter- and intra-assay repeatability for viraemia around 50 copies/mL (Garcia-Diaz et al., 2013, Hopkins et al., 2015, Ruelle et al., 2012, Swenson et al., 2014). Discrepancies were identified in most studies between the two widely used commercial assays, the TaqMan v2.0 and the RealTime. An explanation could lie with co-amplification of cellular HIV-1 DNA along with HIV-1 RNA with the former platform (Fernandes et al., 2010, Taylor et al., 2013). Residual viraemia is quantified with ultra-sensitive assays frequently employing modified protocols of these assays. Hence, technical variation should be factored in when comparing data derived from different platforms.

Taken together these findings, residual viraemia appeared to be mainly the result of transient bursts of virus production which were quickly suppressed by ART. However, virus replication may have been allowed in a small subset of patients due to sub-optimal drug levels or dysregulation of immune checkpoints, such as PD1. In this context, residual viraemia and LLV are generated by similar mechanisms but to a different extent.

In prospective work it would be interesting to investigate if the association between residual viraemia and viral reservoir persists with measurement of HIV-1 DNA in cells harbouring intact viruses lacking fatal defects such as large deletions and hypermutations which are found in the majority of reservoirs. Recently, *Bruner et al.* developed a novel method of quantification of intact HIV-1 DNA load (Intact Proviral DNA Assay, IPDA) which overcomes underestimation issues observed with quantitative viral outgrowth assays (Bruner et al., 2019, Simonetti et al., 2020). Replication-competent HIV-1 DNA appeared to correlate with HIV-1 DNA in total CD4 cells in a cohort of suppressed patients, but treatment duration and history were unknown (Papasavvas et al., 2020). Application of IPDA in our cohort of

carefully selected patients would shed more light as to the degree of ongoing replication contribution to residual viraemia.

Another aspect that warrants further investigation is the unexpected increase of PD1 expression on memory CD4 and CD8 cells in patients with undetectable HIV-1 RNA compared to those with residual HIV-1 RNA presence in plasma. We would wish to confirm this finding in a larger longitudinal cohort including patients on InSTI regimens. The aim would be to better characterise memory CD4 and CD8 subsets by measuring additional checkpoint receptors and surface markers, such as CCRX5 for circulating Tfh and the T cell factor 1 (TCF) which differentiates between terminally exhausted (TCF1-) and self-renewing (TCF1+) T cells (Blank et al., 2019).

The preliminary data of this work and the advancements in knowledge and techniques since I started my studies could lead to exciting future research.

Appendix

This appendix contains:

- Figure A.1, which depicts HIV-1 RNA level over time in patients with detected viraemia at one or both sample points (Chapter 4)
- Table A.1, which illustrates the variability in the definition of LLV in literature by giving examples of LLV ranges used by different authors (Chapter 2)
- Table A.2, which tabulates changes in the NRTI backbone in our study cohort (Chapter 3)
- Table A.3, which details the steps of the backward selection process of the multivariate analysis and the effect of removing each variable on the model as performed in SPSS version 25 (Chapter 3).

Figure A.1: Reproducibility of HIV-1 RNA level (copies/mL) in subjects with detected viraemia at one or both sample points.
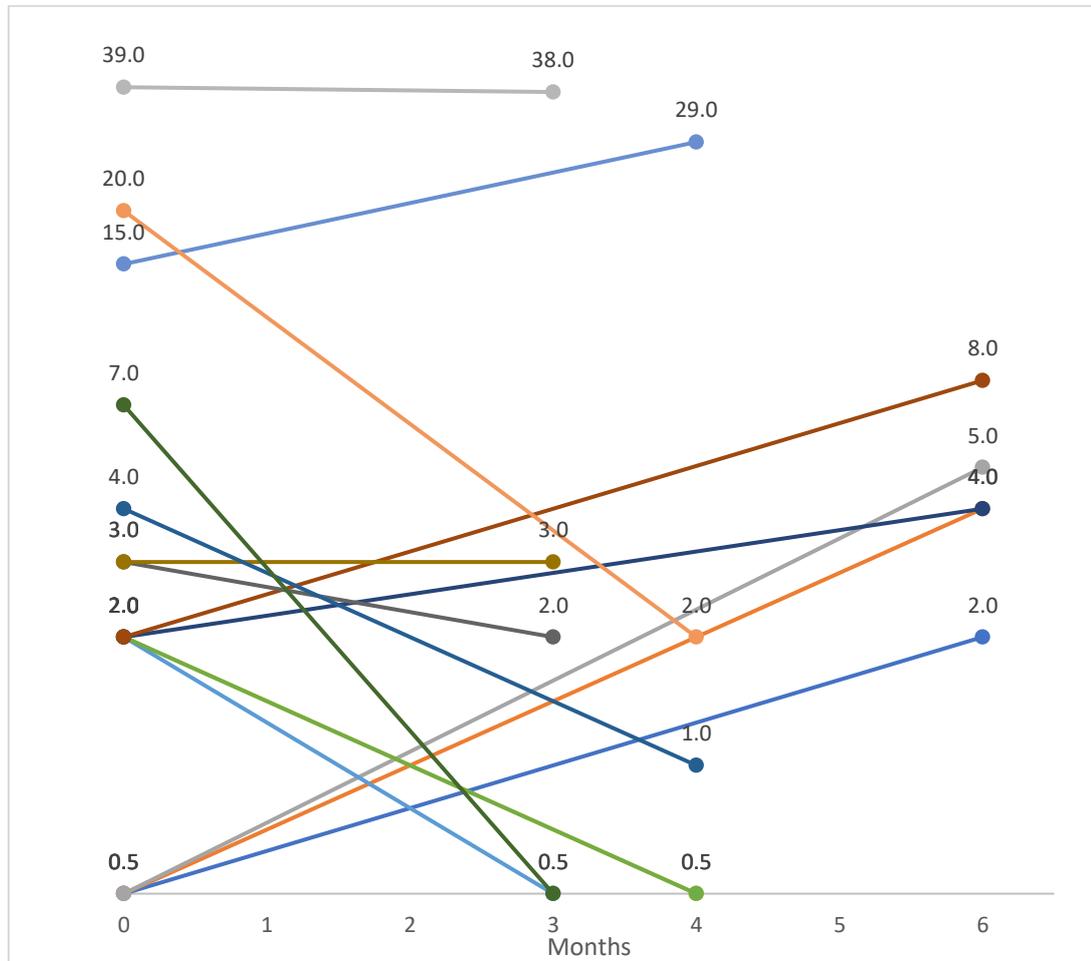
Each line represents an individual. Starting dot at 0 months represents baseline sampling (S0) and finish dot represents second sampling (S1).

Table A.1: Examples of low level viraemia definitions in literature.

First author	LLV range (copies/mL)	Country	VL assay or database	Reference
Dravid	21-999	India	NucliSENS Easy Q real-time nucleic acid sequence-based amplification (NASBA)	(Dravid et al., 2018)
Rupérez	150-999	Mozambique	Abbott m2000 RealTime (0.2 mL sample volume)	(Ruperez et al., 2015)
Chao	75-5,000	California	Kaiser Permanente's laboratory database	(Chao et al., 2012)
Charpentier	21-49	France	CAP/CTM v2.0	(Charpentier et al., 2012)

LLV: low level viraemia; VL: viral load; CAP/CTM v2.0: COBAS AmpliPrep/COBAS TaqMan HIV-1 test version 2.0

Table A.2: Changes in the nucleoside reverse transcriptase inhibitors backbone.

No	First combination	Second combination	Reason for 1st change	Third combination	Reason for 2nd change	Forth combination	Reason for 3rd change
1	AZT/3TC/EFV	TDF/FTC/EFV	Simplification				
2	ABC/3TC/EFV	TDF/FTC/EFV	CVD risk				
3	ABC/3TC/EFV	TDF/FTC/EFV	Low CD4 count				
4	AZT/3TC/EFV	TDF/FTC/EFV	Lipodystrophy				
5	AZT/3TC/EFV	TDF/FTC/EFV	Low CD4 count				
6	AZT/3TC/EFV	TDF/FTC/EFV	Simplification				
7	AZT/3TC/EFV	TDF/FTC/EFV	Update				
8	AZT/3TC/EFV	ABC/3TC/EFV	Update	TDF/FTC/EFV	Hepatitis C infection		
9	AZT/3TC/EFV	ABC/3TC/EFV	Update				
10	AZT/3TC/EFV	DDI/3TC/EFV	Anaemia	TDF/3TC/EFV	Lipodystrophy	TDF/FTC/EFV	Hypercholesterolaemia
11	AZT/3TC/LPV/r	AZT/ABC/LPV/r	Neuropathy	AZT/ABC/DRV/r	Diarrhoea		
12	AZT/3TC/SQN	AZT/3TC/ATV/r	Hypercholesterolaemia	ABC/3TC/ATV/r	Lipodystrophy		

AZT: zidovudine; 3TC: lamivudine; EFV: efavirenz; TDF: tenofovir; FTC: emtricitabine; ABC: abacavir; CVD: cardio-vascular disease; DDI: didanosine; LPV: lopinavir; r: ritonavir; DRV: darunavir; SQN: saquinavir: ATV: atazanavir

Table A.3: Steps of the backward selection process of the multivariate analysis and the effect of removing each variable on the model.

Step No	Independent variables	Odds ratio	95% CI	*p*-value
1	Age	0.96	0.89-1.03	0.258
	Third agent (PI vs. NNRTI)	0.58	0.08-4.27	0.589
	Time from suppression to recruitment	0.89	0.70-1.13	0.349
	Total HIV-1 DNA in PBMC	2.79	0.78-9.94	0.114
	CD8	1.04	0.97-1.10	0.267
	PD1 on CD8+/CD45RA-/CCR7+	0.99	0.92-1.06	0.749
	PD1 on CD8+/CD45RA-/CCR7-	0.98	0.92-1.04	0.461
	PD1 on CD4+/CD45RA-/CCR7-	0.97	0.92-1.02	0.213
2	Age	0.96	0.89-1.03	0.233
	Third agent (PI vs. NNRTI)	0.54	0.08-3.88	0.543
	Time from suppression to recruitment	0.89	0.70-1.14	0.359
	Total HIV-1 DNA in PBMC	2.85	0.80-10.12	0.106
	CD8	1.04	0.97-1.10	0.268
	PD1 on CD8+/CD45RA-/CCR7-	0.97	0.93-1.02	0.199
	PD1 on CD4+/CD45RA-/CCR7-	0.97	0.92-1.02	0.175
3	Age	0.96	0.89-1.03	0.253
	Time from suppression to recruitment	0.91	0.72-1.14	0.400
	Total HIV-1 DNA in PBMC	2.34	0.78-6.99	0.127
	CD8	1.04	0.98-1.11	0.245
	PD1 on CD8+/CD45RA-/CCR7-	0.98	0.94-1.02	0.243
	PD1 on CD4+/CD45RA-/CCR7-	0.97	0.92-1.02	0.187
4	Age	0.95	0.87-1.00	0.093
	Total HIV-1 DNA in PBMC	2.52	0.86-7.40	0.093
	CD8	1.04	0.98-1.11	0.236
	PD1 on CD8+/CD45RA-/CCR7-	0.98	0.94-1.02	0.250
	PD1 on CD4+/CD45RA-/CCR7-	0.97	0.92-1.01	0.153
5	Age	0.94	0.88-1.00	0.054
	Total HIV-1 DNA in PBMC	2.37	0.82-6.80	0.109

Step No	Independent variables	Odds ratio	95% CI	p-value
	CD8	1.04	0.98-1.11	0.192
	PD1 on CD4+/CD45RA-/CCR7-	0.95	0.91-0.99	0.032
6	Age	0.95	0.89-1.01	0.072
	Total HIV-1 DNA in PBMC	3.42	1.32-8.83	0.011
	PD1 on CD4+/CD45RA-/CCR7-	0.96	0.93-1.00	0.050

CI: confidence interval; PI: protease inhibitor; NNRTI: non-nucleoside reverse transcriptase inhibitor

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