THE UNIVERSITY OF LIVERPOOL

Department of Clinical Infection, Microbiology and Immunology
Institute of Infection and Global Health

HIV TREATMENT SIMPLIFICATION: OUTCOMES OF SWITCHING HIV-1 PATIENTS TO PROTEASE-INHIBITOR MAINTENANCE MONOTHERAPY

Thesis submitted in accordance with the requirements of
The University of Liverpool for award of degree of Doctor in
Philosophy

By

Adam Abdullahi

DECLARATION OF AUTHORSHIP:

I, Adam Abdullahi, hereby declare that the work described here forth is my own and was generated by myself under the auspices of the Anna Maria Geretti Blood Borne Virus research group at the Institute of infection and Global Health, University of Liverpool, Liverpool, United Kingdom. Where material has been obtained from any sources other than my own, I confirm that I have clearly indicated. I also declare that this work has not been submitted for any awards at any other institutions other than for a doctorate degree in Infectious Diseases and Global Health at the University of Liverpool in the month of November, in the year 2020.

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G. Villa, R. Phillips, C. Smith, D. Owusu, **A. Abdullahi**, H. Austin, D. Awuah, L. Sayeed, D. Chadwick, S. Bhagani, A.M. Geretti. Liver steatosis in HIV-positive patients with and without HBV infection accessing care in a programmatic setting in Africa. *Manuscript in preparation*.

DEDICATION

To my paternal and maternal grandfathers who both saw the inception of my PhD journey but not its completion, may your kind souls rest in eternal peace

And

To all the HIV positive women in Africa who find themselves in circumstances beyond their control

PREAMBLE

It really does take a village to raise a child

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ABSTRACT:

HIV treatment simplification: Outcomes of switching HIV-1 patients to protease-inhibitor maintenance monotherapy

Adam Abdullahi

The work in this thesis characterised the virological outcomes of HIV-1 patients on second-line ART switching to boosted-darunavir maintenance monotherapy in sub-Saharan Africa and explored their determinants, with the aim of providing evidence to inform practice and policy.

Firstly, I took advantage of samples and data collected within a trial of maintenance monotherapy that was conducted in Yaoundé, Cameroon, between August 2014 and July 2015. The trial population was composed of HIV-1 positive adults who were receiving suppressive second-line ART with two NRTIs and a ritonavir-boosted protease inhibitor (PI/b). Patients were randomised to either a switch to maintenance monotherapy with ritonavir-boosted darunavir (DRV/r) for 48 weeks or to continue their current triple ART regimen.

My first question was to investigate the virological outcomes and the relationship between viraemia and drug resistance. I used stored samples to investigate the presence of drug-resistance associated mutations (RAMs) in peripheral blood mononuclear cells (PBMC) collected at study entry (while patients were virologically suppressed on triple ART) and in follow-up plasma samples collected at the time of virological rebound on DRV/r monotherapy. I analysed the viral genomes by Sanger sequencing and ultra-deep sequencing (UDS) and used phylogenetics to assess their relatedness. The resistance analyses focused on reverse transcriptase and protease; in a subset of patients I also sequenced the gag gene to identify mutations in cleavage sites and other regions. I then used the resistance data alongside the available demographic, clinical and laboratory data to identify predictors of virological outcomes by statistical modelling. The results were interesting. I found that presence of RAMs at study entry affecting the NRTIs and the NNRTIs were predictive of a reduced (rather than increased) risk of virological rebound during follow-up on DRV/r monotherapy and the effect was independent of adherence levels. I also found that despite a high prevalence of viraemia during DRV/r monotherapy, there was no emergence of new protease resistance even by sensitive UDS, thus confirming data from previous studies conducted in Europe. While this observation was reassuring as it indicated no loss of treatment options in the monotherapy arm, I wondered whether there were other adverse consequences of frequent viraemia during DRV/r monotherapy.

I thus investigated the kinetics of soluble CD27 (sCD27), a marker of immune activation and inflammation that a previous study from our group had linked to residual viraemia in subjects on suppressive ART. I tested prospective samples taken before and four weeks after the switch from triple ART to DRV/r

monotherapy and observed that median sCD27 levels increased significantly after simplification, with the effect driven by a subset of the patients. I used various control populations to place the findings into context using different control populations. Hence, I propose that sCD27 may serve as an indicator of a risk of viraemia, a hypothesis that needs to be investigated prospectively.

One additional research question was related to Hepatitis B Virus (HBV). My research hypothesis was that in a HBV hyperendemic setting like Cameroon, discontinuation of HBV active agents in people with HIV-1 could abolish a prophylactic effect against HBV acquisition and reactivation. To address this question, I measured markers of HBV infection prospectively in MANET trial participants. The results showed discontinuation of NRTIs was associated with a risk of both *de-novo* acquisition and reactivation of HBV. This represents another factor that makes DRV/r monotherapy undesirable for Cameroon and sub-Saharan Africa in general.

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ABBREVIATIONS

/r	Boosted with ritonavir
3TC	Lamivudine
ABC	Abacavir
AE	Adverse event
AIDS	Acquired immunodeficiency syndrome
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
ANRS	French Agence Nacional de Recherche sur le SIDA
Anti-HBc	hepatitis B core antibody
ART	Antiretroviral therapy
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
ATV	atazanavir
AZT	Zidovudine
BIC	Bictegravir
Bp	Base pair
BMI	Body mass index
CAB	Cabotegravir
CCR5	CC-chemokine receptor 5
CD4	Cluster of Differentiation 4
CD8	Cluster of Differentiation 8
cccDNA	Closed covalent circular DNA
CI	Confidence interval
CIRCB	International Reference Centre Chantal Biya
CKD-EPI	Chronic kidney disease epidemiology collaboration-derived equation
COBI	Cobicistat
CPC	Centre Pasteur Cameroon
CRF	Circulating recombinant form
Ct	Cycle threshold
CXCR4	CXC-chemokine receptor 4
CYP450	Cytochrome p450 enzymes
D4T	Stavudine
DDC	Zalcitabine
DDI	Didanosine
DHSS	Department of Health and Human Services
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DRC	Democratic Republic of Congo
DRV	Darunavir

DTG	Dolutegravir
EACS	European AIDS Clinical Society
EDTA	Ethylenediaminetetraacetic acid
EFV	Efavirenz
eGFR	Estimated glomerular filtration rate
ELISA	Enzyme-linked immunosorbent assay
EVG	Elvitegravir
FBS	Fetal bovine serum
FDA	FDA U.S Food and Drugs Administration
FTC	Emtricitabine
Gag	Group specific antigen protein
gp41	Glycoprotein 41
gp120	Glycoprotein 120
gp160	Glycoprotein 160
HBeAb	Anti-HBe
HBeAg	Hepatitis B envelope antigen
HBsAb	Anti-HBs
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HIV-1	Human immunodeficiency virus type 1
HIV-2	Human immunodeficiency virus type 2
HSV-2	Herpes simplex virus 2
IAS-USA	International AIDS Society-USA
INT	Integrase
INSTI	Integrase strand inhibitor
IQR	Inter-quartile range
KATH	Komfo Anokye Teaching Hospital
LLOD	Lower limit of detection
LLOQ	Lower limit of quantification
LPV	Lopinavir
LTR	Long terminal repeat
MA	Matrix
MANET	Monotherapy in Africa, New Evaluations of Therapy trial
MCV	Maraviroc
MGS	Male genital schistosomiasis
mRNA	Messenger ribonucleic acid
Nef	Negative factor protein
NC	Nucleocapsid
NIBSC	National Institute for Biological Control and Standards
NGS	Next-generation sequencing
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse-transcriptase inhibitor
NVP	Nevirapine
OR	Odds ratio
ORF	Open reading frame
UD	Undetected
UDS	Ultra-deep sequencing
PBMC	Peripheral blood mononuclear cell

PBS	Phosphate buffered saline
p7	Nucleocapsid
p17	Matrix
p24	Caspid
PLHIV	people living with HIV
PI	Protease inhibitor
PI/b	Boosted protease inhibitor
PIC	Pre integration complex
PCR	Polymerase chain reaction
PR	Protease
r	ritonavir
RAL	Raltegravir
RAM	Resistance associated mutation
RAxML	Randomized axelerated maximum likelihood
rcDNA	relaxed circular DNA
RPV	Rilpivirine
RNA	Ribonucleic acid
RT	Reverse transcriptase
SAE	Severe adverse event
sCD27	Soluble cluster of differentiation 27
SIV	Simian immunodeficiency virus
SSA	Sub-Saharan Africa
STI	Sexually transmitted infection
Tat	Transactivator protein
TAM	Thymidine analogue mutation
TAM-1	Thymidine analogue mutation type 1
TAM-2	Thymidine analogue mutation type 2
T20	Enfuvirtide
TAF	Tenofovir alafenamide
TFV	Tenofovir
TDF	Tenofovir disoproxil fumarate
UK	United Kingdom
UNAIDS	United Nations Agency for AIDS
URF	Unique recombinant form
VAS	Visual analogue scale
Vif	Viral infectivity factor
Vpr	Viral protein regulatory
Vpu	Viral protein u
WHO	World Health Organisation
ZDV	Zidovudine

CHAPTER ONE - GENERAL INTRODUCTION

1.1 The identification of AIDS and the discovery of HIV

A clinical condition later defined as the Acquired Immune Deficiency Syndrome (AIDS) was first identified in 1981 when, in Los Angeles and New York, homosexual men of perceived normal health status were reported to be at risk of *Pneumocystis pneumonia* and *Kaposi's sarcoma*[1,2]. Similar opportunistic conditions were soon reported in other parts of the world[2,3]. A form of AIDS presenting as a fatal illness characterised by weight loss and diarrhoea and locally called "slim disease" was documented In rural Uganda in 1985;[4] however, it was observed that slim disease was not exclusive to homosexual men but was found equally in men and women.

The aetiological agent of AIDS was first discovered in 1983, when Françoise Barré-Sinoussi with her mentor, Luc Montagnier at the Pasteur institute in Paris isolated a retrovirus from the lymph node biopsy of a patient with cervical lymphadenopathy[5]. The new retrovirus was named Lymphadenopathy-Associated Virus [LAV][6]. A few months later, Robert Gallo and colleagues at the National Cancer Institute of Bethesda in Maryland identified a new retrovirus from the peripheral blood lymphocyte of patients with AIDS, which they named Human T-Lymphotropic virus type III (HTLV-III). In 1985, the full genome of LAV and HTLV-III were characterised indicating that they were variant forms of the same virus[7]. In May 1986, the International Committee of the Taxonomy of Viruses (ICTV) named the virus Human Immunodeficiency Virus (HIV). The discovery of HIV by Françoise Barré-Sinoussi and Luc Montagnier led to the joint award of the Noble Prize in Physiology or Medicine in 2005.

Serological evidence of infection with a second type of HIV emerged from studies of commercial sex workers in Senegal in 1985[8]; subsequent isolation of HIV-2 was documented in 1986 from a Cape Verdean patient[9]. In 1987, an AIDS-like disease which had a faster course than that observed in

humans was reported in Asian rhesus macaques held at a primate research centre in Boston, where they were co-hosted with other non-human primates including African monkeys such as African green monkeys and sooty mangabeys[10]. This led to the characterisation of a lentivirus termed Simian Immunodeficiency Virus (SIV). Further research later led to the identification of several other SIV variants from different old-world primate species including chimpanzees, sooty mangabeys and African Green Monkeys. Observations from wild primate populations indicate that, although generally less pathogenic, SIVs, like HIV, can be associated with progressive CD4 cell loss, lymphatic tissue destruction, and premature death in their Simian African hosts[11].

1.2 Origin of HIV

HIV-1 and HIV-2 originated as a result of zoonoses, i.e., transmission of animal viruses into humans. The progenitor of HIV-1 is a primate lentivirus named SIV_{cpz} which is found in some subspecies of chimpanzees in Cameroon; cross-species transmission likely occurred via blood contact during primate hunting or butchering[12]. The first retrospectively documented case of HIV-1 seropositivity in humans was in a blood sample collected from a man in Leopoldville, colonial West Central Africa in 1959. While possible sporadic cases of HIV-1 infection were documented before the 1970s[13], the HIV-1 pandemic started in the late 1970s. Scientific evidence indicates that HIV-1 started its spread from Kinshasa in the present day Democratic Republic of the Congo (DRC)[14], aided by increasing urbanisation, global travel, and health care interventions (i.e., re-use of needles)[15].

The progenitor of HIV-2 is found in sooty mangabeys and is named SIV_{smm}[17]. SIV_{smm} is also the progenitor of SIV_{mac}[18]. Serological data suggest that HIV-2 may have been circulating in West Africa as early as 1966[19]. HIV-2 is endemic in West Africa but is not as readily transmitted and generally not as pathogenic as HIV-1[20]; several African countries (e.g., Senegal, Gambia) have reported declining prevalence rates of HIV-2 in recent years[19].

1.2.1 Genetic variants of HIV

HIV-1 strains are classified into four groups named M, N, O and P and are further sub-classified on the principle of phylogeny (figure 1.1). Group M variants are responsible for the global pandemic. Group N and O were discovered in 1998 and 1990 respectively[22,23]. Group O accounts for <1% of global infections, whereas group N is less prevalent than group O; both are found in West Central Africa. In 2009, an additional group was identified in a Cameroonian woman[24] and assigned to group P. The four genetically distinct groups represent separate crossings of the species barrier: Groups M, N and O have their precursors in chimpanzees in Southern Cameroon[25], whereas Group P originated from gorillas in the same region.

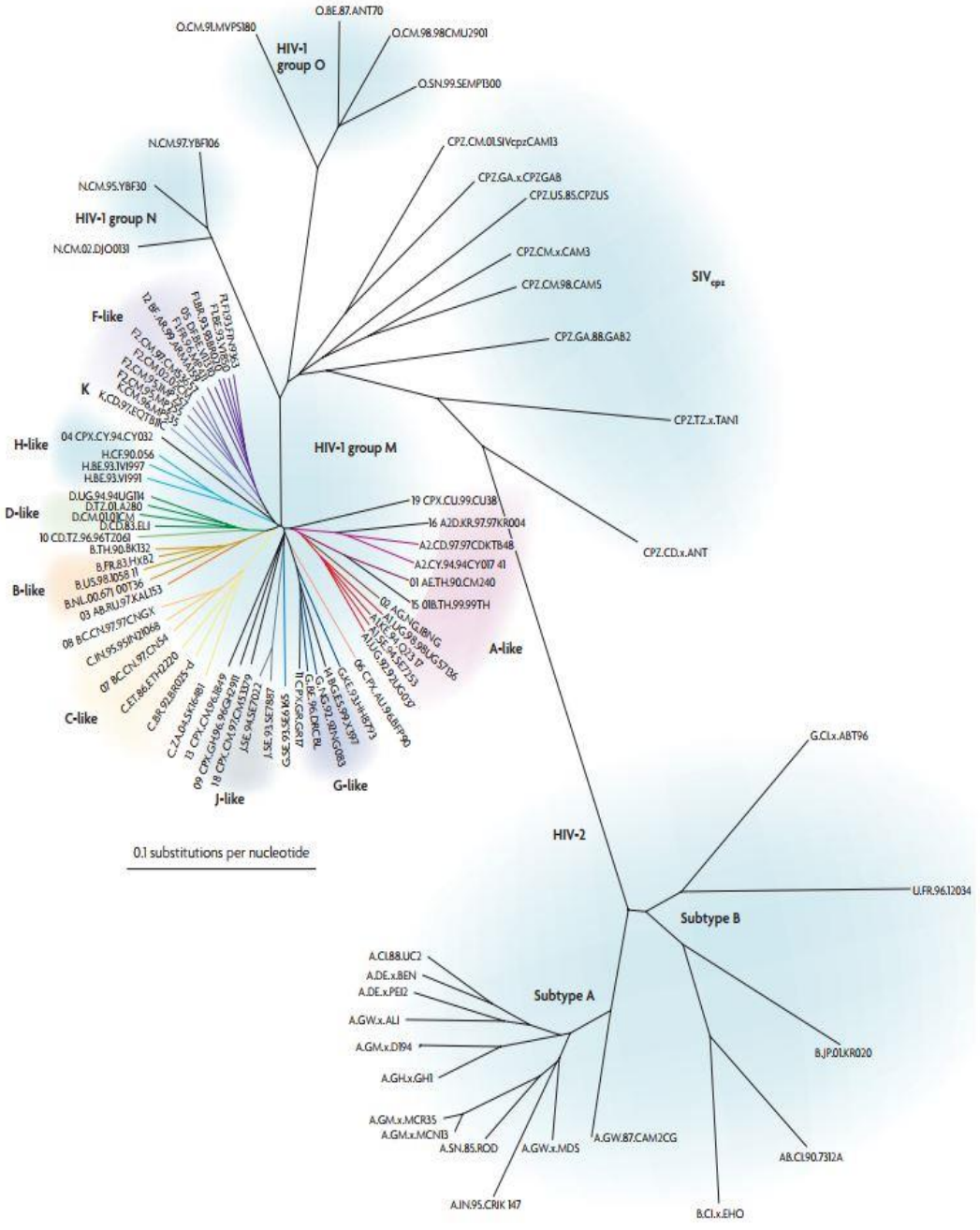


Figure 1.1: Phylogenetic tree showing the relatedness of HIV-1 group M, group N and group O variants, HIV-2 variants, and SIV_{cpz}. The tree was derived from 87 full-length sequences.

Adapted from [20]

HIV-1 group M has undergone remarkable genetic diversification, which continues to occur in areas of high-level virus transmission. To put this into context, HIV-1 as a virus evolves around 10^6 times quicker than human DNA[24]. HIV-1 genetic diversity is also driven by the ability of different virions to infect the same cell and give rise recombinants, which, depending on

spread and epidemiological characterisation, are termed circulating recombinant forms (CRFs) and unique recombinant form (URF). CRF are identified by full genome sequencing in at least three epidemiologically unlinked individuals and account for at least 20% of HIV-1 infections globally [26]. Currently, nine subtypes of HIV-1 Group M have been substantively characterised: (A-D, F-H, J, K). Additionally, various sub-subtypes (A1-A4, F1, F2)[27] and at least 55 CRFs also circulate [28]. Subtypes differ by 15% and 25% in the amino acids of the *gag* and *env* genes respectively[29,30].

The greatest diversity of HIV-1 strains is found in Central Africa. Beyond this, specific strains can often be linked to specific geographical regions with five strains namely A, B, C, CRF01_AE and CRF02_AG dominating the global landscape (Figure 1.2).

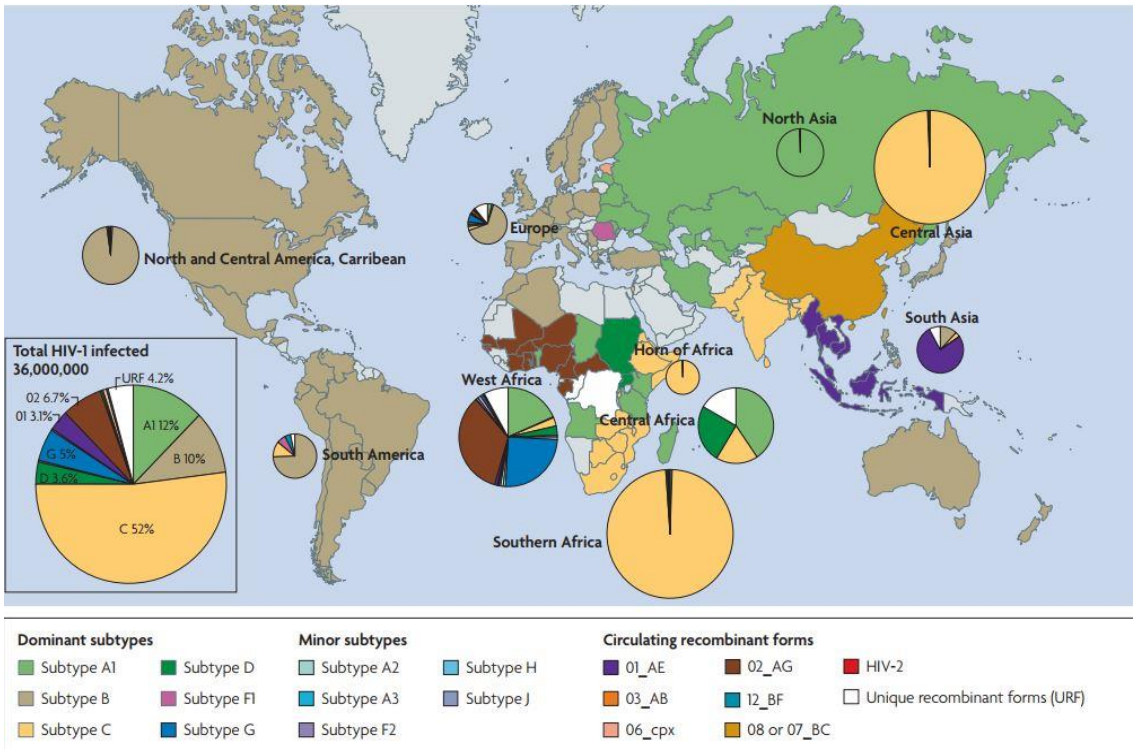


Figure 2.1: Map showing the diversity of HIV-1 strains in the current epidemic. The countries are colour coded based on the main circulating Group M HIV-1 variant in each region; the pie charts indicate the relative proportions of each variant in each of the regions. The grey area has a low prevalence of HIV-1 or were not represented in the literature.

Adapted from [20]

1.3 Epidemiology of HIV-1 infection

There is a high burden of HIV-1 infections worldwide, with a marked impact in low-resource settings of sub-Saharan Africa (SSA) and Southeast Asia.

UNAIDS estimated numbers[31,32]

At the end of 2018: infected globally; 37.9 million people; died from AIDS-related illnesses; 770,000 people

Since the start of the epidemic: ever infected globally; 77.3 million people; died from AIDS-related illnesses; 35.4 million people

There has been a significant decrease (by 47%) in the number of incident HIV-1 infections in recent years, with 1.8 million estimated incident infections in 2018 compared to a peak incidence of 3.4 million in 1996. SSA carries a disproportionate burden accounting for nearly three quarters of global infections, although prevalence varies geographically. The highest prevalence is found in Swaziland (27%)[33]. Numerically, however, the highest number of affected individuals reside in South Africa and Nigeria, accounting for 25% and 13% of all people living with HIV (PLHIV) in SSA, respectively[32]. Much has been achieved across SSA since the commitment of world leaders at the United Nations General Assembly in 2006 to scale-up interventions towards attainment of a universal HIV care, support and prevention services[34].

1.4 Biology of HIV-1

HIV is a member of the Retroviridae family of viruses, genera Lentivirus[35]. A defining characteristic of retroviruses is that their genetic information is stored in two molecules of single stranded ribonucleic acid (RNA) and the virus replication cycle requires reverse transcription of genomic RNA into deoxyribonucleic acid (DNA).

1.4.1 The HIV particle

Comparable to other retroviruses, cryogenic micrograph images of HIV-1 (Figure 1.3) shows the virus particle as a spherical entity of approximately

100nm in diameter[36]. The outer membrane of HIV-1, called the envelope, originates from the membrane of the infected cell, from which it inherits a lipid bilayer structure. The envelope contains the viral surface glycoprotein (gp) 120 and the viral transmembrane glycoprotein named gp41. Each spike on the surface of a virion is a trimer of gp120 molecules attached non-covalently to three gp41 molecules[37]. In addition to the envelope (Env) proteins, the lipid bilayer also contains several host cell derived membrane proteins such as actin, ubiquitin and major histocompatibility complex antigen[38]. Underneath the envelope, the matrix shell, which is composed of an estimated 2000 copies of the matrix protein, surrounds the capsid, which is made of the capsid protein and contains two copies of single stranded positive sense RNA in complex with the viral enzymes protease, reverse transcriptase (RT) and integrase[39].

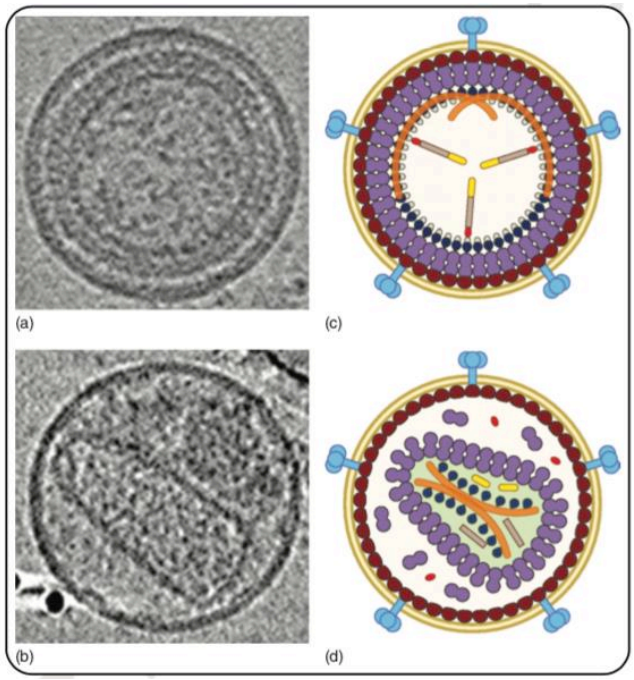


Figure 1.3: Cryogenic micrographs and schematic images of the immature (a, c) and mature (b, d) HIV-1 particle.

Adapted from[36,40]

The genome of HIV-1 (Figure 1.4) is approximately 9.8kb in size. It is flanked by 2 long terminal repeats (LTR) sequences and is made of nine open reading frames (ORFs) encoding 15 proteins[41]. These proteins include the three poly-protein precursors, Gag, Gag-Pol and Env, which require cleavage into their mature forms: Gag encodes the matrix (MA) and capsid (CA) structural

proteins; Pol encodes the viral enzymes protease, RT and integrase; and *Env* encodes gp160 from which gp120 and gp41 are derived. The other six ORFs encode accessory and regulatory proteins; Tat, Rev, Vif, Vpr, Nef and Vpu, which are responsible for the regulation of virus genome expression, production and release.

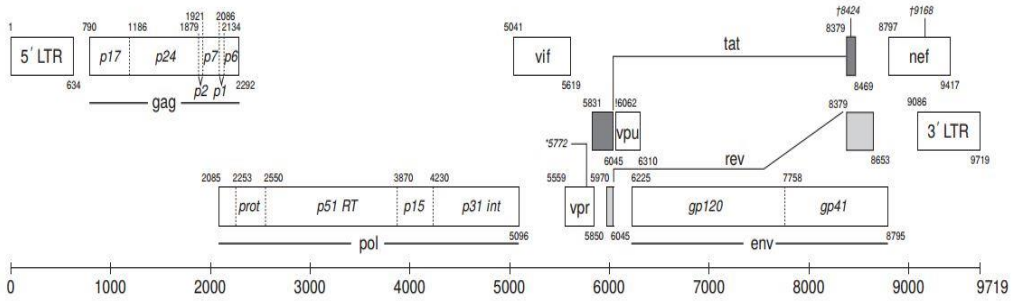


Figure 1.4: Organisation of the HIV-1 genome, with rectangles depicting the open reading frames. The start position of the nucleotide "a" in the "atg" start codon is shown in the upper left corner with the number on the right indicating the stop position.

Adapted from [42]

1.4.2 HIV-1 proteins

1.4.2.1 Structural proteins

Gag

The *gag* gene encodes a polyprotein precursor that is cleaved by the viral protease enzyme to yield the mature structural proteins matrix (p17), capsid (p24), nucleocapsid (p7) and p6[43]. The cleavage of the $Pr55^{gag}$ precursor also yields two spacer peptides, termed p1 and p2, which are thought to influence the order and rate of Gag protein processing.

Matrix protein (p17)

The MA protein forms the shell between the envelope and the capsid and is involved in directing virus components to the plasma membrane for assembly[44].

Capsid protein (p24)

The CA protein plays an important role in both the early steps of virus replication (viral RNA release from the capsid) and in the later steps of virion assembly and maturation.

Nucleocapsid protein (p7)

The NC protein plays an important role in facilitating structural rearrangement of the viral genomic material during replication through its nucleic acid chaperone function[45].

p6

The p6 protein play a role in the incorporation of Vpr into virion particles during assembly[49].

Pol

The polymerase gene encodes the enzymes that play key roles in the life cycle of HIV, reverse transcriptase (RT), protease (PR) and integrase (INT).

RT

The RT enzyme mediates the conversion of the viral genomic RNA into a double-stranded DNA. RT has three enzymatic activities: RNA-dependent RNA polymerase, DNA-dependent DNA polymerase and ribonuclease H[48], which are required for the synthesis of DNA from the viral genomic RNA template[49]

PR

The PR enzyme is responsible for the processing of Gag and Gag-Pol polyproteins during viral maturation[52]

INT

The INT enzyme is responsible for the integration of the linear double stranded DNA formed by RT into the host chromosome[54].

Env

Env encodes the polypeptide precursor gp160, which is cleaved by cellular host proteases to yield gp120 and gp41[54] (Figure 1.3 c,d).

1.4.2.2 Accessory proteins

In addition to the three ORF encoding for the structural polyproteins, six other ORFs exist in the HIV-1 genome encoding for accessory or regulatory proteins that do not require any further modification, these proteins are: vpr, vpu, vif, nef, tat and ref. Tat and ref are regarded as regulatory proteins and termed trans-activating proteins.

Vpr

The viral protein regulatory is part of the viral particle and plays a key role in controlling the cell cycle, viral genome expression and import of HIV-1 DNA into the nucleus.

Vpu

The viral protein u controls the degradation of CD4[55] and aids the budding of new virus particles from the cell membrane.

Vif

The viral infectivity factor enhances virus infectivity by counteracting the effect of the host restriction factor APOBEC3G [56]. APOBEC are cellular proteins that induces hyper mutation of Guanine to Adenosine (G to A) on the positive strand of HIV-1 DNA leading to defective virions. [57]

Nef

The negative factor protein plays an essential role in viral pathogenesis by acting at multiple phases of the virus/host cell interactions.

1.4.2.3 Regulatory proteins

Tat

The trans-activator protein is responsible for transactivating the LTR promoter to allow mRNA transcription[58].

Rev

The regulator of virion expression protein drives the export of mRNA from the nucleus to the cytoplasm[59].

1.5 Virus replication cycle

The viral replication cycle of HIV-1 can be divided into the early and late phases. The early phase involves binding, fusion, start of reverse transcription, nuclear import and integration of the HIV-1 DNA into the host chromosome (figure 1.5.) The late phase involves the expression of viral genes from the integrated viral genome, assembly, maturation and release of a mature virion.

1.5.1 Viral Entry

HIV-1 binds to the CD4 cell receptor on the surface of target cells, including primarily lymphocytes and macrophages. Binding of the gp120 envelope protein to the CD4 receptor triggers a conformational change which allows binding of gp120 to a co-receptor[60]. The α chemokine CXC receptor 4 (CXCR4) or the β chemokine co-receptor 5 (CCR5) are the two main co-receptors used by HIV-1[61]. The significance of co-receptor binding has been substantiated by the discovery that a 32 base pair deletion in the CCR5 gene, described as CCR5Δ32, either prevents infection (homozygous) or reduces disease progression (heterozygous)[62–64]. CCR5Δ32 results into the introduction of a premature stop codon into the locus of the CCR5 receptor preventing its expression on the cell membrane. The allele frequency is about 10% for heterozygousity and 1% for homozygousity across Europe and Western Asia, with a North to South gradient of reducing prevalence in the population[65]. Co-receptor binding exposes and activates the hydrophobic fusion peptide in the outer amino acid domain of the transmembrane envelope protein gp41, which allows its N-terminal fusion peptide to be inserted into the target's cell membrane. With the viral and cellular membranes in close contact, this leads to the formation of a fusion pore[66] through which the viral capsid is released into the host cell cytoplasm.

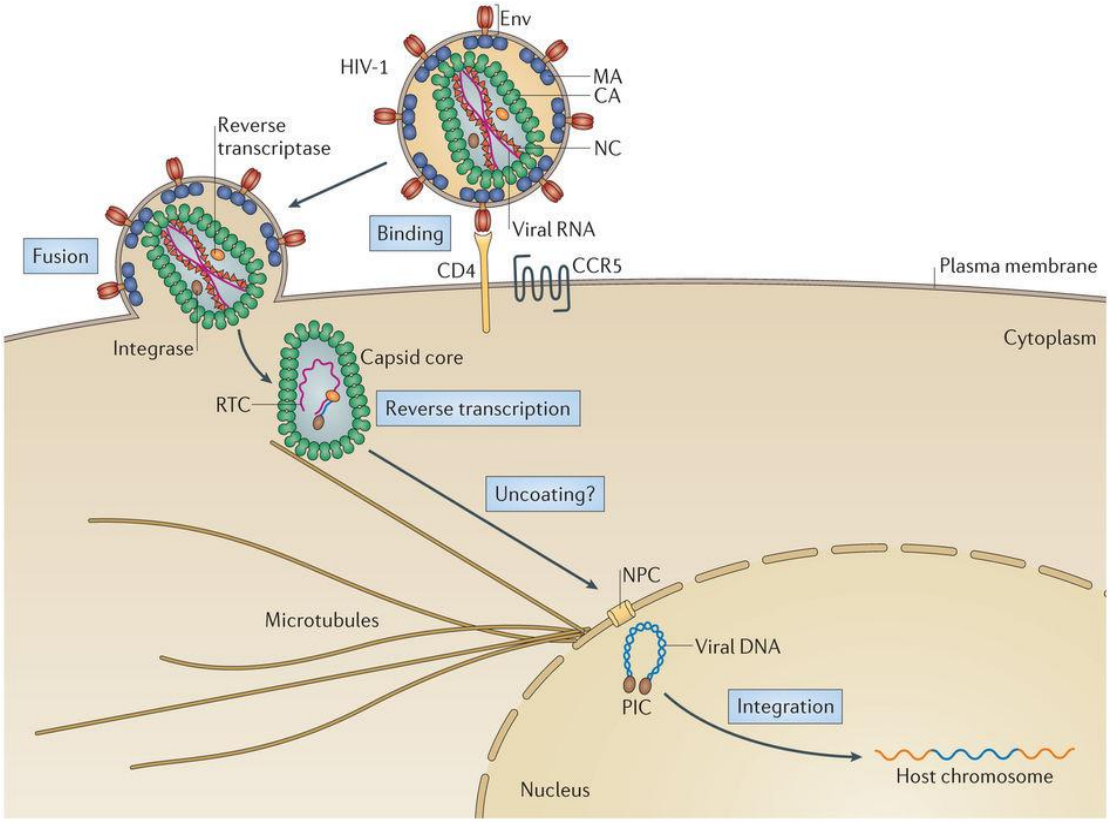


Figure 1.5: The features of the early stages of HIV-1 replication cycle. Recent evidence indicates that uncoating and reverse transcription are completed in the nucleus.
Adapted from [63]

1.5.2 Uncoating

Following entry, the capsid is disassembled, a process described as "uncoating". The exact timing and location of the uncoating process is still subject to scientific debate[67]. Recent data indicate that, contrary to previous understanding, uncoating and reverse transcription are completed in the nucleus rather than the cytoplasm[68].

1.5.3 Reverse transcription

RT transcribes the two single stranded viral RNA molecules into double stranded (ds) DNA. The steps in the reverse transcription process includes the generation of complementary antisense (minus) DNA strand to form the DNA/RNA hybrid, RNA degradation from the RNA/DNA hybrid by RNaseH and the generation of a complete dsDNA through the action of the DNA-dependent DNA polymerase[69].

1.5.4 Nuclear translocation and entry

In the nucleus, the viral dsDNA is contained within a pre-integration complex (PIC) together with host cell factors including LEDGF/p75 and viral proteins including RT, INT, MA, CA and Vpr[70]. The exact mechanism through which the nuclear import of HIV-1 occurs is yet to be fully understood[44]. In contrast to oncoviruses that require degradation of the nuclear membrane during mitosis, HIV-1 and other lentiviruses are actively transported across the nuclear membrane[71] permitting HIV-1 to infect non-dividing cells such as dendritic cells and macrophages.

1.5.5 DNA Integration

The linear dsDNA resulting from reverse transcription follows one of the following pathways:

i) integration into the host chromosome
ii) short-term persistence as unintegrated linear dsDNA
iii) circularisation to form 1-LTR-circles
iv) circularisation to form 2-LTR-circles

While each of these four forms are observable in culture, effective infection requires the integration of the linear dsDNA into the host chromosome by the INT enzyme[72]. The series of well-coordinated events leading to integration can be divided into two phases: 3' processing and strand transfer. The first step occurs immediately after synthesis of the dsDNA[73]. The INT enzyme catalyses the modification of each 3' terminus of the linear dsDNA by excising two nucleotides and producing a pre-integration substrate ending with CA-3' sequence. The strand transfer step, which is also catalysed by the INT enzyme, involves the cleavage of specific target sites on the host cell DNA via nucleophilic activity, leading to the exposure of five nucleotides at each end; the 3' viral DNA end is then covalently bound to the 5' cellular DNA end. In the final step, cellular repair enzymes remove unpaired nucleotides at the 5' end and seal the single-strand gaps ligating the 5' end of viral DNA to cellular DNA[74].

1.5.6 Viral transcription and gene expression

Following integration, the provirus of HIV-1 is transcribed in a well organised process[75] with the cis and trans-acting elements i.e., Tat and Rev proteins playing key roles. Transcription of the proviral DNA yields the full genomic RNA of about 10,000bp and shorter RNA segments. These are categorised into three groups based on the degree of splicing. Partially spliced mRNAs encode Env, Vif, Vpr and Vpu. Fully spliced mRNAs encode Tat, Rev and Nef, and unspliced transcripts encode Gag and Gag-Pol polyproteins. The envelope protein is generated in the rough endoplasmic reticulum and Golgi complex where it undergoes extensive N-glycosylation, before transportation to the plasma membrane.

1.5.7 Assembly, budding and release

Assembly involves the packaging of both viral and cellular factors leading to the formation of immature virions. The immature virion buds out from the host producer cells and acquire their lipid envelope from the host cell plasma membrane and shortly after or during this, the newly budded virions mature into infectious virions[76].

1.5.8 Maturation

Virus maturation can occur simultaneously during budding or immediately after budding. At first, newly budding virions are immature and non-infectious and require protease-mediated cleavage to transform the virions into mature and active forms. The viral protease cleaves at least 10 sites in the Gag and Gag-Pro-Pol polyproteins at specific sites into functional subunits; MA, CA, NC, p6, PR, RT and IN. During the maturation process, cleaved proteins are morphologically rearranged to produce infectious virions, characterised by formation of conical capsid core[69].

1.6 Mechanisms of HIV genetic diversification

HIV-1 is characterised by profound genetic diversity given that initial infection is typically initiated by a single founder virus. This diversity allows adaptation to pressure from antiretroviral agents and the immune system[77,78]. The extensive diversity is due to:

i) Error-prone reverse transcriptase enzyme
ii) High replication rate (up to 10^9 virions per day in an untreated patient)
iii) Viral recombination
iv) Errors by RNA polymerase II
v) Hypermutation induced by cellular proteins of the APOBEC family

After transmission to a new host, multiple mechanisms contribute to continuous genetic evolution of HIV. One key molecular mechanism is the highly error prone RT enzyme[79]. The RT enzyme, unlike cellular DNA polymerases, has a high error rate and no proof-reading activity. The lack of proof-reading activity means incorporation of the wrong nucleotide triphosphate cannot be corrected by RT. Nucleotide misincorporations become fixed as a point mutation in progeny viruses[36]. The rate of nucleotide substitutions by the RT is ~$3x10^{-5}$ per nucleotide per cycle of replication, equalling one nucleotide substitution per genome in a single replication cycle. This phenomenon is further amplified by the high viral turnover of ~ 10^8-10^9 viral particles per day in the absence of antiviral therapy. Another mechanism is the ability of RT to "hop" or "switch" from one template to the other during replication, which can result in deletions and insertions[80,81]. Further to this, when different viral strains co-infect the same cell, the ability of the RT to switch between templates can lead to recombination[69]. Genomic diversification of the HIV genome is further driven by the cellular proteins of the APOBEC family, which are packaged into virions and cause G-to-A mutations during plus DNA strand synthesis following degradation of the RNA[69]. An additional source of viral genetic diversity is driven by the error-prone cellular RNA polymerase II enzyme, which is responsible for transcription of proviral DNA. Although RNA polymerase II also lacks proof reading ability, its error rate is thought to be lower than that of RT. The variants generated through these multiple mechanisms exist in a host as "quasispecies", i.e a population of genetically similar but non-identical virus strains. Overall, the low replication fidelity provides an adaptive advantage. Viral progeny could be incapacitated and not be able to continue replication or display varying levels of fitness including ability to escape selective

pressure. These quasispecies harbour mutations that can reduce susceptibility to antiretroviral drugs and escape immune response. As the drug resistance-conferring substitutions typically exist as single mutations, treatment with combination antiretroviral therapy can achieve effective viral suppression.

1.7 HIV-1 pathogenesis

1.7.1 HIV-1 transmission

HIV-1 can be transmitted through the following routes[36]:

i) vertically from mother to child during pregnancy, delivery or breastfeeding
ii) percutaneous or mucous membrane exposure to infected blood or blood products
iii) Intimate sexual contact involving mucosal penile-vaginal contact, penile-anal contact and in very rare cases, oral sex.

The risk of HIV transmission varies significantly. Blood transfusion from a HIV-positive donor to a HIV-negative donor poses the highest risk (~90%) followed by mother-to-child transmission (~23%). Risk of transmission is lower for exposure via parenteral routes with percutaneous needle injuries (0.23%) and drug injection paraphernalia contaminated by infected blood (0.63%)[82]. Contrary to belief in some regions, especially across some parts Africa, HIV is not transmitted by saliva, sweat or faeces[83].The most common route of HIV-1 transmission is through intimate sexual contact [84] and the most important vehicle for transmission is seminal fluid. HIV-1 is detected in semen as free viral particles[85] and can be detected a few weeks following infection[86]. For sexual exposure, anal intercourse poses the highest risk, with approximately 138 infections per 10000 episodes of unprotected receptive anal sex (1.38%)[82]. This is followed by insertive anal sex (11 infection per 10000 episodes of act; 0.11%), receptive penile-vaginal intercourse (8 infections per 10000 episodes of act; 0.08%) and insertive penile-vaginal intercourse (4 infections per 10000 episodes of act; 0.04%). The presence of concomitant infections such as Herpes Simplex Virus type 2 (HSV-2) and

other sexually transmitted infections (STI), which may cause genital inflammation enhances the risk of transmission and acquisition of HIV-1[87].

There is a direct relationship between the concentration of virus in the blood[88] and genital secretions[89] and the risk of HIV transmission. In principle, this would indicate that decrease in HIV viral load would reduce the risk of HIV transmission. Evidence indicates that initiation of antiretroviral therapy (ART) leads to the suppression of HIV-1 RNA in plasma within 3-6 months. A robust body of scientific evidence, including the PARTNER 1 and 2 studies[90,91], supports the conclusion that patients who are on stable ART and show a suppressed plasma HIV-1 RNA do not transmit the infection to their sexual partners if they engage in condomless sex. In the PARTNER studies, 888 heterosexual and 340 men who have sex with men (MSMs) sero-discordant subjects were recruited and provided 1238 eligible couple years of follow-up. The HIV positive partner had HIV-1 RNA <200 copies/ml. The subjects reported condomless sex a median of 37 times per year (IQR: 15-71) over median follow-up period of 1.3 years (IQR: 0.8-2.0)[90]. There was no documented case of HIV transmission between couples. In the final analysis of the MSMs recruited to the PARTNER studies, 782 gay couples 1593 provided eligible couple years of follow-up and results showed no phylogenetically linked incident infection among sero-discordant couples[91]. This scientific evidence underpins the U=U (undetectable equals untransmissible) message currently endorsed by the UNAIDS and illustrates the benefits of diagnosing and treating HIV early to prevent transmission and disease progression.

1.7.2 Clinical stages

The natural course of HIV-1 infection in the absence of ART can be divided into three stages (Figure 1.6):

i) primary infection

ii) asymptomatic phase

iii) AIDS phase

Primary infection comprises the acute stage prior to the development of antibodies, and the recent stage that follows seroconversion and spans up to six months after the initial infection. The acute phase is characterised by high levels of plasma HIV-1 RNA ("viral load") and a high risk of transmission[88], and is accompanied by a sharp decline in the CD4 cell count. The life-long HIV-1 DNA reservoir is established during this early phase[92]. Approximately two to four weeks after HIV exposure[93] coinciding with peak viraemia[94], at least half of newly infected subjects develop symptoms. These typically consist of a febrile illnesses with fatigue, headache, night sweats, myalgia, arthralgia, lymphadenopathy, diarrhoea, sore throat, and rash, collectively termed the acute retroviral syndrome[95]. Symptoms resolve spontaneously[36]. The non-specific nature of the acute retroviral syndrome requires a high level of suspicion to ensure an accurate diagnosis is made[96]. Diagnosis during acute infection is based on diagnostic tests targeting direct virus detection, typically HIV-1 RNA (or HIV-1 DNA) and p24 antigen. HIV-1 RNA appears in plasma around day 11 after infection, and is followed within ~6 days by the p24 antigen, which transiently appears in the blood when HIV-1 RNA levels rises >10,000 copies/ml[97]. and before the appearance of antibodies. Seroconversion usually occurs within 3-6 weeks following infection and 1-3 weeks following the onset of symptoms[93], and is followed by a decline in viral load and at least partial recovery of the CD4 cell count. The viral load decreases to a "set-point" which is usually reached within six months of the infection. In untreated infection, the viral load set point varies between infected individuals and is a strong predictor of the subsequent rate of CD4 cell decline and disease progression[98].

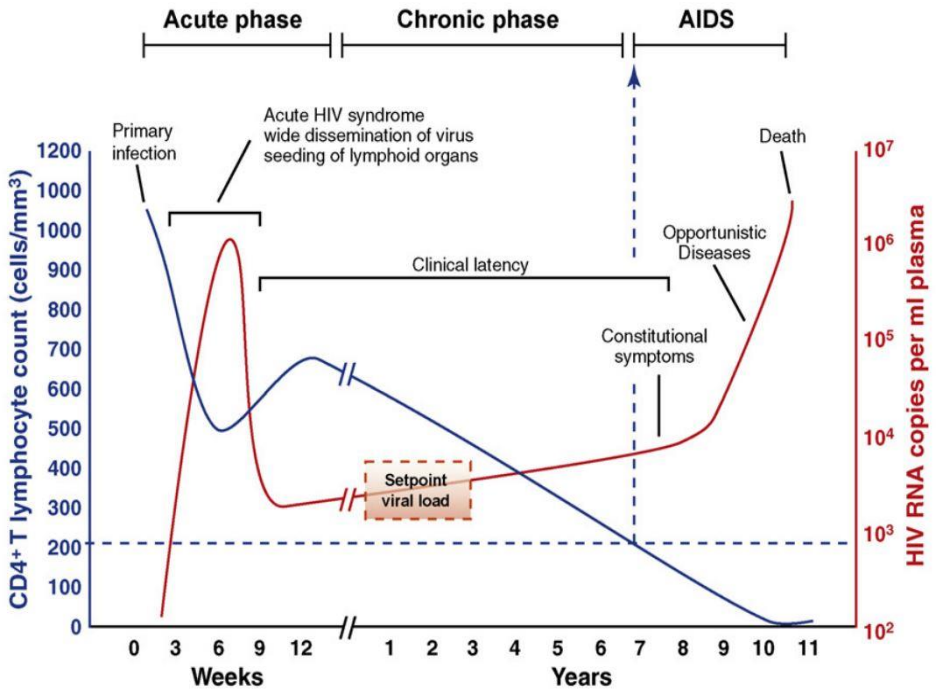


Figure 1.6: Time course of HIV-1 infection showing the immunological and virological pattern of acute, chronic and AIDS stages of HIV-1 infection. Acute HIV-1 infection lasts between 6-12 weeks with flu-like symptoms, a peak viral load and a decrease in $CD4^+$ cells. The chronic asymptomatic/ stage of infection lasts 6-10 years where the viral load reaches a nearly stable set point: the viral load slowly increases and the CD4 cell count slowly decreases over several years. The onset of AIDS typically follows a CD4 cell decline below 200 cells/mm^3 and is characterised by a sharp increase in virus replication and an accelerated decline in CD4 cells; in the absence of treatment the AIDS phase usually leads to death within around 2 years.

Adapted from [96]

The chronic phase of HIV-1 infection is usually a long asymptomatic or minimally symptomatic period. Yet, viral replication occurs at high level in the lymphoid tissues (~10^8 virions produced per day) with adaptation of the viral population to evade host immune responses, leading to progressive immune dysfunction and CD4 cell loss. The onset of opportunistic infections and cancers, as well as direct damaging effects of HIV-1 replication, signals progression to the AIDS stage (Figure 1.6), which characterised by an increase in viral load.

HIV-1 pathogenesis is dependent on two interrelated mechanisms. In addition to the progressive immunosuppression that allows for the onset of opportunistic infections and cancers, HIV-1 promotes a continual state of immune activation and inflammation in the infected host. The dynamic of HIV immune activation is characterised by an increase in multiple inflammatory mediators such as soluble CD14 (a marker of monocyte activation), an

inverted CD4/CD8 ratio (reflecting loss of CD4 cells and expansion of CD8 cells), and exhaustion and senescence of T cells[99]. This state of continuous inflammation is thought to be responsible for the multi-organ disease observed in HIV patients. It Is of note that the mucosal surface of the gastrointestinal tract serves as an immunological barrier against infection[100]. HIV infection disrupts the physiological architecture of the gut and enables translocation of microbial products into systemic circulation[62]. This phenomena further stimulates the innate immune system causing further damage to the gut and persistent immune activation[101].

1.7.3 Viral latency

Most HIV-1 infected cells die quickly due to the cytopathic effect of the virus and antiviral host responses;[102] a subset of CD4 T cells survives, reverting back to a resting state while carrying integrated HIV-1 DNA. During latency, HIV-infected memory CD4 T cells do not sustain virus replication but retain the capacity to produce infectious virus upon activation. This reservoir of infection is long-lived and not susceptible to the activity of antiretroviral drugs or immune surveillance[103]. How the reservoir is maintained during effective ART has been the matter of extensive research. The mainstream view is that the reservoir is maintained by T cell proliferation (either homeostatic or due to antigenic stimulation) without the need for virus production[104]. One other possibility is that in some patients ongoing virus replication in sites of suboptimal drug penetration or drug activity (“sanctuary sites”) may allow replenishment of the reservoir during ART[105].

One important aspect of HIV-1 latency is that a large fraction of integrated provirus is defective (e.g., due to hypermutation and deletions). *In-vitro,* only <1% of integrated proviruses are able to induce infectious virus production following stimulation[106]. It is estimated that during effective ART, approximately 300 cells per 10^6 memory CD4 T cells harbour integrated HIV-1 DNA but only ~1 per 10^6 contains replication competent provirus[107]. It should be noted that despite their defective nature, integrated HIV-1 DNA sequences have the capacity to express HIV-1 genes due to the presence of

an intact LTR promotor region[106]. The resulting products may play a role in maintaining antigenic stimulation and immune activation.

1.7.4 Biomarkers of HIV-1 infection

1.7.4.1 CD4 cell count

The CD4 cell count is the main indicator of disease progression in people with HIV[108]. The greatest risk of opportunistic infections and cancers occurs at a CD4 count <200 cells/mm^3 [109]. Recovery occurs with ART although the lowest ever CD4 cell count (nadir) remains an important predictor of disease outcomes.

1.7.4.2 Plasma HIV-1 RNA load

The amount of free virus particles circulating in blood is represented by the copy number of the viral RNA per millilitre of plasma (viral load). This is the key biomarker of HIV replication and can be accurately quantified using commercially available assays[110]. The role of viral load as a prognostic marker has long been established[111]. The viral load has an inversely proportional relationship to the CD4 cell count[112], rate of disease progression[113], and risk of transmission[89]. The viral load is also the key measure of treatment success whereby the goal of ART is to achieve a viral load below the lower limit of quantification of routine viral load assays and maintain virological suppression long term.

1.7.4.3 HIV-1 DNA

Measuring the amount of HIV-1 DNA present in peripheral blood mononuclear cells (PBMC) is not part of the routine management of HIV-1 infection, but it is often used in the research context to provide an indication of the size of the reservoir[114]. Most assays measure the total amount of HIV-1 DNA, therefore capturing integrated as well as unintegrated variants[115]. Specialised assays measure only integrated provirus or LTR circles[116].There is evidence to indicate that during virologically suppressive ART, there is a good correlation between total and integrated HIV-1 DNA load measured in PBMC[114].

1.8 Antiretroviral therapy

1.8.1 Anti-retroviral drug classes and drug agents

The first antiretroviral agent for treating HIV-1 infection was zidovudine (3'-azido-2',3-dideoxythymidine, abbreviated AZT or ZDV), a nucleoside reverse transcriptase inhibitor (NRTI) that was introduced in 1987[120], soon followed by other agents with a similar mode of action. However, monotherapy and dual therapy with NRTIs had disappointing results due to loss of activity as a result of the emergence of drug resistance[119]. The turning point in HIV-1 treatment came with the introduction of the first protease inhibitors (PIs) saquinavir and indinavir and the first non-nucleoside reverse transcriptase inhibitor (NNRTI) nevirapine in 1995. It was demonstrated that the combination of three antiretrovirals with at least two different mechanisms of action led to sustained suppression of virus replication without emergence of resistance in patients fully compliant to treatment. Currently approved antiretroviral agents target viral entry, reverse transcription, integration and viral maturation (figure 1.7) and clinicians now have access to several drugs (table 1.1) that can be administered in combination to treat HIV-1. More than 25 antiretroviral drug agents are currently approved for use and a multitude of experimental drug agents are in advanced stages of development existing within previous drug classes or classes with a novel mechanisms of action (Table 1.2), including long acting injectable agents such as cabotegravir[120]. These drug agents also exist as co-formulations to increase convenience of administration and improve treatment compliance (Table 1.3). Some antiretroviral agents require a pharmacological booster to enhance their effect, either ritonavir or cobicistat. PIs and the first-generationx= INSTI elvitegravir are co-administered in conjunction with a booster to inhibit the metabolic activity of the hepatic CYP3A4 enzyme and increase plasma levels.

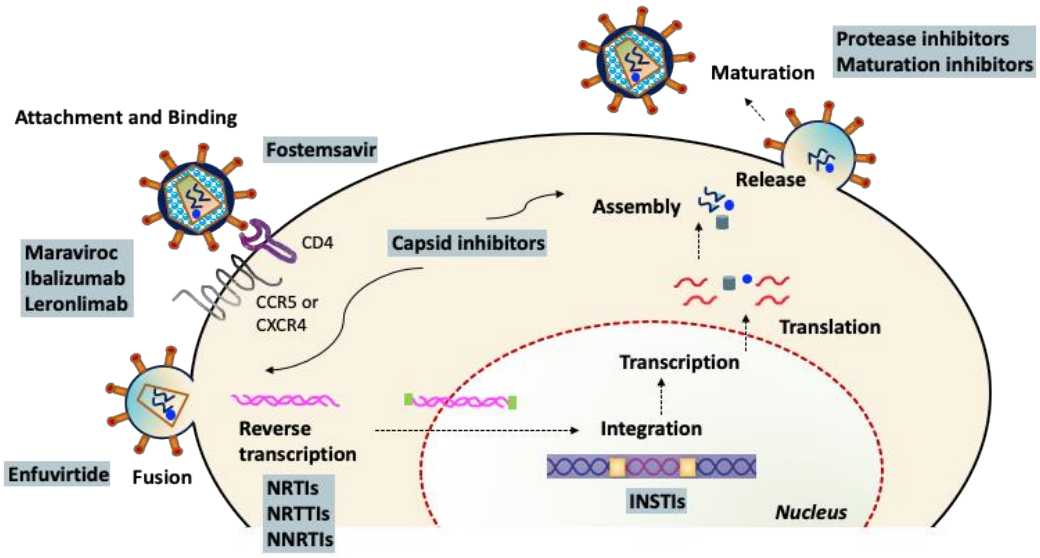


Figure 1.7: A simplified schematic representation of the HIV-1 life cycle showing the classes of drugs and their target sites. Abbreviations; NRTI=nucleo(t)side reverse transcription inhibitor; NNRTI=non-nucleoside reverse transcription inhibitor; NRTTI=Nucleoside reverse transcriptase translocation inhibitors; INSTIs=integrase strand-transfer inhibitors.

Adapted from[69]

Table 1.1: Antiretroviral drug agents currently approved for HIV treatment (early 2020)

Target	Drug class	Agents	Acronym	Use in clinical practice
Entry	CCR5 antagonists	Maraviroc	MVC	Treatment failure / toxicity
	Post-attachment inhibitors	Ibalizumab	IBA	Treatment failure
	Fusion inhibitors	Enfuvirtide	T-20	Treatment failure
Reverse transcription	Nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs)	Abacavir	ABC	Common
		Emtricitabine	FTC	Common
		Didanosine	DDI	Discontinued
		Lamivudine	3TC	Common
		Stavudine	D4T	Discontinued
		Zalcitabine	DDC	Discontinued
		Zidovudine	AZT / ZDV	Generally uncommon
		Tenofovir alafenamide	TAF	Common
		Tenofovir disoproxil fumarate	TDF	Common
	Non-nucleoside reverse transcriptase inhibitors (NNRTIs)	Doravirine	DOR	Limited experience[a]
		Efavirenz	EFV	Common
		Etravirine	ETR	Treatment failure
		Nevirapine	NVP	Uncommon
		Rilpivirine	RPV	Common
Integration	Integrase strand transfer inhibitors (INSTIs)	Bictegravir	BIC	Limited experience[a]
		Dolutegravir	DTG	Common
		Elvitegravir	EVG	Common
		Raltegravir	RAL	Common
Maturation	Protease inhibitors (PIs)	Atazanavir	ATV	Common
		Darunavir	DRV	Common
		Fosamprenavir	FPV	Uncommon
		Lopinavir	LPV	Common
		Indinavir	IDV	Discontinued
		Nelfinavir	NFV	Discontinued
		Ritonavir	RTV / r	Only as a booster
		Saquinavir	SQV	Discontinued
		Tipranavir	TPV	Uncommon

[a]Recently approved

Adapted from [69]

Table 1.2. Experimental anti-retroviral drugs in advanced clinical development

Target	Drug class	Agents	Key properties of drug agent
Entry	Gp120 inhibitors	Fostemsavir (FTR)	• Binds to gp120 and inhibits attachment to the CD4 receptor
	CCR5 inhibitors	Leronlimab (PRO-140)	• Blocks the CCR5 co-receptor • Parental formulation and long-acting
Reverse transcription	Nucleoside reverse transcriptase translocation inhibitors (NRTTIs)	Islatravir (MK-8591 or EFdA)	• Inhibits RT translocation on the nucleic acid substrate and causes DNA chain termination through multiple mechanisms • Oral and parental formulations; long acting
Integration	INSTIs	Cabotegravir (CAB)	• Oral and parental formulations; long acting
Gag function	Maturation inhibitors		• Bind to gag polyprotein, inhibiting the last proteolytic cleavage (between CA and SP1)
	First generation: Bevirimat		• Lack of efficacy against naturally occurring strains with polymorphisms in CA and SP1 • Development halted
	Second generation: Various GSK compounds		• GSK-3532795: poor tolerability and drug resistance; development halted • GSK-2838232: requires boosting for once-daily dosing
	Capsid inhibitors	GS-6207	• Inhibits multiple processes essential for viral replication by modulating capsid assembly, disassembly and transport • Predicted to show a high barrier to drug resistance; parental formulations; long acting

Adapted from [69]

Table 1.3: Co-formulated antiretroviral drug agents

Agent class					Trade
NRTI	**NNRTI**	**INSTI**	**PI**	**Booster**	**name**
Zidovudine, Lamivudine					Combivir
Abacavir, Lamivudine					Kivexa (Epzicom)
Abacavir, Lamivudine		Dolutegravir			Triumeq
TDF, Emtricitabine					Truvada
TAF, Emtricitabine					Descovy
TDF, Emtricitabine	Efavirenz				Atripla
TDF, Emtricitabine	Rilpivirine				Eviplera (Complera)
TAF, Emtricitabine	Rilpivirine				Odefsey
TDF, Lamivudine	Doravirine				Delstrigo
TDF, Emtricitabine		Elvitegravir		Cobicistat	Stribild
TAF, Emtricitabine		Elvitegravir		Cobicistat	Genvoya
TAF, Emtricitabine		Bictegravir			Biktarvy
TAF, Emtricitabine			Darunavir	Cobicistat	Symtuza
			Lopinavir	Ritonavir	Kaletra
			Darunavir	Cobicistat	Rezolsta (Prezcobix)
			Atazanavir	Cobicistat	Evotaz
Lamivudine		Dolutegravir			Dovato
	Rilpivirine	Dolutegravir			Juluca

Abbreviations: NRTI = nucleoside and nucleotide reverse transcriptase inhibitor; NNRTI = non-nucleoside reverse transcriptase inhibitor; INSTI = integrase strand transfer inhibitor; PI = protease inhibitor; TDF = tenofovir disoproxil fumarate; TAF = tenofovir alafenamide.

Adapted from [69]

1.8.2 Mechanism of HIV antiretroviral drug action

Entry Inhibitors

Fusion inhibitors

Enfuvirtide is the only currently available fusion inhibitor. It exerts its effect by binding to the heptad repeat region (HR1) of gp41 thereby preventing the hairpin formation that drives fusion of the viral envelope and host cell membrane[121].

CCR5 antagonists

Maraviroc is the only current approved CCR5 antagonist. Maraviroc is a non-competitive allosteric antagonist of CCR5[122] that binds to the CCR5 chemokine receptor and causes a conformational change to inhibit the binding of CCR5 to the V3 crown of gp120[123] and therefore, entry into host cell. As maraviroc binds to CCR5 but not to CXCR4, maraviroc inhibits CCR5-tropic viruses but not CXCR4 tropic viruses[123]. This means that pre-existing CXCR4 tropic viruses may expand and lead to infection or virological failure.

Post attachment inhibitor

Ibalizumab is the only currently approved post attachment inhibitor. Ibalizumab is a humanised monoclonal antibody that binds to the extracellular C2 domain of the CD4 receptor preventing the interaction between gp120 bound to CD4 and the CCR5 or CXCR4[123].

Reverse transcriptase inhibitors

Nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs)

NRTIs are synthetic substrate analogues of naturally occurring deoxynucleoside triphosphates (dNTPs) that lack a 3' hydroxyl group on the ribose sugar. NRTIs acts as competitive inhibitors and their incorporation into the nascent elongating DNA chain causes termination[125]. NRTIs require metabolic conversion by cellular host-kinases to the corresponding triphosphate form[126]. Nucleoside analogues require tri-phosphorylation; the

nucleotide analogue tenofovir, requires bi-phosphorylation[127]. Currently, tenofovir, lamivudine, emtricitabine, abacavir and to a lesser extent, zidovudine all remain in clinical use (Table 1.1) whereas didanosine, stavudine and zalcitabine are no longer in clinical use due to drug toxicity[123].

Non-nucleoside reverse transcriptase inhibitors (NNRTIs)

All NNRTIs are non-competitive allosteric inhibitors of the RT enzyme and exert their catalytic activity by binding to a hydrophobic pocket in RT which is distinct from the active site[128]. This causes a conformational change in the active site that inhibits the catalytic activity of the enzyme and prevents DNA polymerization[129]. NNRTIs are active against HIV-1 but not HIV-2. Approved NNRTIs are shown in Table 1.1, comprising first and second-generation agents.

Protease inhibitors (PIs)

Protease inhibitors are competitive inhibitors that bind with high affinity to the active site of the PR enzyme, preventing binding and catalytic cleavage of the gag and gag-pol polyproteins. PIs are metabolised by hepatic and gastrointestinal cytochrome p450 enzymes (CYP450). which reduces bioavailability. They are typically administered with a pharmacological booster, ritonavir or cobicistat. Ritonavir and cobicistat inhibit CYP450, increasing plasmatic concentrations and half-life, thus increasing potency and barrier to resistance, and reducing the frequency of dosing[130]. Approved PIs are shown in Table 1.1.

Integrase strand inhibitors (INSTIs)

The HIV-1 integrase gene contains 288 amino acids encoded by the 3′ end of the pol gene. INSTIs prevent the activity of the integrase enzyme by binding to its active site and preventing integration of the linear viral DNA into the host chromosome by blocking the transfer of dsDNA into the host chromosome[69]. Raltegravir, elvitegravir, dolutegravir and bictegravir are approved INSTIs and cabotegravir is in advanced development (table 1.2).

Elvitegravir requires a pharmacological booster and is co-administered with cobicistat.

1.8.3 Principles of HIV-1 drug resistance

At any given time, within the HIV quasispecies of a host, certain virus strains dominate which reflects a balance between viral fitness and ability to evade selective pressures. Variants carrying one or occasionally more mutations that reduce susceptibility to antiviral drugs emerge spontaneously during virus replication[131]. Such spontaneously generated variants typically have lower fitness than variants without the mutations (described as “wild-type”), and therefore, in the absence of selective drug pressure, they exists only at very low frequency and are rarely detectable in circulation[132]. In the event virus replication continues to occur under selective drug pressure, for instance due to poor compliance with treatment, the resistant variants gain selective advantage and expand to become dominant and detectable within the viral quasispecies. As the HIV-1 genome is highly plastic, continuous replication under drug pressure drives further evolution and the acquisition of additional mutations on the viral genome. Some of the mutations increase drug resistance and cross-resistance, whereas others restore viral fitness and are termed “compensatory” mutations. Upon discontinuation of drug pressure, the resistant variants are rapidly outgrown by wild-type virus and become undetectable by routine testing methods. However, the mutated strains often persist at low frequency and can be detected in circulation using ultrasensitive techniques. In addition, the variants become archived in the latent HIV-1 DNA reservoir from which they can resume replication at later stages.

Resistance-associated mutations (RAMs) are classed as primary or major and secondary or accessory. Primary mutations directly reduce drug susceptibility while accessory mutations may contribute resistance effects or act as compensatory mutations. The number of RAMs required to confer drug resistance is dependent of the antiretroviral drug agent. In some cases, a single RAM is sufficient to abolish drug activity (e.g., the K103N mutation in RT confers high-level resistance to the NNRTIs efavirenz and nevirapine). In

other cases, drug resistance increases with the accumulation of multiple RAMs. How easily HIV-1 can escape from a drug depends on multiple factors that together characterise the “barrier to resistance” of that agent. Boosted PIs are the drug class with the highest barrier to resistance, followed by second-generation INSTIs[130,133]. It is important to note that because there are reciprocal interactions between resistance pathways, the barrier to resistance of an ART regimen is more than the simple sum of the barrier to resistance of each component in the regimen.

1.8.4 Monitoring for HIV-1 drug resistance

The presence of drug resistance can be evaluated by analysing:

i) The nucleic acid sequence of the virus to detect mutations that are known to confer phenotypic or clinical drug resistance; this is termed “genotyping”

ii) The ability of the virus to replicate in vitro in the presence of drugs of interest; this is termed “phenotyping”.

Genotyping involves making inferences about the drug susceptibility of a virus isolate by analysing the sequence of interest, which is typically aided by resistance predictive algorithms. Several algorithms exist, with the main programmes being the HIV Stanford drug resistance algorithm (HIVdb) (http://hivdb.stanfordd.edu/index.html); Rega Institute resistance algorithm (Rega) (http://regaweb.med.kuleuven.be/software/rega_algorithm/) and the French Agence Nacional de Recherche sur le SIDA (ANRS) resistance algorithm (http://www.hivfrenchresistance.org/). These algorithms employ a rule-based system to provide drug activity scores [134]. Genotyping can employ Sanger or population sequencing, which produces a consensus sequence derived from the dominant quasispecies in a sample. The method has a detection threshold of >15-20% and cannot detect variants that occur at low frequency in a sample. Ultra-deep sequencing (UDS) or next-generation sequencing (NGS) methods are able to both detect low frequency mutants and provide a quantification of the variant in a patient sample, which is defined as the variant frequency. The most commonly used NGS platform is

currently the Illumina platform. UDS mostly relies on first producing an amplicon by polymerase chain reaction (PCR). This process can introduce errors which may falsely suggest the presence of mutations[135]. A number of strategies are adopted to obtain reliable data, including filtering sequences with low quality reads and setting a threshold for the interpretation of variant frequency, typically at 1% or 2% of the reads. Some methods have omitted the PCR step during UDS to circumvent some of the PCR associated error[136].

Nomenclature of HIV-1 drug resistance

Resistance mutations such as the lamivudine-selected substitution M184V are represented using a number to denote the position of the mutated amino acid in the protein preceded and followed by letters indicating the “wild type” and the resistant-conferring amino acid, respectively (Figure 1.8)

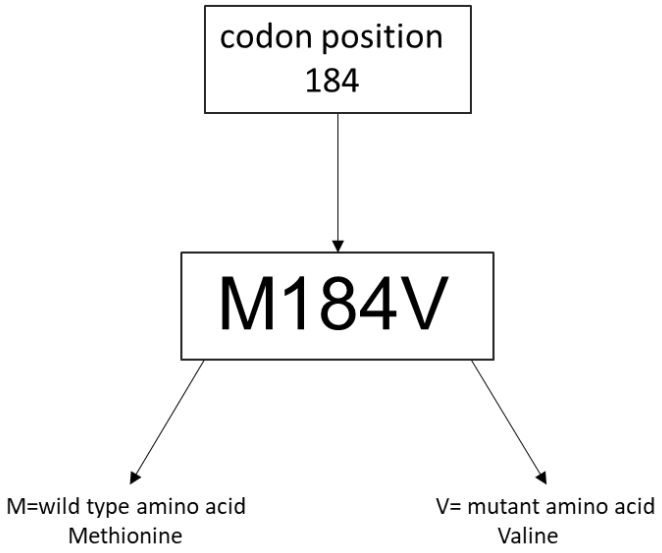


Figure 1.8: Representation of the nomenclature used for HIV-1 drug resistance-associated mutations

1.8.4.1 Resistance to nucleoside and nucleotide reverse transcriptase inhibitors

There are two mechanisms of NRTI resistance. Discriminatory mutations allow the RT to discriminate between synthetic dideoxy-NRTI chain terminators and the natural dNTPs, preventing binding and incorporation into the nascent viral DNA chain (Figure 1.9). Common discriminatory mutations occur at amino acid positions 65, 70, 74, 115 and 184. M184V and M184I are positioned in a highly conserved subdomain of RT close to the active site

of the enzyme. They are selected primarily by lamivudine and emtricitabine and confers a high level of phenotypic resistance to both agents. M184V/I also cause low-level phenotypic resistance to abacavir (Figure 1.10). In contrast, these mutations increase susceptibility to zidovudine and tenofovir. K65R is another important discriminatory mutation. It is selected by tenofovir and abacavir and decreases susceptibility to all NRTIs except zidovudine[137].

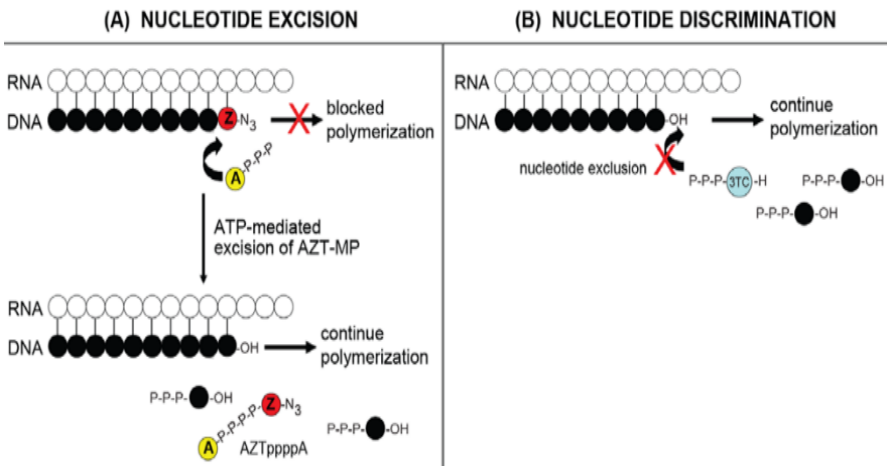


Figure 1.9: Schematic illustration of the mechanisms of NRTI resistance. A) Nucleotide excision: mutation in reverse transcriptase such as the thymidine analogue mutation T215Y aid the ATP-mediated removal of the incorporated NRTI (e.g., AZT) from the nascent DNA chain and allow elongation to resume. B) Nucleotide discrimination: mutation such as the M184V enable the reverse transcriptase to discriminate between naturally dNTPs and dideoxy-NRTI chain terminators preventing NRTI incorporation into the elongating DNA chain.

The second mechanism of NRTI resistance is defined as primer unblocking or nucleotide excision. This mechanism allows the phosphorylytic excision of the incorporated NRTI triphosphate allowing elongation of the DNA chain to resume[138]. A classic example is the zidovudine mutation T215Y. Adenosine triphosphate (ATP) usually acts as a pyrophosphate donor in the excision (Figure 1.9).

Major Nucleoside RT Inhibitor (NRTI) Resistance Mutations

	Discriminatory					TAMs						MDR	
	184	65	70	74	115	41	67	70	210	215	219	69	151
Cons	M	K	K	L	Y	M	D	K	L	T	K	T	Q
3TC	**VI**	**R**										**Ins**	**M**
FTC	**VI**	**R**										**Ins**	**M**
ABC	VI	**R**	E	**VI**	**F**	L			W	FY		**Ins**	**M**
TFV	***	**R**	E		F	L		R	W	FY		**Ins**	**M**
ZDV	***	***	*	*		**L**	**N**	**R**	**W**	**FY**	QE	**Ins**	**M**

Major Non-Nucleoside RT Inhibitor (NNRTI) Resistance Mutations

	100	101	103	106	181	188	190	230
Cons	L	K	K	V	Y	Y	G	M
DOR	I	EP		**AM**I	CIV	**L**CH	**SE**	**L**
EFV	**I**	E**P**	**NS**	**AM**	CIV	**LC**H	**ASEQ**	**L**
ETR	**I**	E**P**			**CIV**	L	ASEQ	**L**
RPV	**I**	**EP**			**CIV**	**L**	ASEQ	**L**
NVP	**I**	**EP**	**NS**	**AM**	**CIV**	**LCH**	**ASEQ**	**L**

Figure 1.10: Major resistance mutations for NRTI and NNRTIs. Mutations are classed as discriminatory, thymidine analogue mutations (TAMs) and multi-drug resistance mutations Each mutation is denoted by their amino acid position and letter code. The consensus of the wild-type virus in reference to HXB2 is depicted in the top row and the amino acid change is shown for each drug below. Underlining and bold shows high-level clinical/phenotypic resistance; bold alone shows moderate-level clinical/phenotypic resistance. Plain amino acid code shows low-level resistance while asterisks (***) represent increased susceptibility to the drug in the presence of mutation. Abbreviations; lamivudine (3TC), emtricitabine (FTC), abacavir (ABC), TFV (tenofovir), ZDV (zidovudine); cons= wild type consensus amino acid; MDR= multi-drug resistance; ins=insertions.

Adapted from [139]

Thymidine analogue mutations (TAMs) are selected by the thymidine analogues zidovudine and stavudine and have resistance effects for all NRTIs with the exception of lamivudine and emtricitabine. They emerge along two pathways: TAM-1 and TAM-2[140]. TAM-1 mutations comprise M41L, L210W, and T215Y. TAM-2 mutations comprise D67N, K70R, T215F and K219Q/E. The TAM-1 pathway is associated with higher levels of resistance and cross-resistance[141]. TAMs appear in sequential order and their accumulation over time correlates with growing zidovudine and stavudine resistance and cross-resistance to tenofovir and abacavir. TAMs tend to markedly reduce viral fitness by affecting the active site of a key viral enzyme. Due to this reason, NRTIs often retain significant antiviral activity despite the presence of RAMs, and the effect is enhanced by the concomitant presence of M184V[142]. This reflects the fact that M184V inhibits primer unblocking[143].

1.8.4.2 Resistance to non-nucleoside reverse transcriptase inhibitors

The most common NNRTI RAMs are listed in Figure 1.10. First-generation NNRTIs are characterised by a low barrier to resistance and a single RAM is sufficient to cause high-level resistance[139]. Cross-resistance within the class is broad. Second-generation NNRTIs (etravirine and doravirine) have a higher barrier to resistance and often retain activity against certain NNRTI-resistant mutants if used in combination with other active agents[139]. NNRTI RAMs reduce drug susceptibility without a significant impact on viral fitness[65].

1.8.4.3 Resistance to protease inhibitors

PIs can be divided into first generation agents (ritonavir, saquinavir, indinavir and nelfinavir); second generation agents (fosamprenavir, lopinavir and atazanavir) and third generation agents (tipranavir and darunavir). More recent PIs have a higher affinity for the enzyme active site and in the presence of a booster are often able to accommodate a number of mutation without losing antiviral activity[144]. Major RAMs can occur at multiple positions within the 99-amino acid residue of the PR[145] (Figure 1.11). Whereas a single mutation can reduce susceptibility to some PIs, significant resistance to boosted PIs typically requires the accumulation of multiple mutations. Boosted PIs have the highest barrier to resistance among available antiretroviral drugs and it is common for patients to show lack of RAMs despite ongoing viraemia. Lopinavir, tipranavir and darunavir have the highest genetic barrier of any available antiviral drug, and ≥ three mutations are required to confer high level resistance [146]. The activity of darunavir can be predicted using a mutational score[147]that comprises V11I, V32I, L33F, I47V, I50V, I54L/M, T74P, L76V, I84V, and L89V[147].

Cons	30 D	32 V	46 M	47 I	48 G	50 I	54 I	76 L	82 V	84 I	88 N	90 L
ATV/r		**I**	IL	V	**VM**	**L**	**VTAM**		ATFS	**V**	**S**	M
DRV/r		**I**		**VA**		**V**	**LM**	**V**	F	V		
FPV/r		**I**	**IL**	**VA**		**V**	**VTALM**	**V**	ATFS	**V**		M
IDV/r		**I**	**IL**	VA			**VTA**	**V**	**ATFS**	**V**	S	M
LPV/r		**I**	IL	**VA**	VM	**V**	**VTALM**	**V**	**ATFS**	**V**		M
NFV	**N**		**IL**	VA	**VM**		**VTALM**		**ATFS**	**V**	**DS**	**M**
SQV/r					**VM**		**VTAM**		AT	**V**	S	**M**
TPV/r		I	IL	**VA**			**VTAM**		**LT**	**V**		

Figure 1.11: Major resistance mutations for protease inhibitors. Each mutation is denoted by their amino acid position and letter code. The consensus of the wild-type virus in reference to HXB2 is depicted in the top row and the amino acid change is shown for each drug below. Underlining and bold shows high-level clinical/phenotypic resistance; bold alone shows moderate-level clinical/phenotypic resistance. Plain amino acid code shows low-level resistance. Abbreviations; ritonavir-boosted atazanavir (ATV/r), ritonavir-boosted darunavir (DRV/r), ritonavir-boosted fosamprenavir (FPV/r), ritonavir-boosted indinavir (IDV/r), ritonavir-boosted lopinavir (LPV/r), nelfinavir (NFV), ritonavir-boosted saquinavir (SQV/r) and ritonavir-boosted tipranavir (TPV/r); cons= wild type consensus amino acid

Adapted from [139]

In addition to RAMs occurring in PR, an alternative pathway of PI resistance is driven by mutations in *gag*, including mutations occurring primarily in the cleavage sites (CS) but also involving non-cleavage sites[148]. CS mutations can act as compensatory mutations[149], and can also confer resistance either in isolation or in combination with PR mutations[150]. The sequence of cleavage of the PR is shown in figure 1.12.

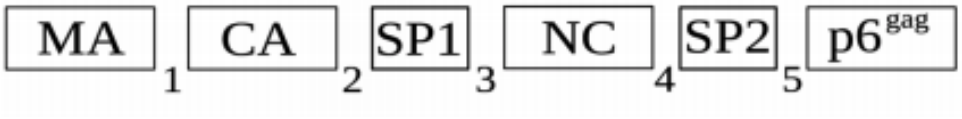


Figure 1.12: Cleavage steps of the Gag polyprotein. Each box indicates each cleavage steps in the order of cleavage. Abbreviations: MA = matrix; CA = capsid; SP1 = spacer peptide 1; NC = nucleocapsid; SP2 = spacer peptide 2.

1.8.4.4 Resistance to Integrase strand transfer inhibitors

Raltegravir and elvitegravir have a low barrier to resistance, whereas dolutegravir and bictegravir can retain significant activity against mutated viruses. Mutations associated with INSTIs (Figure 1.13).

Major Integrase Inhibitor (INSTI) Resistance Mutations										
	66	92	118	138	140	143	147	148	155	263
Cons	T	E	G	E	G	Y	S	Q	N	R
BIC	K	Q	**R**	KAT	SAC			**HRK**	H	**K**
DTG	K	Q	**R**	KAT	SAC			**HRK**	H	**K**
EVG	**AIK**	**Q**	**R**	**KAT**	**SAC**		**G**	**HRK**	**H**	**K**
RAL	AI**K**	**Q**	**R**	**KAT**	**SAC**	**RC**		**HRK**	**H**	K

Figure 1.13: Major resistance mutations for integrase strand transfer inhibitors. Each mutation is denoted by their amino acid position and letter code. The consensus of the wild-type virus in reference to HXB2 is depicted in the top row and the amino acid change is shown for each drug below. Bold: reduced susceptibility or virological response. Plain text: reduced susceptibility in combination with other INSTI-resistance mutations. Abbreviations: raltegravir (RAL), elvitegravir (EVG), dolutegravir (DTG); cons= wild type consensus amino acid

Adapted from [139]

There is a high potential for cross-resistance between INSTIs. For instance, a mutational combination of Q148H/R/K ± G140S/A confers the highest level of resistance and cross-resistance[151]. This is mediated by the high resistance effects of mutations at position 148 combined with the compensatory effects of mutations at codon 140, which restores viral fitness.

1.9 Principles of antiretroviral therapy

Treatment guidelines by the World Health Organization (WHO) and specialist societies such as the European AIDS Clinical Society (EACS) and the Department of Health and Human Services (DHSS) in the US recommend the start of ART soon after diagnosis and regardless of the CD4 cell count[152–154]. The HIV-1 treatment paradigm adopted in the late 1990s, involving the use of two agents from the NRTI class and a third agent from a different class[155], continues to be preferred, although the preferred individual agents have evolved over time to reflect the introduction of agents with greater efficacy and tolerability.

Table 1.4. WHO, North America and European recommended regimens for starting ART in adults with HIV infection (2018)[152,154,156]

WHO	DHHS	IAS-USA	EACS
TDF + (3TC or FTC) + DTG	BIC/TAF/FTC[a]	DTG/ABC/3TC[a]	DTG/ABC/3TC[a]
TDF + (3TC or FTC) + (EFV or PI/b)	DTG/ABC/3TC[a]	DTG + TAF/FTC	DTG + (TAF or TDF)/FTC
AZT/3TC + EFV	DTG + (TAF or TDF)/FTC	EVG/COBI/TAF/FTC[a]	EVG/COBI/(TAF or TDF)/FTC[a]
TDF + (3TC or FTC) + RAL	EVG/COBI/(TAF or TDF)/FTC[a]	RAL + TAF/FTC	RAL + (TAF or TDF)/FTC
	RAL + (TAF or TDF)/FTC		RPV + (TAF or TDF)/FTC[a]
			DRV/ (RTV or COBI) + (TAF or TDF)/FTC

[a] single tablet regimen.

Abbreviations: DHSS= Department of Health and Social Services; IAS-USA= International AIDS Society-USA; EACS= European AIDS Clinical Society;=ABC = abacavir; 3TC = lamivudine; DTG = dolutegravir; TFV = tenofovir (TDF or TF); FTC = emtricitabine; TAF = tenofovir alafenamide; BIC = bictegravir; RAL = raltegravir; EVG = elvitegravir; COBI = cobicistat; DRV = darunavir; RTV = ritonavir; ATV = atazanavir; DOR = doravirine; EFV = efavirenz; RPV = rilpivirine.

Most people with HIV-1 initiate ART with a combination of three antiretroviral agents typically taken once a day; treatment recommendations are similar in resource limited[154] and resource-abundant[153] settings although options are generally more restricted. NRTIs are part of preferred first-line and second-line regimens and are often termed the "backbone" of a regimen[69]. For several years, the preferred third agent for starting ART were the NNRTIs efavirenz and nevirapine. Because of the high barrier to resistance, PI/b were and are still often preferred for starting ART in patients with compliance issues or certain forms of drug resistance[157]. More recently, the preferred third agent for starting ART is an agent from the INSTI class. For patients with failure of first-line ART, second-line regimens are typically composed of 2 NRTIs + PI/b or dolutegravir[158]. Subsequent lines of therapy require tailoring based on the treatment history.

1.9.1 Simplification strategies

Current antiretroviral therapies are able to effectively suppress viral replication, prevent HIV transmission, restore immune function and enhance quality of life. However, a desire to limit long-term exposure to potential drug toxicity, enhance adherence, and reduce the cost of treatment, combined with the availability of novel potent agents, has sparked interest in exploring simplified treatment regimens. Two approaches are considered. The first is the initiation of ART with a dual regimen in selected patients. More established is the concept of maintenance therapy, whereby patients established on suppressive triple ART switch to a simpler regimen. Simplification strategies that have been explored in clinical studies include monotherapy with PI/b or dolutegravir, and dual combinations with either a PI/b or a second-generation INSTI with a second agent. Specialist societies such as the EACS[152] and DHSS[153] endorse the use of dolutegravir and rilpivirine as a dual combination in selected patients. The use of PI/b monotherapy has been explored in clinical trials (Table 1.5). Results have generally shown a lower rate of HIV-1 RNA suppression when compared to triple ART, but no emergence of PR RAMs.

1.9.2 Literature search strategy and criteria

The literature review of previously published evidence on the using of protease inhibitor monotherapy was performed amongst HIV patients already suppressed on first or second-line ART. A search of two electronic database, i.e. Embase and PubMed was conducted. In brief, search terms included “protease inhibitors”, “monotherapy”, “lopinavir”, “darunavir” and “atazanavir”. Studies were limited to randomised control trials with no language restriction. Trials included, compared PI/r to dual or triple-ART with any combination of drug agents. Trials were excluded if patients were unsuppressed at baseline or were naïve to ART.

Table 1.5: Summary characteristics of relevant clinical trials evaluating the use of boosted protease inhibitor monotherapy.

Study	Drug	Location	Year	N	Randomisation arms	Main inclusion criteria	Main exclusion criteria	Primary endpoint	Main findings
MANET[a]	DRV/r	Cameroon	2014-2015	120	DRV/r monotherapy vs. continuation of triple PI/b-based ART (2:1)	On second-line PI/b-based triple ART for ≥12 weeks; confirmed viral load <60 copies/ml (4-12 weeks apart)	Previous failure on PI/b-based ART; HBsAg positive; AIDS-defining diagnosis; clinically significant disease; pregnancy or breast feeding	% patients with <400 copies/ml at week 24	72/81 (88.9%) vs. 37/39 (94.9%)
MOBIDIP[159]	LPV/r DRV/r	Cameroon, Burkina Faso, Senegal	2014-2015	265	PI/b monotherapy vs. PI/b+3TC dual therapy (1:1)	On second-line PI/b triple ART for ≥48 weeks; No change in ART in the last 3 months; confirmed viral load < 200 copies/ml (last 6 months); CD4 count > 100 cells/mm^3; adherence ≥90% at last control	History of PI failure; HBsAg positive; HIV encephalitis; pregnancy or breast feeding.	% patients with treatment failure at week 96, comprising virological failure (>500 copies/ml), treatment intensification, or PI/b discontinuation	33/133 (24.8%) vs. 4/132 (3.0%)
MONET[160]	DRV/r	Europe Russia	2007-2008	256	DRV/r monotherapy vs. triple DRV/r-based ART (1:1)	On triple ART for ≥24 weeks; confirmed viral load <50 copies/ml (≥6 months apart); CD4 count > 200 cells/mm^3	History of virological failure; history of major PR RAMs; HBsAg positive; pregnancy or breastfeeding.	% patients with treatment failure at week 48, comprising viral load >50 copies/ml and discontinuation of DRV/r	20/127 (15.7) vs. 19/129 (14.7%)
MONOI[161]	DRV/r	France	2007-2008	225	DRV/r monotherapy vs. triple DRV/r-based ART (1:1)	Established on triple ART; viral load <50 copies/ml at screening and <400 copies/ml in the last 18 months (≥4 measurements); CD4 count >200 cells/mm^3; nadir CD4 count >50 cells/mm^3	History of PI failure; HBsAg positive; pregnancy or breastfeeding; AIDS defining diagnosis within 30 days of screening; HIV related neurological disease	% patients with treatment success at week 48, when treatment failure comprised viral load >400 copies/ml, discontinuation of study arm and withdrawal from study	104/113(92.0) vs 98/112 (87.5)
PROTEA [162]	DRV/r	Europe Israel	2012-2014	273	DRV/r monotherapy vs. triple DRV/r-based ART (1:1)	Established on triple first-line ART with viral load <50 copies/ml for ≥ 48 weeks; CD4 count > 200 cells/mm^3;	History of virological failure on any ART; history of major PR RAMs; HBsAg positive	% patients with <50 copies/ml at week 48	118/137 (86.1) vs 129/136 (94.4)

						nadir CD4 count > 100 cells/mm^3			
PIVOT[163]	LPV/r or DRV/r	United Kingdom	2008-2010	587	DRV/r monotherapy vs. triple PI-based ART (1:1)	Established on triple ART for ≥24 weeks with no change in treatment; viral load <50 copies/ml at screening and <50 copies/ml for ≥24 weeks prior to screening; CD4 count > 100 cells/mm^3	History of PI resistance; previous change for unsatisfactory virological response; pregnancy; treatment of acute opportunistic infection within previous three months; HBsAg positive and active or planned HCV treatment.	Loss of future treatment option defined as new intermediate-level or high-level resistance to one or more drugs to which the patient's virus was sensitive at trial entry using Kaplan-Meier estimate	6/296 (2.0%) vs 2/291 (0.7%%)
OK[164]	LPV/r	Spain	2004-2005	205	DRV/r monotherapy vs. triple LPV/r-based ART (1:1)	Established on LPV/r ART for ≥4 weeks and had viral load < 50 copies/ml for ≥6 months; CD4 count > 100 cells/mm^3	Previous failure on PI/b-based ART; HBsAg positive	% patients without therapeutic failure at week 48, comprising viral load >500 copies/ml, treatment intensification, PI/b discontinuation and loss to follow-up	92/103 (89%) vs 92/102 (90%)
KalMO[165]	LPV/r	Brazil	2004-2005	60	LPV/r monotherapy vs. triple NNRTI-based ART (1:1)	Established on triple ART for ≥ 6 months; viral load < 80 copies/ml; CD4 count > 200 cells/mm^3; nadir CD4 count > 100 cells/mm^3	Previous documented intolerance or history of failure on LPV/r; pregnancy, lactation, any LPV/r contraindicated treatment and previous AIDS defining diagnosis.	% patients with <80 copies/ml at week 96	24/30 (80.0%) vs 26/30 (86.7%)
DREAM[166]	LPV/r	France	2009-2011	197	LPV/r monotherapy vs. triple ART comprising of TDF+FTC+EFV (1:1)	Established on triple ART with viral load < 50 copies/ml for 12 months; CD4 count > 200 cells/mm^3; nadir CD4 count > 100 cells/mm^3 at screening.	Previous failure on PI/r; history of PI resistance; HBsAg positive; pregnancy or breast feeding; liver cirrhosis; hypersensitivity to efavirenz or LPV/r.	% of patients without virological failure (<50 copies/ml at week 96 or without AIDS defining diagnosis)	63/98 (64%) vs 70/99 (71%)

KALESOLO[167]	LPV/r	France	2005-2006	186	LPV/r monotherapy vs. triple NNRTI-based ART (1:1)	Established on triple ART with viral load < 50 copies/ml for 6 months with no changes in the last 3 months;	Previous failure on PI/r; opportunistic infection in the last 6 months; HBsAg positive	% patients with <50 copies/ml at week 48	73/87 (84%) vs 87/99 (88%)
MODAt[168]	ATV/r	Italy	2010-2012	103	ATV/r monotherapy vs. triple ATV/r-based ART (1:1)	Established on 2 NRTIs +ATV/r at least 48 weeks; viral load <50 copies/ml at screening and <400 copies/ml in the 24 weeks; nadir CD4 count > 100 cells/mm^3 at screening.	Previous failure on PI/r; History of PI resistance; pregnancy and breast feeding; HBsAg positive;	% patients with treatment success at week 48, comprising viral load <50 copies/ml, treatment intensification, PI/b discontinuation, loss to follow up	37/51 (73%) vs 44/52 (85%).

[a]Data presented in this thesis, not yet published.

Data presented was analysed using per intention-to-treat analysis i.e. missing data was characterised as failure.

Abbreviations: NRTI = nucleoside and nucleotide reverse transcriptase inhibitor; NNRTI = non-nucleoside reverse transcriptase inhibitor; PI/b=boosted protease inhibitor; DRV/r=ritonavir-boosted darunavir; LPV/r= ritonavir-boosted lopinavir; ATV/r= ritonavir-boosted atazanavir; ART=antiretroviral therapy; HBV=hepatitis B virus; HCV=hepatitis C virus; HBsAg=Hepatitis B surface antigen; AIDS=acquired immune deficiency syndrome; TLOVR= time to loss of virological response; RAMS=resistance associated mutations;

1.10 HIV-1 in sub-Saharan Africa

HIV-1 affects more people in SSA than in any other region of the world, accounting for 70% of the global burden[169] and providing a crude indication of the correlation between HIV infection and poverty[170]. By the end of 2018, there were approximately 25.9 million people estimated to be living with HIV[31] in SSA, with 20.6 million in Eastern and Southern Africa and 5.0 million in Western and Central Africa. Numerically, most people with HIV are in South Africa (25%) and Nigeria (13%). HIV-1 related data for Cameroon is shown in table 1.6

Table 1.6: HIV data for Cameroon.

Characteristic	Cameroon
National adult population (>15 years) [a]	14,188,000
First AIDS related case	1985 [171]
National HIV incidence (general population) [a]	1.0%
National adult (15-45 years) HIV prevalence [a]	3.6%
PLHIV [a]	540,000
Incident infection [a]	23,000
AIDS related death [a]	18,000
% accessing ART [a]	52%
% accessing ART and virologically suppressed [a]	Unreported

[a] UNAIDS estimated numbers as of 2018.

1.11 Hepatitis B Virus in people with HIV-1

Globally, hepatitis B virus (HBV) presents as one of the most common infections with one-third of the world's population showing evidence of exposure indicated by seropositivity for hepatitis B core antibody (anti-HBc)[173]. It is estimated that around 257 million people are chronically infected as indicated by seropositivity for hepatitis B surface antigen (HBsAg). People with chronic HBV infection are at risk of liver cirrhosis, hepatic decompensation and hepatocellular carcinoma[174] and each year nearly 900,000 people die of HBV-related complications[175]. In 2014, at the 67th World Health Assembly, the WHO reaffirmed its resolution to eliminate viral hepatitis as a public health concern by 2030, highlighting the need for improved immunization, diagnosis and treatment[174]. As HIV and HBV are spread through similar routes, their epidemiological pattern overlaps. Co-infection with HIV-1 and HBV is associated with an increased risk of rapidly

progressing liver disease[176], making it paramount that both infections are adequately diagnosed, monitored and treated[156,174]

1.11.1 Hepatitis B Virus

HBV is a small enveloped virus of ~ 42nm and has a partially double stranded circular DNA genome of 3.2 kilobases (Figure 1.14) with four overlapping opening reading frames: surface, core, pol and X. The viral proteins comprise the surface antigen (HBsAg), the core protein and the e antigen (HBeAg), the polymerase enzyme, and the X protein. HBV is classified into 10 genotypes A-J, multiple subtypes, and the variants show geographical distribution. Genotype A subtype A1 and genotype E predominate across SSA. The natural history of HBV infection varies widely and is driven by both viral and host factors. Following infection, the risk of becoming a chronic carrier of HBsAg depends on age: 80-90% of children infected in first year of life and 30-50% of those infected before the age of six years develop chronic HBV infection[177], whereas the risk is <10% among immunocompetent adults. Importantly, people who successfully clear acute infection retain the viral genome inside hepatocytes and may relapse if they become immunocompromised.

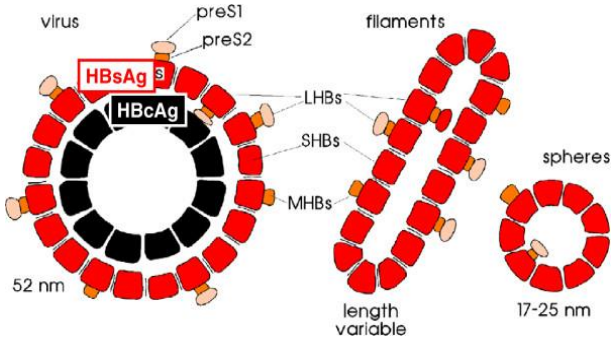


Figure 1.14: A model schematic of the HBV virion showing infectious (Dane particles) and non-infectious HBV virion particles. The infectious Dane particle is shown on the left with the PreS/PreS1 and S proteins and the non-infectious filamentous and spherical entities are shown on the right.

Adapted from [178]

1.11.2 HBV replication cycle

The replication cycle of HBV is shown in Figure 1.15. Key features are that the virus establishes a “minichromosome” in the nucleus of hepatocytes in the form of covalently closed circular DNA (cccDNA), following repair of the

relaxed circular DNA (rcDNA) by host enzymes. The cccDNA serves as the transcriptional template for the virus, encoding pregenomic RNA and various messenger RNAs (mRNAs)[179]. In the cytoplasm, the viral pregenomic RNA undergoes reverse transcription into DNA by the viral polymerase enzyme, in a process that is similar to that observed with HIV and is similarly prone to errors. Thus, there are several parallels between HIV and HBV. Both viruses exist as a quasispecies in their hosts. They can be effectively inhibited by nucleoside and nucleotide analogues that act as inhibitors of DNA synthesis via chain termination. Several antiviral agents used to treat HIV-1 also have activity against HBV, including tenofovir, lamivudine and emtricitabine. Comparably with HIV, current treatment interventions are able to control HBV replication but unable to eliminate the virus[180]. This is because both viruses establish persistent forms in the nucleus of target cells which are not susceptible to antiviral therapy and fuel virus rebound upon treatment discontinuation. There are also important differences, however. Virus escape from antiviral therapy occurs more promptly with HIV-1 than HBV and monotherapy with a potent NRTI such as tenofovir is sufficient to induce long-term HBV control.

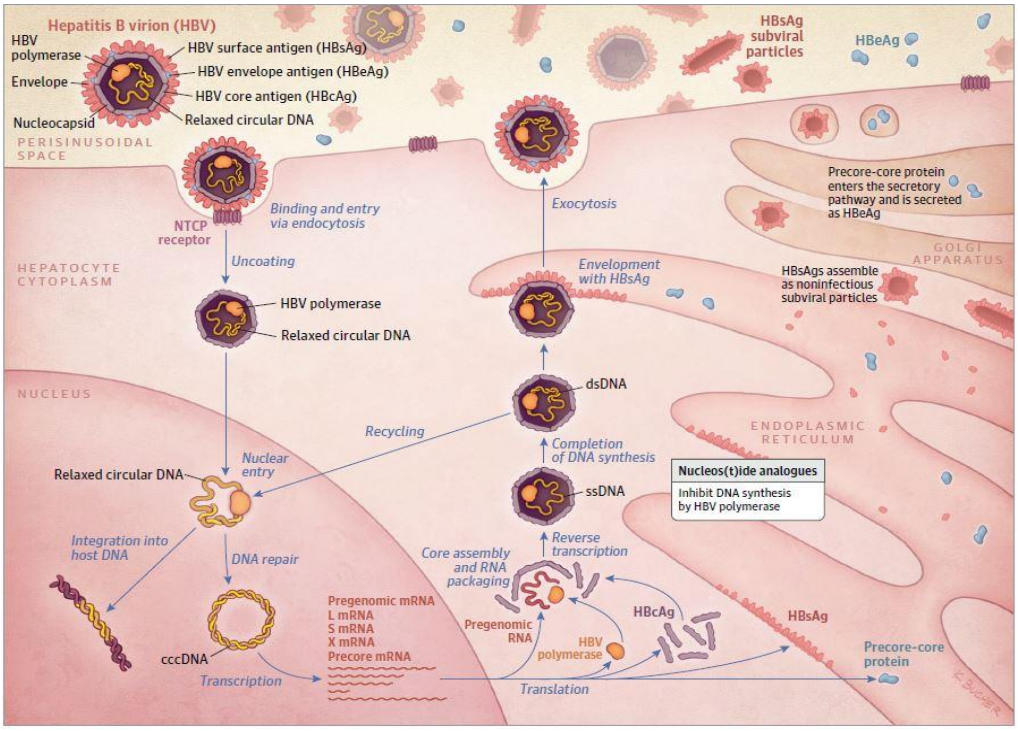


Figure 1.15: HBV binds to the sodium taurocholate co-transporting polypeptide (NTCP) receptor on the surface of hepatocytes. This drives endocytosis and the release of its DNA-containing nucleocaspid into the cytoplasm followed by nuclear transport. Within the nucleus, the viral DNA in its relaxed circular form is converted into a closed covalent circular DNA (cccDNA). Integration of the HBV DNA into host genome can also take place. The

cccDNA functions as a “minichromosome” and template for viral RNA transcription. cccDNA has a long half-life and persists within the nucleus of the host hepatocyte and serves as a reservoir for viral reactivation.

Adapted from [179]

1.11.3 Biomarkers of HBV infection

Multiple serum biomarkers are used to profile HBV infection (Table 1.7 and 1.8).

Table 1.7: Virological, biochemical and serological markers of HBV infection.

Marker	Definition of marker	Significance
HBsAg	surface antigen	Current infection (chronic if persistent for >6 months))
anti-HBs	antibody to surface antigen	Typically, immunity from either natural infection or vaccination
anti-HBc IgM	IgM to core antigen	Acute infection or flare of replication in chronic infection
anti-HBc IgG/Total	IgG antibody to the core antigen	Past infection or chronic infection
HBeAg	e antigen	In an untreated person, high HBV replication and infectivity
anti-HBe	antibody to e antigen	Seroconversion from HBeAg positive
AST/ALT	Liver transaminases	Hepatic inflammation and necrosis
HBV DNA	viral DNA	Level of viral replication

Table 1.8: Interpretation of HBV status

Interpretation	HBsAg	Anti-HBs	Anti-HBc	HBeAg	HBV DNA	Detail of interpretation
Current infection	+ve	Usually -ve	+ve	+ve or -ve	+ve	HBsAg for >6 months defines chronic infection;
Resolved/past infection	-ve	Usually +ve	+ve	-ve	Usually -ve	HBV DNA may be intermittently detected at low level
Vaccinated	-ve	+ve	-ve	-ve	-ve	HBV vaccine contains HBsAg only
Non-immune	-ve	-ve	-ve	-ve	-ve	Individuals have not previously been vaccinated and are susceptible to infection following exposure to HBV

Abbreviation; HBV= Hepatitis B virus; HBsAg = Hepatitis B surface antigen; anti-HBc=HBV core antibody; anti-HBs=HBV surface antibody.

1.11.4 Prevention and treatment of HBV in the context of HIV infection

HIV-1 positive individuals are at higher risk of HBV co-infection due to similar percutaneous and sexual routes of transmission. Thus, international recommendations indicate that patients with HIV infection be screened at diagnosis for HBV infection and immunity and vaccinated if found to be susceptible to HBV[174]. Recommendations also indicate that patients with both HIV and HBV should receive tenofovir plus emtricitabine or lamivudine as part of ART[154,182].

1.11.5 HBV in Africa

HBV is the most common cause of end-stage liver disease across Africa[183,184]. Whilst HBV prevalence is generally heterogeneous across the region, prevalence of HBV across SSA is estimated at >8%[185]. HBV is believed to be mainly transmitted in SSA through horizontal routes which typically involves inadvertent childhood transmission (within household or through unsafe cultural practices)[185]. This commonly occurs before HIV infection occurs later in life. There is a significant overlap between HIV and HBV with an estimated 2.7 million[186]. Recent pooled analysis has shown an estimated HIV/HBV co-infection rate of up to 12.4% in Western and Central Africa[187]. In line with other guidelines, WHO has since 2010, indicated the use of two agents with activity against HBV and HIV typically, tenofovir and lamivudine or emtricitabine, as part of ART regimens for HIV/HBV co-infected patients[174]. Although screening HIV-positive patients for HBV is recommended, this is not implemented systematically across the SSA region[185]. There is also a variable rate of infant HBV vaccination coverage across SSA, although rates have improved in many countries. In Cameroon for example, reported infant vaccination coverage (three doses) was 85% for Cameroon in 2016[188]. It is noteworthy that adult catch-up vaccination programmes do not exist across the region.

1.12. Aims and objectives of this thesis

1.12.1 Aim of the thesis

The aim of this research work was to investigate the factors that determine the outcome of antiretroviral therapy in populations across Africa in order to provide evidence to guide treatment and implementation strategies and inform practice.

1.12.2 Objectives

1.12.2.1 Objective 1

To analyse the virological outcomes of patients switching from suppressive second-line ART to DRV/r monotherapy within the MANET trial and investigate the correlation between different adherence measures

1.12.2.2 Objective 2

To evaluate the drug resistance outcomes of HIV patients switching to DRV/r monotherapy, including the presence of archived drug resistance at study entry prior to switch to DRV/r monotherapy

1.12.2.3 Objective 3

To investigate the levels of soluble CD27 as a marker of immune activation in the MANET population at study entry and following treatment simplification to DRV/r monotherapy

1.12.2.4 Objective 4

To investigate the evolution of serological markers of HBV infection among patients switching from triple ART regimen to a simplified NRTI-sparing regimen with DRV/r monotherapy within the MANET trial

CHAPTER TWO - MATERIALS AND METHODS

2.1 Study populations

2.1.1 MANET Trial, Cameroon

The study population in Cameroon comprised 120 HIV-1 positive adults who participated in the Monotherapy in Africa, New Evaluations of Treatment (MANET) clinical trial (NCT02155101). MANET was a pilot, open-labelled randomised trial that took place at the Yaoundé Central Hospital between August 2014 and July 2015. The study protocol, listing the full inclusion and exclusion criteria, the endpoints, and the schedule of assessment is included in Appendix 1. Eligible subjects had received second-line antiretroviral therapy (ART) with 2 nucleos(t)ide reverse transcriptase inhibitors (NRTIs) plus either lopinavir/ritonavir (LPV/r) or atazanavir/ritonavir (ATV/r) for ≥12 weeks, showed plasma HIV-1 RNA suppression <60 copies/ml at two consecutive measurements 4 to 12 weeks apart, had a CD4 count >100 cells/mm^3, and tested hepatitis B surface antigen (HBsAg) negative. Patients underwent a first screening to determine the viral load and HBsAg status; those with a viral load <60 copies/ml that tested HBsAg-negative were asked to attend a second screening visit when they underwent consenting for study participation and a full clinical and laboratory assessment. Eligible subjects were randomised 2:1 to either switch to monotherapy with once daily darunavir (two tablets of 400 mg totalling 800 mg daily) plus ritonavir (one tablet of 100 mg daily) for 48 weeks (n=81), or to continue triple ART (2 NRTIs plus a PI/b) and remain in observation for 24 weeks (n=39). Patients attended for planned study visits at weeks 4, 12, 24, 36 and 48 and blood samples were taken. At all study visits, plasma samples were taken; additional serum samples were taken at screening 1 and PBMC samples were taken at screening 2. Further to designated study visits, patients collected a prescription from a pharmacy every 4 weeks, when they also underwent an assessment of adherence using a visual analogue scale (VAS) and pill count (Adherence forms shown in Appendix 2). Pill count was expressed as the percentage of doses taken and calculated by counting the number of pills left over and dividing the number of

pills taken by the number of pills expected to be taken over the prescription period. Adherence since the previous visit was also self-reported via a VAS and graded from 0% (complete non-adherence) to 100% (complete adherence) on an ordinal number scale in 10% increments. Travel expenses to attend study visits and pharmacy visits were reimbursed. The MANET study was approved in July 2013 by the by the University of Liverpool Ethics Committee and the Cameroon National Ethics Committee. The study was overseen by an independent data safety board.

The MANET trial was designed by Professor Anna Maria Geretti. For the work presented in this thesis (Chapters 3, 4, 5 and 6), I had access to the study database, which was then subjected to cleansing and data verification, and to a repository of samples that was shipped from Cameroon to the University of Liverpool.

2.1.2 HEPIK and OPTIMISE cohort studies, Ghana

The study populations in Ghana comprised 165 HIV-1 positive adults from two observational cohort studies named HEPIK and OPTIMISE, both led by Professor Geretti. Patients recruited into the two cohorts attended the HIV outpatient clinic of the Komfo Anokye University Teaching Hospital (KATH) in Kumasi between 2010 and 2018. HEPIK aimed to assess the natural history of hepatitis B virus (HBV) co-infection in people with HIV. OPTIMISE aimed to assess the feasibility and clinical utility of same-day, point-of-care plasma HIV-1 RNA (viral load) quantification in people on ART. The HEPIK study and the OPTIMISE study received approval by the Committee of Human Research, Publication and Ethics of the Kwame Nkrumah University of Science and Technology in Kumasi, in September 2010 and January 2018, respectively and ethics from the University of Liverpool was not required. Both studies performed a range of investigations to determine factors associated with HIV-1 and HBV control. I did not directly participate in the HEPIK study, but I participated directly in the OPTIMISE study during a field trip that took place over four weeks in February and April 2018. My role during the field trip involved the processing of samples for storage, test the viral load at point of care using the Cepheid Xpert platform (see below), and perform HBsAg and

hepatitis C virus (HCV) antibody testing by rapid lateral flow assays. For the work presented in this thesis (Chapter 5), I used samples that were shipped from Ghana to the University of Liverpool.

2.1.3 United Kingdom study populations

Some of the work presented in this thesis (Chapter 5) used stored samples from three observation cohort studies of HIV-1 positive adults named PROGRESS, ERAS and EVOCK, which were based in the United Kingdom (UK). The studies were led by Professor Geretti. PROGRESS was approved by the Moorfields and Whittington Research Ethics Committee in June 2009. ERAS was approved by the South East London Research Ethics Committee in June 2010. EVOCK was approved by the South Central-Berkshire Research Ethics Committee in June 2012. PROGRESS recruited HIV-1 positive adults with a new diagnosis of HIV-1 infection that did not meet the CD4 cell count cut-off for starting ART (as applicable at the time) to determine the host and viral determinants of the rate of CD4 cell count decline in the absence of ART. The study was based in the HIV outpatient clinic of the Royal Free Hospital in London. ERAS was a multicentre study that investigated factors associated with HIV-1 persistence during ART. The study had strict inclusion and exclusion criteria. Patients had started ART with two NRTIs plus efavirenz or nevirapine, achieved a viral load <50 copies/ml within six months, and during subsequent follow-up showed no episode of viral load rebound >50 copies/ml while undergoing at least two viral load measurements per year; recruitment was stratified by duration of ART to span ≥10 years. EVOCK was a multi-centre study that recruited HIV-1 positive patients with and without co-infection with the hepatitis C virus (HCV), with the aim of studying host and viral determinants of HCV control, and the effect of interferon-alpha on HIV-1 replication.

2.1.5 Ethics Approvals and Material Transfer Agreements

Ethics committee approvals are shown in Appendix 3. MTAs regulating the transfer agreement of samples collected in Cameroon and Ghana shown in Appendix 4.

2.2 Sampling and laboratory testing not part of experimental work described in the thesis

2.2.1 MANET

Centre Pasteur of Cameroon

Full blood counts, CD4 cell counts, serum biochemistry (alanine aminotransferase [ALT], aspartate aminotransferase [AST], total bilirubin, creatinine with estimated glomerular filtration rate [eGFR], sodium, potassium, calcium, blood lipids) and HIV-1 RNA load were measured at the Centre Pasteur of Cameroon (CPC) in Yaoundé. The viral load was routinely determined with the Biocentric real-time PCR assay as described by Rouet et al. of the French ANRS HIV research group[189]. The assay employs manual nucleic acid extraction with the QIAGEN QIAamp viral RNA kit, followed by amplification and quantification on the Applied Biosystems ABI Prism 7300 platform. It provides reliable quantification of group M HIV-1 strains across the wide diversity observed in Cameroon, with a lower limit of quantification (LLOQ) of 60 copies/ml[190]. With group O infection or in case of Biocentric assay failure, CPC used the Abbott Real-Time HIV-1 assay, which has a quantification range of 40 to 10^7 copies/ml and an LLOQ of 40 copies/ml[191].

International Reference Centre Chantal Biya

As part of MANET screening, HBsAg testing in serum was performed at the International Reference Centre Chantal Biya (CIRCB) in Yaoundé using the Determine lateral flow assay (Inverness Medical, Stockport, United Kingdom)[192]. PBMC were prepared from whole blood in EDTA using Ficoll gradient centrifugation. Blood was first diluted with phosphate buffered saline (PBS) to 15ml, overlaid in 50ml Falcon tubes containing 10ml of Ficoll solution, and centrifuged at 500g for 30 minutes with no brakes. The PBMC layer was transferred into a new tube, made to 10ml with PBS, and centrifuged at 400g for 10 minutes with brakes. The supernatant was discarded without disturbing the pellet, resuspended in 10ml of PBS and centrifuged at 400g for 10 minutes. The supernatant was discarded, and the pellet was resuspended in

3ml of cold freezing medium containing 90% Foetal Bovine Serum (FBS) and 10% dimethyl sulphoxide (DMSO); this was gently mixed by inverting the tube several times. CIRCB also performed preparation of plasma and serum for storage. For plasma separation, whole blood in EDTA was centrifuged at 2000xg for 10 minutes. For serum separation, whole blood was allowed to clot for 1 hour in upright tubes followed by centrifugation at 2000xg. PBMC, plasma and serum were stored at -80^{o}C within 2 hours of collection and kept under temperature monitoring until shipment on dry ice to the UK.

University of Liverpool

Total cellular HIV-1 DNA was quantified in PBMC by in-house real-time PCR targeting a conserved consensus region of the LTR as described[116,193]. A standard curve generated from four points of a HIV-1 LTR DNA plasmid was used for quantification. The 90% and 50% detection thresholds were 40 and 20 copies per 10^6 PBMC respectively.

2.2.2 HEPIK and OPTIMISE

Plasma samples and serum samples from HEPIK and OPTIMISE participants were obtained from whole blood in EDTA and whole clotted blood, respectively according to the procedures described above. Samples were stored at -80^{0}C within 2 hours of collection and kept under temperature monitoring until shipment on dry ice to the UK. For HEPIK, plasma HIV-1 RNA was quantified at the University of Liverpool by researchers of Professor Geretti's group using the Abbott RealTime assay, with automated extraction on the M2000sp platform which employs magnetic micro particle technology, and real-time amplification and detection on the M2000rt system (Abbott, Wiesbaden, Germany). The process introduces an internal control into the samples, which is processed together with the calibrators and controls (high positive, low positive and negative). As described, the assay has a quantification range of 40 to 10^7 copies/ml and an LLOQ of 40 copies/ml[191].

For OPTIMISE, I quantified the viral load at KATH using the GeneXpert HIV-1 Viral Load assay (Cepheid, Sunnyvale, USA). The assay uses real-time PCR to quantify HIV-1 Group M, Group N and Group O strains. The system is designed to combine automated and integrated sample preparation, nucleic acid extraction, amplification and detection in individual disposable cartridges, with results available within 90 minutes[194]. Using 1mL of plasma, the quantification range is 40 to 10^7 copies/ml, with an LLOQ of 40 copies/ml. The assay also reports qualitative target detection below the LLOQ, with a described lower limit of detection (LLOD) for 1mL input of 22 copies/ml[195].

2.2.3. UK Study Populations

Whole blood in EDTA and whole clotted blood were sent by courier to the central laboratory, which was based either at the Royal Free Hospital in London or at the University of Liverpool. Here, the blood was processed for plasma and serum separation as described and stored -80^{o}C under temperature monitoring[193]. The viral load in these samples was measured at the University of Liverpool using the Abbott RealTime assay as described[116].

2.3 Laboratory experimental work described in the thesis

2.3.1 HIV-1 DNA sequencing

PBMC collected from MANET patients at study entry, when the viral load was suppressed <60 copies/ml, were used to detect resistance-associated mutations (RAMs) in reverse transcriptase (RT) and protease (PR) by Sanger sequencing.

2.3.1.1 DNA extraction from PBMC

Total DNA was extracted manually using the QIAamp DNA Blood Mini kit (QIAGEN, Crawley, UK). To improve DNA recovery, 20µL of proteinase K was added to 200µL of buffer AL and 200µL of sample in a microcentrifuge tube. After incubation at 56°C for 10 minutes, 200µL of 100% ethanol (EtOH) was added and the sample was mixed thoroughly by vortexing. The mixture was then transferred into the QIAamp Mini column, placed in 2ml collection tubes and centrifuged at 8000rpm for one minute. The column then underwent two

wash steps. The first step involved addition of 500µL of AW1 to the column which was followed by spinning at 8000rpm for one minute and discarding of the flow-through. The second wash step involved addition of 500µL of AW2 to the column followed by spinning at 14000rpm for three minutes to dry the membrane filter. The column was then transferred to a 1.5ml microcentrifuge tube and 200µL of buffer AVE equilibrated at room temperature was added. The column was centrifuged at 8000rpm for one minute and the elute was stored at -80^{o}C until use.

2.3.1.2 Amplification of the polymerase region of HIV-1 DNA

HIV-1 DNA was amplified according to the ANRS "split" protocol[196]. The protocol amplifies RT and PR separately, prior to sequencing of both regions in a single reaction. The two amplicons comprise the entire PR [amino acids 1-99; HXB2 position 2253-2549] and part of RT [1-259, HXB2 position 2556-3334] using nested PCR.

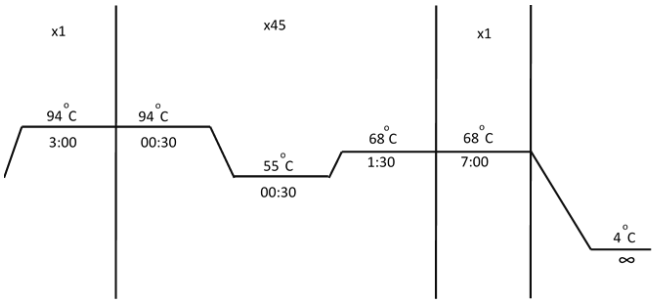
The 1st round PCR reaction was setup as follows:

Protease:

Protease	
Reagent	Amount per sample (µL)
Platinum PCR Super Hi-Fi mix	45
5'PRO1 (10µM)	1
3'PRO1 (10µM)	1
Extract DNA	5
Total	52

Reverse Transcriptase	
Reagent	Amount per sample (µL)
Platinum PCR Super Hi-Fi mix	45
MJ3 (10µM)	1
MJ4 (10µM)	1
Extract DNA	5
Total	52

The conditions of the 1st round PCR reaction were as follows:

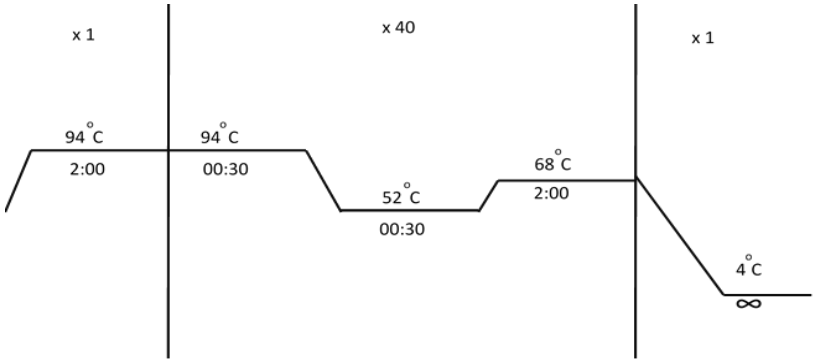


The nested PCR reaction was set up as follows:

Protease	
Reagent	Amount per sample (µL)
Platinum PCR Super Hi-Fi mix	45
5'PRO2 (10µM)	1
3'PRO2 (10µM)	1
First PCR product	5
Total	52

Reverse Transcriptase	
Reagent	Amount per sample (µL)
Platinum PCR Super Hi-Fi mix	45
A35 (10µM)	1
NE135 (10µM)	1
First PCR Product:	5
Total	52

The conditions of the nested PCR reaction were as follows:



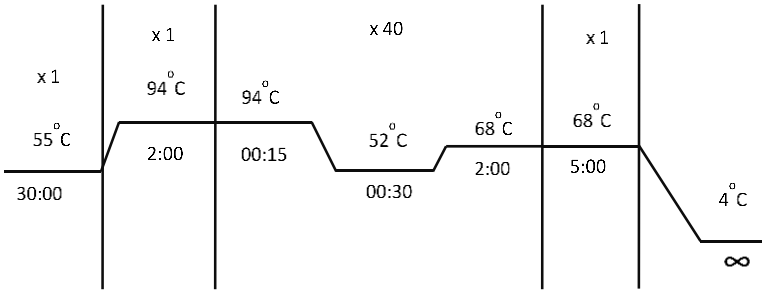
2.3.1.3 Amplification of the polymerase region of plasma HIV-1 RNA

A nested reverse transcription (RT) PCR protocol was used to amplify the entire PR [amino acids 1-99; HXB2 position 2253-2549] and two-thirds of RT [amino acid 1-335; HXB2 position 2550-3554] as a single 1200bp amplicon. For each sample, 1200bp amplicons were generated in triplicates. Primers used for amplification are shown in Table 2.1. The Invitrogen SuperScript® III One-Step RT-PCR System/ Platinum® Taq DNA polymerase High Fidelity Kit (Life Technologies) was used for single step cDNA synthesis and amplification. The method uses a mixture of Superscript III Reverse Transcriptase and Platinum Taq High Fidelity DNA polymerase. Nested PCR was performed using Platinum Taq High Fidelity DNA polymerase (Life Technologies).

The 1st round PCR reaction was set up as follows:

Reagent	Amount per sample (µL)
2x reaction mix	25
Primer RES1 (10µM)	1
Primer RES2 (10µM)	1
Superscript III RT/Platinum taq Hi-Fi mix	1
Water	12
Sample RNA extract	10
Total	50

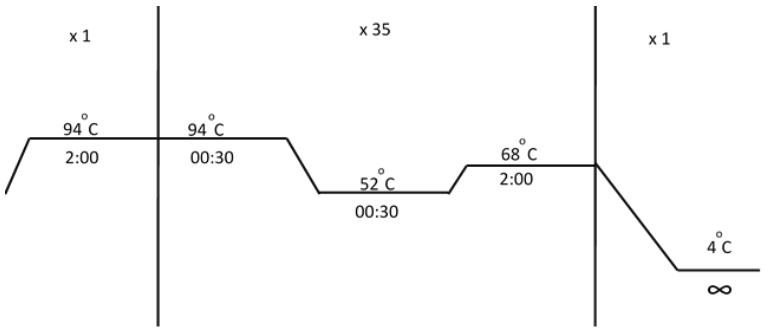
The conditions of the 1st round PCR reaction were as follows:



The nested PCR reaction was set up as follows:

Reagent	Amount per sample (µL)
Water	0.5
Primer RES3 (10µM)	1
Primer RES4 (10µM)	1
Platinum PCR Super Hi-Fi mix	45
First PCR product	2.5
Total	50

The conditions of the nested PCR reaction were as follows:



2.3.1.4 Agarose gel electrophoresis

Following amplification electrophoresis on 1.5% gel was used to separate amplified product by size and visualise DNA fragments. 5µL of SYBR safe was added to the prepared molten agarose gel after microwave heating and cool off before pouring into a sealed gel tank with an approximate depth of 5-10mm. Gel combs were inserted into the tank before pouring and the gel was allowed to solidify. After gel solidification in the gel cast, combs were removed, and the cast was submerged in 0.5x TBE buffer in the electrophoresis chamber. Samples were diluted 1:5 using a loading dye buffer (Invitrogen, Warrington, UK) and loaded into each well before separation by electrophoresis for one hour at 140 volts.

2.3.1.5 Sanger sequencing of HIV-1 DNA

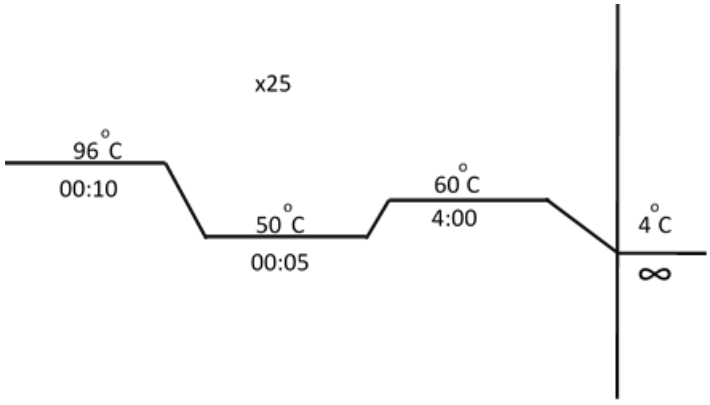
Amplicons were first purified using the QIA PCR purification kit (QIAGEN), which is optimised for purification of PCR products of 100-10000 base pairs. A total of 200µL of Buffer PB was added to 40µL of amplicon followed by 10µL of sodium acetate. After mixing thoroughly, the product was added to the QIA column, and this was centrifuged for one minute to allow binding of DNA to the column. Then, 750µL of buffer PE was added to the column and this was centrifuged for one minute to wash. The flow-through was discarded and the column was centrifuged for one additional minute to ensure all the PE buffer had been expelled from the column. The column was then eluted with 50µL of buffer EB and centrifuged for one minute. DNA was quantified in 1µL of purified DNA product by Nanodrop® ND-1000 UV-vis spectrophotometer (ThermoScientific, Wilmington, USA). Gel electrophoresis with SYBR safe (Sigma-Aldrich Ltd, Gillingham, UK) staining and visualisation of gel on

ultraviolet transilluminator was used to confirm presence of product by running a 5µL aliquot of purified DNA diluted along with 1µL DNA molecular marker (Invitrogen® Life technologies, Warrington UK). Purified DNA products were diluted to 40 ng of DNA per well for sequencing.

Products were sequenced by Sanger sequencing using the 3730xL (Applied Biosystems, Warrington, UK) genetic analyser. Cycle sequencing reactions were setup using the BigDye® Terminator Cycle Sequencing Ready Reaction v3.1 kit (Applied Biosystems, Warrington, UK) as shown below:

Reagent	Amount per sample (µL)
BigDye Terminator v3.1 Ready Reaction Mix	2
Primer (10µM)	1
BigDye Terminator 1x Sequencing buffer	9
Purified DNA amplicon product	8
Total	20

Thermo cycling conditions for the cycle sequencing were as follow:



Following cycle sequencing, reactions were purified by precipitation and centrifugation at 2000xg of the DNA product with a mixture containing 52µL of 100% EtOH and 2µL of 3M acetate ($C_2H_3O_2$), followed by an additional cleanse step with 150µL of EtOH. Purified DNA pellets were resuspended in 10µL of Hi-Di formamide for 30 minutes and a plate septum was used to seal the plate before a brief centrifugation step to homogenise the mix. Plates were secured into the adapter and placed into the 48 capillary 3730xL genetic

analyser. Samples were processed with a 36-cm capillary array using the POP conformational Analysis Polymer, POP-7.

Sequences generated on the 3730xL genetic analyser were analysed by SeqScape software v.5.2 (Applied Biosystems, Warrington, UK). The SeqScape platform has a multifunction to allow import, assembly, editing and analysis of the sequences. ABI files created by the genetic analyser were imported into the SeqScape and the resulting ABI format chromatograms files were analysed manually by observing the peaks and nucleotide changes in reference to the HXB2 sequence (Figure 2.1). The consensus sequences were exported as FASTA formats and analysed for the presence of RAMs in RT and PR.

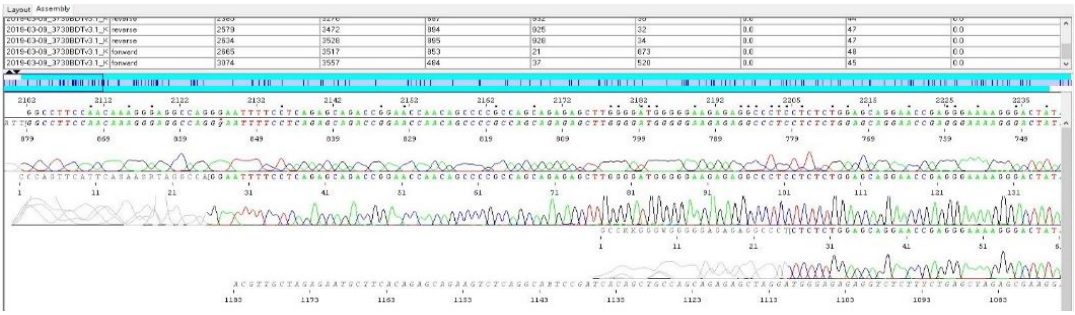


Figure 2.1: Chromatograms showing sequence analysis of a portion of the 1300bp of the HIV-1 polymerase from the PBMC of one of the subjects. The light blue line indicates clear range of the genome section of HIV-1 and the dark blue indicates unknown variant changes in reference to the HXB2 genome.

2.3.2 HIV-1 RNA sequencing

Plasma collected from MANET patients at the time of viral load rebound, were used to sequence HIV-1 RNA and detect the presence of RAMs in RT and PR using next generation sequencing (NGS) on the Illumina MiSeq platform. In addition, a subset of plasma samples underwent Sanger sequencing of the gag gene.

2.3.2.1 RNA extraction from plasma

Total RNA was extracted manually using the QIAamp viral RNA kit (QIAGEN), which applies the extraction protocol developed by Boom, Sol and Wertheim-van-Dillen[197]. A total of 140µL of plasma was used if HIV-1 RNA levels were

>10000 copies/ml. If levels were <10000 copies/ml, samples were enriched by ultra-centrifugation using Optima XPN (Beckman Coulter, High Wycombe, UK) at 40,000 RPM (274,000xg) for 15 minutes at 4°C. Following ultra-centrifugation, supernatant was discarded, and pellets were reconstituted into 150µL. For extraction, samples were added to buffer-AVL containing carrier-RNA, mixed thoroughly and incubated at room temperature for 10 minutes. Following incubation, 560µL of 100% EtOH was added to the mixture. After vortexing, the mixture was transferred to the QIAamp Mini column, placed in 2ml collection tubes and centrifuged at 8000rpm for one minute. The column then underwent two wash steps. The first step involved addition of 500µL of AW1 which was followed by spinning at 8000rpm for one minute and discarding of the flow-through. The second wash step involved addition of 500µL of AW2 to the column followed by spinning at 14000rpm for three minutes to dry the membrane filter. The column was transferred to a 1.5ml micro centrifuge tube and 60µL of buffer AVE equilibrated at room temperature was added. The elute was stored at -80^{o}C until further use.

Table 2.1: Primers employed for amplification during experimental proceedings

Primer	Sequence 5' - 3'	Direction
RES3[a]	ATG GYT CTT GAT AAA TTT GAT ATG TCC	Reverse
RES4 [a]	AGA CAG GCT AAT TTT TTA GGG A	Forward
SEQ1 [a]	GAG CCA ACA GCC CCA CC	Forward
SEQ2 [a]	CAA TGG CCA TTG ACA GAA G	Forward
SEQ3 [a]	GGA TCA CCA GCA ATA TTC CA	Forward
SEQ5 [a]	TGG GCC ATC CAT TCC TGG CTT	Reverse
SEQ6 [a]	CAT CCC TGT GGA AGC ACA TT	Reverse
SEQ7 [a]	TCT GCT ATT AAG TCT TTT GAT	Reverse
GAGBOUT	GTT GTG TGA CTC TGG TAA CTA GAG ATC CCT CAGA	Forward
GAGFOUT	TCC TAA TTG AAC YTC CCA RAA GTC YTG AGT TC	Reverse
GAGFIN	TCT CTA GCA GTG GCG CCC GAA CAG	Forward
GAGBIN	GGC CAT TGT TTA ACC TTT GGD CCA TCC	Reverse
G00 [b]	GACTAGCGGAGGCTAGAAG	Forward
G50 [b]	CACAGCAAGCAGCAGCTG	Reverse
G70 [b]	ATGAGGAAGCTGCAGAATGGG	Forward
G01 [b]	AGGGGTCGTTGCCAAAGA	Reverse
G05 [b]	TGTTGGCTCTGGTCTGCTCT	Reverse
G35 [b]	CATGCTGTCATCATTTCTTCTA	Reverse
G45 [b]	TTGGACCAACAAGGTTTCTGTC	Reverse
G85 [b]	TGC ACT ATA GGG TAA TTT TG	Reverse
HEPB2[c]	TCT CTG ACA TAC TTT CCA AT	Reverse
HEPBNEST[c]	TTG GGG TGG AGC CCT CAG GCT	Forward
HEPBSEQ [c]	TTG GCC AAA ATT CGC AGT C	Forward
HEPB1731 [c]	CTC CTG CCT CCA CCA ATC	Forward

NEC005	GCCTCAATAAAGCTTGCC	Forward
NEC131	GGCGCCACTGCTAGAGATTTT	Reverse
5'PROT1	TAA TTT TTT AGG GAA GAT CTG GCC TTC C	Forward
3'PROT1	GCA AAT ACT GGA GTA TTG TAT GGA TTT TCA GG	Reverse
5'PROT2	TCA GAG CAG ACC AGA GCC AAC AGC CCC A	Forward
3'PROT2	AAT GCT TTT ATT TTT TCT TCT GTC AAT GGC	Reverse
MJ3	AGT AGG ACC TAC ACC TGT CA	Forward
MJ4	CTG TTA GTG CTT TGG TTC CTC T	Reverse
A35	TTG GTT GCA CTT TAA ATT TTC CCA TTA GTC CTA TT	Forward
NE135	CCT ACT AAC TTC TGT ATG TCA TTG ACA GTC CAG CT	Reverse

[a]=Primers employed in sequencing the HIV-1 polymerase region; [b]= Primers employed in sequencing the HIV-1 gag region; [c]= Primers employed in sequencing the HBV polymerase region

2.3.2.2 Gel extraction of DNA and purification

In preparation for sequencing, gel extraction of DNA was performed from agarose gel slices containing PCR products using the QIAquick gel extraction kit (QIAGEN). The DNA fragment was visualised on a gel and excised using a sharp scapel; 3x buffer QG was added per 1 gel volume. This mixture was incubated at 50^{o}C until the gel was completely dissolved. The dissolved samples were placed in a spin column and centrifuged to allow DNA binding onto the columns. To wash, 750µL of buffer PE was added before a final elution with 50µL of elution buffer. After separation by electrophoresis, amplicons from triplicate amplifications were pooled and then purified using the Agencourt AMpure XP magnetic purification system (Beckman Coulter, High Wycombe, UK). Purified products were quantified using the Qubit dsDNA High Sensitivity Assay Kit on the Qubit 3.0 fluorimeter (Invitrogen). In this purification step, PCR products (totalling 50uL) were added in ratio 1:1 to the AMPure XP reagent to bind DNA fragments from amplicons to the magnetic bead particles. The bound DNA fragments on beads were then separated from other contaminants by placing on the Agencourt SRPI magnetic plate for two minutes before the aspirates were separated and bead-bound DNA was washed twice using 30µL of 70% EtOH. Samples were finally eluted into 30µL of elution buffer, separated from the beads and transferred onto a new plate for quantification. Purified products were sent to Public Health England for sequencing using the Illumina Miseq v2 platform as previously described[198,199]. Following sequencing, generated Miseq paired-end reads from sequence files were compared with HIV reference sequences using

BLAST to identify the optimum reference sequence for mapping using the Burrows-Wheeler Aligner tool as previously described[199]. Following completion of ultra-deep sequencing by PHE, FASQ files were obtained for analysis using the Polymorphism Analysis Sequencing (PASEQ) web-based analysis pipeline. The PASEQ platform has been compared and validated as a sequence variant caller for ultra-deep sequencing[200,201].

2.3.2.3 Subtyping and phylogenetic analysis:

HIV-1 subtyping was initially performed using the Stanford HIV database[202] available at (https://hivdb.stanford.edu/hivseq/by-mutations/). Phylogenetic trees were constructed for sequence quality control, to confirm subtyping and evaluate clusters. Generated sequences were used to estimate maximum likelihood analysis using the randomized axelerated maximum-likelihood (RAxML) implementation housed at the CIPRES server (https://cme.h-its.org/exelixis/software.html)[203], with 1000 bootstrap replicates[204]. The constructed phylogenetic trees using the RAxML implementation were visualised on Figtree v1.4.3 (available from http://tree.bio.ed.ac.uk/software/figtree/). For each sequence generated, ten reference sequences were downloaded from the publicly available GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Generated HIV-1 sequences were aligned by codon using the ClustalW multiple alignment algorithm[205] in MEGA v6[206] and altered manually to ensure sequence gaps did not alter the reading frame to reference HXB2. Codons associated with HIV-1 drug resistance were excised according to the IAS-USA resistance mutation list[207] and aligned consensus sequences were formed from the overlapping sequences.

2.3.3 Sequencing of the gag region of plasma HIV-1 RNA

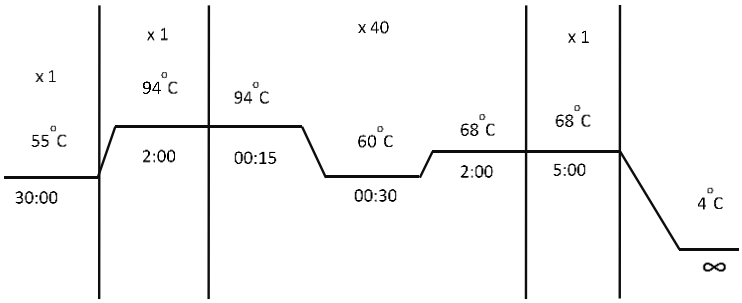
Following nucleic acid extraction from plasma as described above, a nested RT-PCR protocol was used to amplify the Gag-PR domain of HIV-1, covering the entire gag region (HXB2 position 649-2623). Primers used for amplification are shown in Table 2.1. In the 1st round PCR, the Invitrogen SuperScript® III One-Step RT-PCR System/Platinum® Taq DNA polymerase High Fidelity Kit (Life Technologies) was used for cDNA synthesis and amplification in a one-

step reaction, as described above. The nested PCR used the Platinum Taq High Fidelity DNA polymerase (Life Technologies).

The 1st round PCR reaction was set up as follows:

Reagent	Amount per sample (µL)
2x reaction mix	25
Primer RES1 (10µM)	1
Primer RES2 (10µM)	1
Superscript III RT/Platinum taq Hi-Fi mix	1
Water	12
Sample RNA extract	10
Total	50

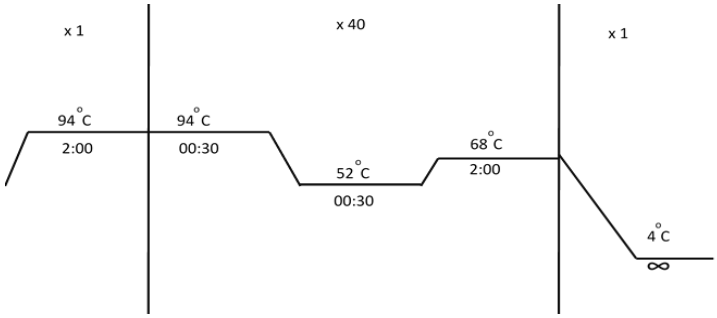
The conditions of the 1st round PCR reaction were as follows:



The nested PCR reaction was set up as follows:

Reagent	Amount per sample (µL)
Water	0.5
GAGBOUT (10µM)	1
GAGFOUT (10µM)	1
Platinum PCR Super Hi-Fi mix	45
First PCR product	2.5
Total	50

The conditions of the nested PCR reaction were as follows:



Sanger sequencing was performed as described above. Sequences were analysed manually by alignment of generated sequences with the HIV HXB2 reference. Mutations were changes relative to the aligned HXB2.

2.4 Detection of soluble CD27

Quantification of sCD27 was performed using a commercially available sandwich ELISA (eBioscience, UK). The assay uses plates pre-coated with anti-CD27 capture i.e monoclonal antibody to human sCD27 and the detection antibody i.e Biotin-Conjugate. As per the manufacturer's instruction, plasma or serum samples were diluted 1:25 with sample diluent; this involved adding 10µL sample to 240µL diluent. This was followed by addition of 50µL diluted sample to the respective wells containing 100µL of water. Samples were incubated for three hours at room temperature. The plate was washed six times with the kit wash buffer using an automated ELISA washer (ThermoFisher) in each wash, a wash buffer volume of 400µL was used with a wait time of 15 seconds before each aspiration step. After washing, 100µL of substrate solution was added and the plate was incubated for 10 minutes at room temperature, followed by addition of the kit stop solution. Optical densities (ODs) of samples were measured using a micro titre plate reader (ThermoFisher) at 450nm. Raw data was entered into an algorithm provided by the manufacturer and sCD27 levels were expressed as units/ml.

2.5 HBV serology

Serum samples collected from MANET patients underwent testing for HBV serological markers comprising HBsAg, anti-HBs, anti-HBc, HBeAg, and anti-HBe using the Architect Chemiluminescent Microparticle Immunoassay

(Abbott, Wiesbaden, Germany). The tests were performed in the accredited NHS Virology Laboratory of Frimley Park Hospital in Surrey. For HBsAg, HBeAg, and anti-HBc, a signal/cut-off (S/CO) >1.00 in a sample specimen was considered reactive. HBeAb with S/CO <1.00 was considered positive and HBsAb >10 IU/ml was considered positive and immune to infection. HBsAg was confirmed by neutralisation and anti-HBc positivity was confirmed on the Cobas 8000 analyzer (Roche Diagnostics, Burgess Hill, UK)

2.6 HBV DNA quantification

HBV DNA quantification was performed at selected time points in the MANET population using the automated Abbott m2000sp/rt RealTime HBV assay (Abbott). This assay extracts DNA from 0.5 ml of plasma using the m2000sp automated sample preparation system. HBV DNA levels were measured from the extracted samples on the m2000rt™ instrument (Abbott)[208]. As describe for HIV-1 RNA quantification, the process introduces an internal control into the samples, which is processed together with the calibrators and controls (high positive, low positive and negative). After sample extraction, the eluate was transferred into 96-well amplification plates together with enzyme and probes and amplified on the m2000rt system. With 0.5 ml input, the assay has a dynamic range of 10 to 10^9 IU/ml and a LLOQ of 10 IU/ml. The assay also reports qualitative target detection below the LLOQ. Calibration of the M2000 system was performed each time a new lot of reagents was used.

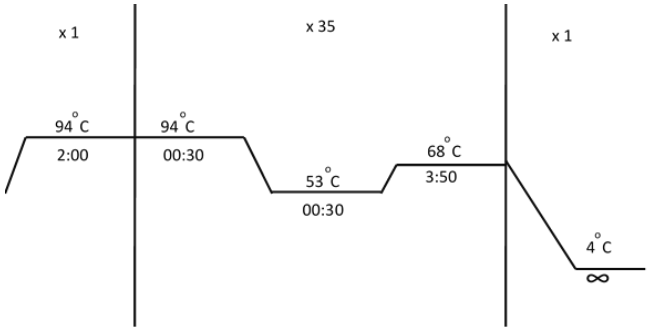
2.6.1 HBV DNA sequencing

The polymerase gene of HBV was amplified by nested-PCR to obtain an amplicon covering the polymerase gene RT domain (amino acid: 1-334) and surface domain (amino acid: 1-226). Platinum® Taq DNA polymerase High Fidelity Kit (Life Technologies) was used for both first-round PCR and nested PCR to obtain a 1300bp amplicon for sequencing using the Sanger method as described above.

The conditions of the 1st round PCR reaction were as follows:

Reagent	Amount per sample (µL)
Water	0.5
Primer HEPBN (10µM)	1
Primer HEPB2 (10µM)	1
Platinum PCR Super Hi-Fi mix	45
Sample DNA extract	2.5
Total	47.5

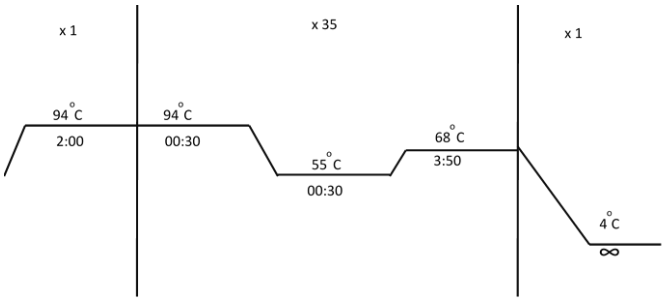
The conditions of the 1st round PCR reaction were as follows:



The nested PCR reaction was set up as follows:

Reagent	Amount per sample (µL)
Water	0.5
Primer HEPBN (10µM)	1
Primer HEPB2 (10µM)	1
Platinum PCR SuperMix high fidelity	45
Total	47.5

The conditions of the nested PCR reaction were as follows:



The Geno2pheno database was used analyse the sequences to determine viral subtypes and resistance profile (http://hbv.geno2pheno.org/index.php).

2.7 Statistical Analysis

All analysis used fully anonymised datasets. Statistical analyses were done with the STATA software version 14 (StataCorp LLC, College Station, USA)

or GraphPad Prism (GraphPad Software, San Diego, USA). The specific testing methods are described in each chapter.

CHAPTER THREE – VIROLOGICAL OUTCOMES OF SWITCHING SUPPRESSIVE SECOND-LINE TRIPLE ANTIRETROVIRAL THERAPY TO MONOTHERAPY WITH RITONAVIR-BOOSTED DARUNAVIR IN THE MANET TRIAL

In this chapter, I analysed the virological outcomes of the MANET trial. This pilot randomised study recruited HIV-1 positive adults that were receiving suppressive second-line antiretroviral therapy with two NRTIs and a boosted protease inhibitor (PI/b) and showed a suppressed plasma viral load. Participants were randomised either to switch to monotherapy with ritonavir-boosted darunavir (DRV/r) or to continue PI/b-based triple ART. The trial was conducted in Yaoundé, Cameroon. After cleansing and lock-up, I used the available dataset to perform the virological outcome analyses. In addition, I conducted an analysis of the correlation between different measures of adherence taken on study. To preserve clarity, I present in-depth resistance analyses in a subsequent chapter.

3.1 INTRODUCTION

In Sub-Saharan Africa, of the estimated 25.6 million people living with HIV, 16.4 million were receiving treatment in 2018[209]. The World Health Organization (WHO) recommends a programmatic approach at treating HIV infection in the region, based upon standardised regimens for first-line and second-line antiretroviral therapy (ART). Recommended regimens comprise two NRTIs (typically tenofovir plus lamivudine) combined with an NNRTI (typically efavirenz), a boosted protease inhibitor (PI/b, typically lopinavir/ritonavir), or more recently an integrase strand transfer inhibitor (INSTI, typically dolutegravir)[154]. In pooled analyses, the rates of virological suppression by intention-to-treat were 65% and 62% after 24 months of NNRTI-based first-line ART[210] and PI/b-based second-line ART[142], respectively.

Whist triple combination regimens have been the reference model for ART, there has been interest in exploring simpler regimens that may reduce toxicity and cost and promote compliance[69,211,212]. Several studies have tested the hypothesis that once viral load suppression is achieved with triple ART, it may be possible to simplify treatment using fewer drugs. In selected populations, promising results have been obtained with dual regimens comprising a PI/b plus lamivudine, an integrase inhibitor, or maraviroc, dolutegravir plus lamivudine or the NNRTI rilpivirine, and the INSTI cabotegravir plus rilpivirine[211,212]. In contrast, simplification with either a PI/b or dolutegravir as monotherapy has generally resulted in increased rates of viraemia relative to continuing triple ART. Boosted darunavir makes an attractive simplification option because of its high potency, high genetic barrier to resistance, and a good tolerability and safety profile[213]. One important feature of maintenance monotherapy with boosted darunavir is the low risk of emergent drug resistance in patients with viraemia, allowing rapid resuppression once the NRTIs are re-introduced.[214,215]

MANET (Monotherapy in Africa, New Evaluation of Therapy) was a pilot randomised clinical trial that was conducted at the Yaoundé Central Hospital, in Cameroon between 2014 and 2016. The aim of the study was to test the feasibility and preliminary virological efficacy of ritonavir-boosted darunavir (DRV/r) as maintenance monotherapy in a population that had previously experienced failure of first-line NNRTI-based triple ART and had subsequently gained virological suppression on second-line PI/b-based triple ART. Patients were randomised either to switch to once daily DRV/r or to continue triple ART. Here we present the analysis of the virological outcomes in the two arms and investigate the correlation between different adherence measures.

3.2 METHODS

3.2.1 Aim of the MANET Trial

MANET was a pilot study that aimed to assess the feasibility and preliminary efficacy and safety of DRV/r monotherapy as a maintenance strategy for patients receiving second-line ART and showing a suppressed viral load in Cameroon. The feasibility assessment was considered critical to enable the design of a larger multicentre study. MANET was approved by the University of Liverpool Ethics Committee and the Cameroon National Ethics Committee and overseen by an independent data safety board. Participants gave written informed consent.

3.2.2 Study population

Details of the MANET study are provided in Chapter 2. Briefly, MANET participants were HIV-1-positive adults who met the following eligibility criteria at screening: (i) on a second-line regimen with 2 NRTIs plus a PI/b for ≥12 weeks; (ii) CD4 count >100 cells/mm^3; (iii) plasma HIV-1 RNA load <60 copies/ml on two measurements taken 4 to 12 weeks apart; (iv) hepatitis B surface antigen (HBsAg) negative; and v) willing to provide written informed consent. Between Aug 2014 and July 2015, 120 patients were randomised (2:1) either to switch to monotherapy with once daily darunavir (two tablets of 400 mg totalling 800 mg daily) plus ritonavir (one tablet of 100 mg daily) for 48 weeks (n=81), or to continue standard triple ART (2 NRTIs plus a PI/b) and remain in observation for 24 weeks (n=39). After screening and randomisation, participants attended planned study visits at weeks 4, 12, 24 (both arms), 36 and 48 (DRV/r arm). Participants collected a new prescription from pharmacy and saw the study nurse every 4 weeks. After week 48, participants in the DRV/r arm returned to standard triple ART. Prior to week 48, an early return to triple ART with two NRTIs + PI/b could occur due to a significant adverse event (AE), or as a result of virological failure (see below). Patients who experienced a first viral load >60 copies/ml during the study were recalled for review, adherence counselling by the study nurse, and a repeat viral load test within 8 weeks of the initial viral load measurement. The protocol

recommended the following management for patients presenting with a confirmed virological rebound >60 copies/ml while in the study:

DRV/r monotherapy arm:

- Viral load ≤400 copies/ml: re-enforce adherence; continue to monitor
- Viral load >400 copies/ml: return to triple ART with 2 NRTIs plus a PI/b

Triple ART arm: follow standard practice, i.e.,

- Viral load <1000 copies/ml: re-enforce adherence; continue to monitor
- Viral load >1000 copies/ml: consider a change of treatment regimen

3.2.3 Study virological objectives

Primary virological objective

- Percentage of subjects with viral load <400 copies/ml after 24 weeks of follow-up following a switch to DRV/r monotherapy vs. continuing triple therapy containing 2 NRTIs + PI/b.

Secondary virological objectives

- Percentage of subjects with viral load <60 copies/ml after 12 weeks of follow-up following a switch to DRV/r monotherapy vs. continuing triple therapy containing 2 NRTIs + PI/b
- Percentage of subjects with viral load <60 copies/ml after 24 weeks of follow-up following a switch to DRV/r monotherapy vs. continuing triple therapy containing 2 NRTIs + PI/b

3.2.4 Adherence measures

Treatment adherence was measured when patients attended study and pharmacy visits. At each study visit, adherence was self-reported via a visual analogue scale (VAS) graded from 0% (complete non adherence) to 100% (complete adherence) in 10% increments[216,217]. At each pharmacy visit, two bottles were dispensed containing 60 tablets of DRV and 30 tablets of ritonavir, respectively. Patients were asked to return the bottles with any left-

over tablet at their next pharmacy visit; at this visit, the pill count was taken whereby the number of pills taken was calculated from the number of pills left over and expressed as a percentage relative to the number of pills expected to be taken since the last visit.

3.2.5 Laboratory testing

Full blood counts, serum biochemistry, plasma HIV-1 RNA load, and CD4 cell counts were measured at the diagnostic laboratory of the Centre Pasteur of Cameroon (CPC) in Yaoundé. Plasma was separated from whole venous blood in EDTA within two hours of collection and stored immediately at -80^{o}C. Plasma HIV-1 RNA was quantified with the Biocentric assay (Bandol, France; lower limit of quantification of 60 copies/ml); in case of assay failure, it was repeated with the Abbott RealTime HIV-1 assay (lower limit of quantification 40 copies/ml). Peripheral blood mononuclear cells (PBMC) were isolated at CIRCB by Ficoll-Hypaque gradient centrifugation and stored at -80^{o}C.

3.2.6 Analyses

In the analysis of baseline characteristics, categorical variables (expressed as proportions and percentages) and continuous variables (expressed as medians with inter quartile ranges [IQR]) were compared between treatment arms using chi-squared test, fisher's exact test, t-test and Wilcoxon Mann Whitney tests, as applicable. For the primary virological outcome analysis, the percentage of subjects with viral load <400 copies/ml was calculated cross-sectionally at week 24 (FDA snapshot method)[218]. The FDA analysis considers virological failure cross-sectionally at designated timepoints where missing data is also considered failure. For the secondary virological outcome analysis, the percentage of subjects with viral load <60 copies/ml was calculated using the composite time to loss of virological response (TLOVR) analysis[218]. The TLOVR analysis, considered outcomes longitudinally over designated timepoints (12 and 24 weeks in this instance). Both primary and secondary virological outcome measures were compared using chi-squared test. Virological rebound was defined as a viral load >60 copies/ml that was either confirmed in a subsequent sample or was the last available on study.

Virological failure was defined as a viral load >400 copies/ml which was either confirmed in a subsequent sample or was the last available on study. A viral load that was above 60 copies/ml but did not exceed 400 copies/ml was described as viraemia. Treatment failure was a composite measure of virological failure and any early discontinuation of study arm due to a significant (as judged by the investigator) adverse event (AE), death, or loss to follow-up. Kaplan-Meier survival curves were plotted to describe time to virological rebound (first viral load measured), virological failure, and treatment failure. For each participant, the adherence measures over the entire duration of participation in the study and before and after the first adherence counselling session were averaged for the purpose of the analysis. The correlation between adherence measured by pill count or by VAS was assessed by Spearman's rank test. Analyses were performed with STATA v.14 (StataCorp).

3.3 RESULTS

3.3.1 Feasibility assessment

A total of 340 subjects established on PI/b-based triple ART for ≥12 weeks underwent the first screening (Figure 3.1); 128/340 (38%) showed a detectable (>60 copies/ml) viral load, with median levels of 1101 copies/ml (IQR 218, 16540). Among 212 subjects with a suppressed viral load at screening 1, 11 (5.2%) tested HBsAg positive. Of the remaining 201 patients considered for inclusion, 32 (15.9%) did not attend screening 2. A further 49 did not meet the inclusion criteria, comprising 25 (12.4%) with a viral load rebound >60 copies/ml between the two screening visits, 11 (5.5%) with significant laboratory abnormalities, 4 (2.0%) reporting pregnancy or planning to become pregnant, and 9 (4.5%) who declined to participate. Overall, the number of patients receiving second-line therapy with 2 NRTIs plus a PI/b and showing a suppressed viral load was 212/340 (62.4%) at screening 1 and 144/169 (85.2%) at screening 2, indicating a rebound rate of 14.8% among the 169 patients with two viral load measurements taken at an interval of 4-12 weeks (median 7 weeks). The number of eligible patients willing to be randomised to monotherapy was 120/129 (93.0%). Retention into follow-up

for 24 weeks after recruitment was 119/120 (99.2%).

3.3.2 Randomised study population

The randomised study population comprised 120 patients. At baseline, they had received ART for a median of 7.5 years, including first-line NNRTI-based ART for a median of 3.0 years and second-line PI/b-based ART for a median of 3.1 years (Table 3.1). The median CD4 count was 467 cells/mm^3; a median nadir CD4 count of 92 cells/mm^3 indicated a history of significant immunosuppression. Most patients (106/120, 88.3%) were receiving once daily tenofovir disoproxil fumarate (here forth referred to as tenofovir) co-formulated with lamivudine plus twice daily lopinavir co-formulated with ritonavir (LPV/r). All had a viral load <60 copies/ml in two measurements separated by a median of 7 weeks (range 6-12). No previous viral load measurements were recorded. When comparing the two treatment arms, patients randomised to DRV/b monotherapy had a slightly longer duration of prior NNRTI exposure (Table 3.1).

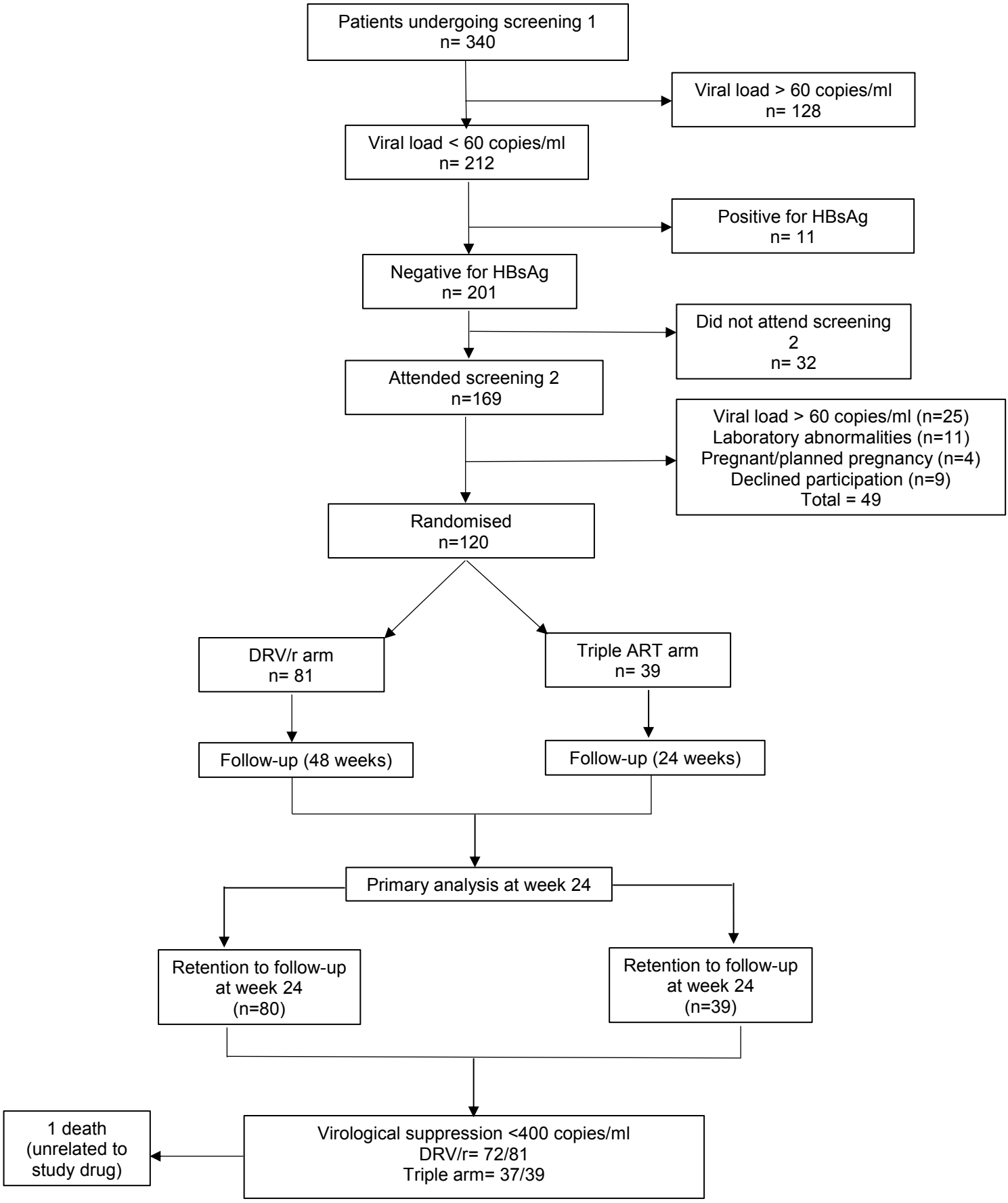


Figure 3.1: Study flow and patient disposition

Table 3.1 Characteristics of the population at study entry

Characteristic	Total	DRV/r	Triple ART	*P*
Total number (%)	120 (100)	81 (100)	39 (100)	
Female, n (%)	91 (75.8)	61 (75.3)	30 (76.9)	0.519
Age, median years (IQR)	44 (38, 52)	45 (38, 51)	45 (37, 52.2)	0.824
Body mass index, median kg/m^2 (IQR)	25.5 (22.1, 29.1)	25.4 (21.8, 28.4)	26.0 (22.5, 29.2)	0.371
Haemoglobin, median g/dL (IQR)	12.3 (11.6, 13.2)	12.3 (11.6, 13.2)	12.4 (11.6, 13.5)	0.834
Estimated glomerular filtration rate >90 ml/min, n (%)	102 (85.0)	67 (82.7)	35 (89.7)	
Estimated glomerular filtration rate 60-89 ml/min, n (%)[a]	18 (15)	14 (17.3)	4 (10.3)	0.417
Time since HIV diagnosis, median years (IQR)	8.5 (5.8, 10.4)	8.8 (5.9, 11.1)	8.0 (5.5, 9.8)	0.166
CD4 count, median cells/mm^3 (IQR)	467 (341, 618)	466 (341, 615)	536 (394, 687)	0.077
Nadir CD4 count, median cells/mm^3 (IQR)	92 (37, 172)	90 (37, 167)	128 (29, 194)	0.427
History of AIDS [b], n (%)	16 (13.3)	12 (14.8)	4 (10.3)	0.353
ART duration, median years (IQR)	7.5 (5.3, 9.4)	7.6 (5.3, 9.8)	6.9 (4.9, 9.2)	0.348
Tenofovir, duration median years (IQR)	2.9 (1.5, 4.7)	2.9 (1.3, 4.6)	1.9 (3.3, 5.3)	0.274
Zidovudine duration, median years (IQR)	2.9 (1.5, 5.4)	2.4 (1.5, 4.0)	3.0 (1.4, 5.8)	0.135
Stavudine duration, median years (IQR)	2.6 (1.3, 4.4)	2.6 (1.3, 4.0)	3.2 (1.3, 4.4)	0.405
NNRTI duration, median years (IQR)	3.0 (1.4, 5.5)	3.1 (1.6, 5.5)	3.0 (1.7, 5.1)	0.027
PI duration, median years (IQR)	3.1 (1.3, 5.3)	3.2 (1.3, 5.8)	3.1 (1.5, 4.9)	0.951
ART regimen at study entry, n (%)				
Tenofovir/lamivudine + lopinavir/ritonavir	106 (88.3)	69 (85.2)	37 (94.8)	
Abacavir + didanosine + lopinavir/ritonavir	6 (5.0)	5 (6.2)	1 (2.6)	
Zidovudine + lamivudine + lopinavir/ritonavir	5 (4.2)	4 (4.9)	1 (2.6)	0.081
Tenofovir + lamivudine + atazanavir/ritonavir	2 (1.7)	2 (2.5)	-	
Tenofovir + abacavir + lopinavir/ritonavir	1 (1.0)	1 (1.2)	-	

[a]eGFR calculated with The estimated glomerular filtration rate (eGFR) was calculated with the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula which adjusts for ethnicity[219]. [b] Comprising tuberculosis (n=8), toxoplasmosis (n=5), Cryptococcus meningitis (n=1), and Kaposi Sarcoma (n=2) Abbreviations: IQR= Interquartile range; AIDS= acquired immune deficiency syndrome; ART= antiretroviral therapy; NNRTI= non-nucleoside reverse transcriptase inhibitor; PI= protease inhibitor.

3.3.3 Virological outcomes

An overview of the MANET study is shown in Table 3.2.

Table 3.2 Overview of the MANET study

	DRV/r	Triple ART
Randomised	81	39
First patient in (date)	22/08/2014	25/08/2014
Last patient last visit (date)	21/06/2016	07/01/2015
Duration of follow-up	48 weeks	24 weeks
Viral load data available at week 24	80 (98.8%)	39 (100%)
Early discontinuation prior to week 24	4 (4.9)	0
Early discontinuation prior to week 48	13 (16.0)	0
Early discontinuation due to viraemia	8/81 (9.9%)[a]	0
Early discontinuation due to adverse event	5/81 (6.2%)[a,b]	0
Serious adverse event	3/81 (4%)[b]	0
Pregnancy	0/81	0
Death	1/81 (1.2%)[c]	0
Loss to follow-up	1/81 (1.2%)[d]	0

[a]Data span 48 weeks. [b]None of the SAEs and AEs leading to early discontinuation were classed as related to the study treatment. [c]A death occurred in a patient who had an early discontinuation at week 19 due to an unrelated SAE and returned to triple ART three weeks prior to death; [d]One patient in the DRV/r arm was lost to follow-up after the week 24 study visit. Abbreviations: DRV/r= ritonavir boosted darunavir; ART=antiretroviral therapy.

In the primary analysis, the percentage of subjects with viral load <400 copies/ml at week 24 was 72/81 (88.9%) on DRV/r and 37/39 (94.9%) on triple ART (p=0.500) (Table 3.3). In the secondary analyses, the percentage with viral load <60 copies/ml at week 12 was 73/81 (90.1%) on DRV/r and 35/39 (89.7%) on triple ART (p=1.000); this reflected 8 and 4 cases of viraemia in the two arms, respectively. Percentages at week 24 were 62/81 (76.5%) and 36/39 (92.3%) respectively (p=0.044); this reflected 15 cases of viraemia and 4 early discontinuations due to a significant AE in the DRV/r arm, and 3 cases of viraemia in the control arm. In the DRV/r arm, over 48 weeks there were 24/81 (29.6%) patients with virological rebound (either confirmed [n=15] or last available [n=9] viral load >60 copies/ml) and 16/81 (19.8%) participants with virological failure (either confirmed [n=9] or last available [n=7] viral load >400 copies/ml). A further 12/81 (14.8%) patients had ≥1 transient episode of viraemia (blip), with most (9/12) experiencing a single blip; the median viral load in this group was 2.2 $\log_{10}$ copies/ml (range 1.9-2.9). Discontinuations of DRV/r prior to week 48 occurred in 6/81 (7.4%) participants owing to a serious adverse event (SAE, n=1), a significant AE (n=4), or loss to follow-up (n=1). Three patients on DRV/r experienced an SAE (Table 3.4). None of the SAEs or significant AEs were classed as drug related. The analysis of time to virological rebound, time to virological failure and time to treatment failure for the DRV/r arm is shown in Figure

3.2. Among patients on triple ART, 4/39 (10.3%) experienced virological rebound, 2/39 (5.1%) met the definition of virological failure over 24 weeks, and a further 4/39 (10.3%) experienced a single blip of 1.9-2.8 $\log_{10}$ copies/ml. No subject experienced treatment failure or an SAE in the triple ART arm.

Table 3.3 Summary of virological outcomes in the two arms of MANET

Endpoint	Study week	DRV/r	Triple ART
VL <400 copies/ml (FDA snapshot)	24	72/81 (88.9%)	37/39 (94.9)
VL <60 copies/ml (TLOVR)	12	73/81 (90.1%)	38/39 (89.7%)
VL <60 copies/ml (TLOVR)	24	62/81 (76.5%)	36/39 (92.3%)
Virological rebound	Through week 24	13/81 16.0%)	4/39 (10.3%)
Virological rebound	Through week 48	24/81 (29.6%)	NA
Virological failure	Through week 24	7/81 (8.6%)	2/39 (5.1%)
Virological failure	Through week 48	16/81 (19.8)	NA
Treatment failure	Through week 24	12/81 (14.8)	2/39 (5.1%)
Treatment failure	Through week 48	22/81 (27.2%)	NA

Abbreviations: TLOVR= time to loss of virological response

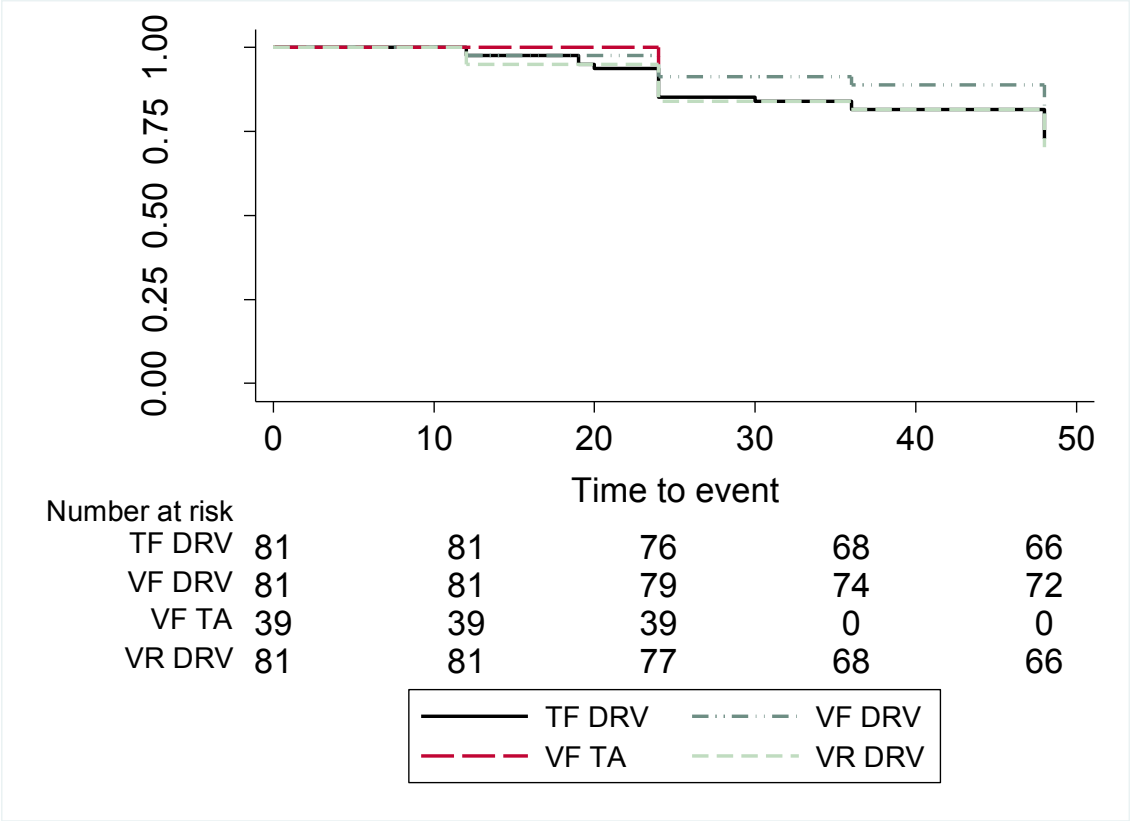


Figure 3.2: Time to virological rebound (first measured viral load >60 copies/ml was plotted), virological failure (VF, either confirmed or last available viral load >400 copies/ml), and treatment failure (TF, either virological failure or discontinuation of study arm prior to study completion for any reason) over 48 weeks of darunavir/ritonavir maintenance monotherapy including patients on triple ART. Virological failure was defined as a plasma HIV-1 RNA load >400 copies/ml, either confirmed in a subsequent sample or last available while the patient remained in the study. Treatment failure comprised virological failure and any early discontinuation of the study arm prior to week 48 for any reason in the darunavir/ritonavir maintenance monotherapy arm.
Abbreviations: DRV= darunavir monotherapy; TF= treatment failure; VF= virological failure; TA= triple arm.

Table 3.4 Summary of serious adverse events in the DRV/r monotherapy arm (n=81)

Patient	Study week of onset	Details	Outcome
1	33	Car accident Hospitalisation	Resolved Continued in study arm
2	0	Acute anxiety Hospitalisation	Resolved Continued in study arm
3	12	Malaria Progressive anaemia Hospitalisation Transfusion reaction Convulsions Suspected sepsis	Discontinued study arm at week 19 Death at week 25

One of the patients with SAE died during the study (Table 3.4). This patient was a 43-year old woman who died at week 25. She had a nadir CD4 count of 7 cells/mm^3 and a CD4 count at study entry of 171 cell/mm^3. The patient was randomised to DRV/r monotherapy and was diagnosed with malaria at week 12, followed by a progressive anaemia leading to hospitalisation for transfusion at week 16. The patient developed a post-transfusion seizure and was returned to standard of care with tenofovir/lamivudine + LPV/r at week 19. The patient's clinical status continued to deteriorate with fever (39.6^oC), nausea, vomiting, fatigue and epigastric tenderness at week 21 and the patient died 4 days later with a diagnosis of a possible transfusion reaction and sepsis. Her viral load had rebounded to 823 copies/ml at week 12 and was 152 copies/ml at week 16.

3.3.4 Adherence measures

Across the entire study population and over the entire study period, mean adherence levels were 97.0% (±4.2) by VAS and 96.5% (±4.1) by pill count. Adherence measure using pill count was approximated to 100% for patients who picked up prescription earlier than expected. The two adherence measures showed a weak positive correlation (Spearman's r=0.31; p=<0.001; Figure 3.3). In patients with virological failure across both arms, mean adherence levels over the entire study period were 96.5% (±4.4) by VAS and 97.0% (±3.3) by pill count, and were 95.9% (±6.2) and 96.7% (±4.2) at week 4, 96.1% (±6.2) and 97.3% (±4.9) at week 12, and 96.1% (±6.1) and 97.0% (±4.0) at week 24, respectively.

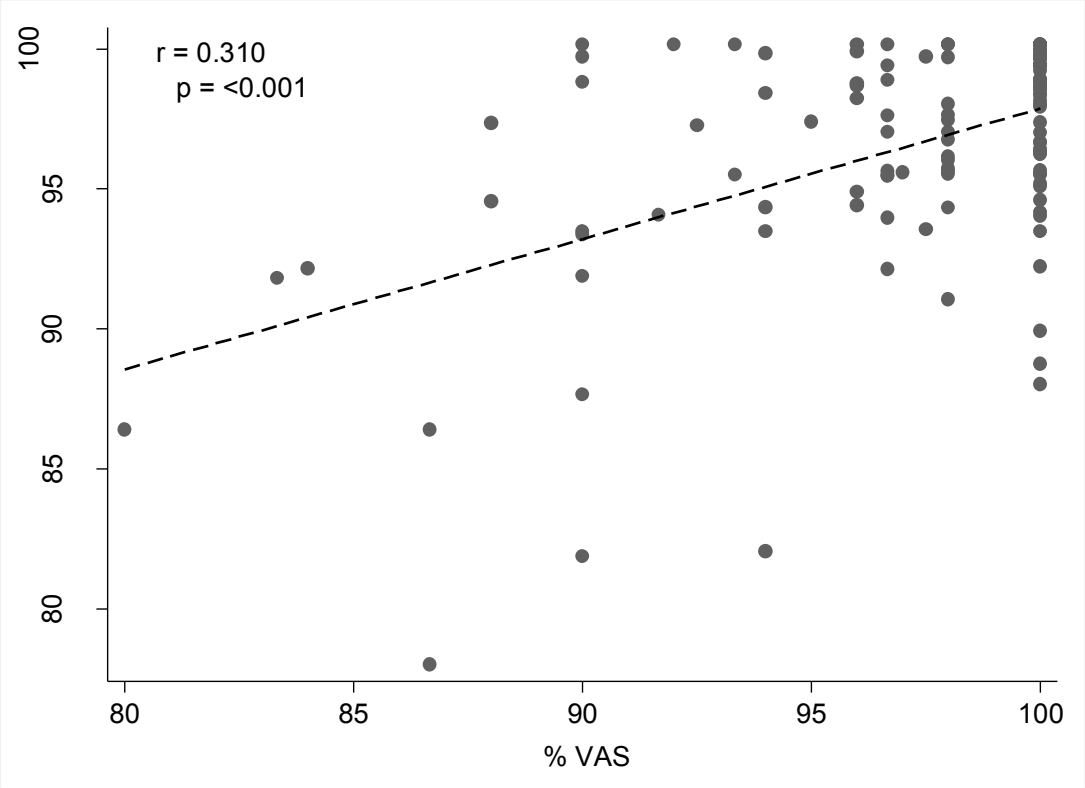


Figure 3.3: Scatter plot showing the correlation between adherence parameters measured by pill count and visual analogue scale averaged over the entire study period. Each dot in the plot represents one observation showing the over the mean study adherence measured by pill count and the corresponding VAS in percentage over the entire period the patient remained in the study. The fitted line is shown.

3.4 DISCUSSION

In its screening phase, the MANET trial produced novel data for Cameroon, indicating that a significant proportion (38%) of patients established on second-line ART with 2 NRTIs and a PI/b (predominantly LPV/r) had a detectable viral load at first screening, and a significant subset (12%) experienced viral load rebound over a median interval of 7 weeks. In two meta-analyses of published data in countries across the SSA region, rates of virological suppression by intention-to-treat were 65% and 62% after 24 months of NNRTI- based first-line ART[210] and PI/b-based second-line ART[142], respectively. Thus, the screening viral load results from the MANET population agree with these data. One further aspect, related to the assessment of feasibility, was that overall interest in participating in the study was high, and eligible patients who declined participation did so largely due to difficulties with attending for frequent clinic visits. The last point should be taken into consideration when designing future studies. Although travel expenses were

reimbursed in MANET, the frequency of visits (every 4 weeks) was high. This was deemed desirable based on the fact that the population was ART experienced and likely to harbour NRTI drug resistance and frequent adherence support was deemed desirable. Retention into follow-up was excellent, although this should be interpreted in context: the study personnel would phone patients to remind them of their appointment and would chase patients that did not attend scheduled visits. Ability to access laboratory testing free of charge was reportedly a significant incentive for the population. The safety profile of DRV/r monotherapy was overall good and in keeping with available data, and there were no discontinuations due to DRV/r-related toxicity. There was one death during the study. This occurred in a patient with malaria who developed progressive anaemia, required a transfusion, and developed complications that were attributed to a transfusion reaction and sepsis.

Several randomised studies have investigated monotherapy with once daily DRV/r as a maintenance strategy for patients showing a suppressed viral load while receiving standard ART. In Western Europe, the studies recruited patients without a previous history of treatment failure[215]. These studies uniformly reported an increased risk of viraemia on DRV/r monotherapy, but a low risk of emerging darunavir resistance-associated mutations, and excellent resuppression rates after re-introduction of NRTIs. A recent study from Burkina Faso, Cameroon, and Senegal, which ran at the same time as MANET, evaluated patients who after failure of first-line ART with 2 NRTIs plus an NNRTI had achieved virological suppression on 2 NRTIs plus a PI/b[159], thus resembling the population of MANET. A total of 133 patients were randomised to maintenance monotherapy with either DRV/r (n=56) or ritonavir-boosted lopinavir (LPV/r). Over 48 weeks, 21% overall experienced virological failure (viral load >500 copies/ml), without appreciable differences between the 2 arms. In MANET, the rates of virological failure in the DRV/r arm over 48 weeks were similar to those in the study from Burkina Faso, Cameroon, and Senegal. Thus, the data from these two studies, totalling 137 patients, indicate that DRV/r monotherapy cannot be recommended for patients established on suppressive second-line PI/b-based ART in sub-Saharan Africa, owing to the need for frequent virological monitoring and prompt treatment intensification to avoid

prolonged viraemia, risk of onward transmission[220], and emergence of resistance on the long term[221].

Interestingly, self-reported adherence by VAS performed better than directly measured pill counts. This is a promising observation given that VAS is an easy tool to implement in resource-limited settings. In the study from Burkina Faso, Cameroon, and Senegal, pill counts were also less likely to detect adherence problems relative to the use of questionnaires [159]. However, there exists conflicting evidence about the relative performance of self-reported versus directly assessed adherence measures in sub-Saharan Africa [222–224], identifying a research need. It is possible that the personal interaction required to administer a VAS (or a questionnaire) may bring benefit when assessing adherence in these settings in comparison to an assessment such as pill count which is subject to reporting bias.

Taken together the findings indicate that a large subset of patients established on second-line PI/b-based ART in the Yaoundé cohort have unsuppressed viral load. Routine virological monitoring is now being implemented in the centre, and there is progress in defining strategies for third-line ART. Although safe and well tolerated, DRV/r monotherapy is not a suitable treatment strategy for patients receiving standard PI/b-based ART in sub-Saharan Africa due to a high risk of both low-level and high levels viraemia. The study improved capacity for conducting clinical trials by establishing training in Good Clinical Practice and in rigorous clinical research methods that were fully compliant with European clinical trial directives. Furthermore, the trial implemented a research consortium that involved 5 different institutions and establishing effective liaisons was an important achievement. The study provided significant training and career development opportunities for the local medical, nursing and scientific staff.

CHAPTER FOUR: RESISTANCE ANALYSIS OF PATIENTS ENTERING THE MANET TRIAL

In this chapter, I studied the presence of drug resistance associated mutations in patients who participated in the monotherapy arm of the MANET trial. This included testing for archived resistance using HIV-1 DNA recovered from peripheral blood mononuclear cells (PBMC) at study entry, and then assessing the presence of resistance in plasma samples collected at the time of HIV-1 RNA rebound on darunavir/ritonavir (DRV/r) monotherapy. I performed sequencing of HIV-1 DNA from PBMC, prepared the plasma HIV-1 RNA samples for ultradeep sequencing (UDS), and analysed the data including the statistical and phylogenetic analyses. The choice of using Sanger sequencing for PBMC samples was on the basis of low virus input expected from the PBMC due to suppressive long-term ART. Some of the work has been published in Geretti AM, Abdullahi A, Mafotsing Fopoussi O, Bonnett L, Defo VF, Moudourou S, Fokam J, Kouanfack C, Torimiro J. An apparent paradox: resistance mutations in HIV-1 DNA predict improved virological responses to antiretroviral therapy. J Antimicrob Chemother. 2019; 74:3011-3015.

4.1 INTRODUCTION

The WHO recommends standardised regimens for first-line and second-line antiretroviral therapy (ART) in sub-Saharan Africa, comprising 2 NRTIs plus an NNRTI, a ritonavir-boosted protease inhibitor (PI/r), or more recently an integrase inhibitor[154]. Pooled estimates indicate rates of virological suppression of 65% and 62% after 24 months of NNRTI-based first-line ART and PI/r-based second-line ART, respectively[225,142]. Sparse virological monitoring and delayed introduction of second-line ART in viraemic patients favour the emergence of drug resistance, and resistance-associated mutations (RAMs) affecting both the NRTIs and the NNRTIs are common at failure of first-line NNRTI-based ART[198,226–229]. The significance of NRTI RAMs

in terms of predicting reduced responses to second-line NRTI-containing ART has been called into question[142,230]. In a meta-analysis of pooled data from sub-Saharan Africa, detection of RAMs at failure of NNRTI-based ART, most commonly M184V and thymidine analogue mutations (TAMs), predicted higher (rather than lower) odds of virological suppression after starting 2NRTIs + PI/r[142]. Thus, it has been inferred that resistance testing underestimates the continued activity of NRTIs such as lamivudine and tenofovir in the presence of M184V and TAMs.

Several randomised studies investigated monotherapy with once daily ritonavir-boosted darunavir (DRV/r 800mg/100mg) as a maintenance strategy for patients showing a suppressed viral load while receiving standard ART. In Western Europe, studies recruited patients without a previous history of treatment failure[231]. In sub-Saharan Africa, a multi-centre study from Burkina Faso, Cameroon, and Senegal investigated 50 patients that after first-line NNRTI-based ART had achieved virological suppression on second-line PI/r-based ART[159]. These studies uniformly reported an increased risk of viraemia on DRV/r monotherapy, but a low risk of emerging darunavir RAMs. These data point to a high barrier to the emergence of resistance with DRV/r once daily, despite relatively high levels of viraemia.

Within this context, the aims of this chapter were to 1) determine the presence of RAMs in HIV-1 DNA derived from PBMC collected in MANET participants at study entry (i.e., when the viral load was suppressed <60 copies/ml); 2) analyse the relationship between archived RAMs and outcomes of DRV/r maintenance monotherapy; 3) determine the presence of RAMs in plasma HIV-1 RNA at the time of virological rebound on DRV/r monotherapy, including the analysis of protease and reverse transcriptase in all patients and the analysis of gag in a subset; and 4) analyse the phylogenetic relationship between sequences derived from HIV-1 DNA and those obtained from HIV-1 RNA.

4.2 METHODS

4.2.1 Study participants

Patients in this analysis took part in the randomised open-label MANET (Monotherapy in Africa, New Evaluations of Treatment) trial at Hôpital Central Yaoundé in Cameroon. As previously described, patients eligible for MANET were receiving 2 NRTIs + PI/r for ≥12 weeks, had a CD4 count >100 cells/mm^3, showed a viral load <60 copies/ml in 2 measurements 4-12 weeks apart (median 7 weeks), and tested hepatitis B surface antigen (HBsAg)-negative. A total of 120 patients were randomised (2:1) to start monotherapy with darunavir (800mg daily) plus ritonavir (100mg daily) for 48 weeks (n=81) or continue standard ART with 2 NRTIs + PI/r for 24 weeks (n=39). The study was approved by the University of Liverpool Ethics Committee and the Cameroon National Ethics Committee and overseen by an independent data safety board. Participants gave written informed consent. This chapter focuses on patients randomised to DRV/r monotherapy. As previously described, in this group patients met the definition of virological failure if during the 48 weeks of the study they showed either confirmed or last available viral load >400 copies/ml.

4.2.2 Laboratory testing

Safety parameters, CD4 cell counts, and viral load were measured at the Centre Pasteur of Cameroon in Yaoundé. The viral load was measured with the Bandol Biocentric assay, with a lower limit of quantification of 60 copies/ml and repeat testing of viral load, the Abbott Real-Time HIV-1 assay was used. In the United Kingdom, total HIV-1 DNA was quantified in PBMC as described[116].

4.2.3 Drug resistance testing

RAMs were detected in HIV-1 DNA and HIV-1 RNA as described in Chapter 2. With PBMC collected at study entry, after nucleic acid extraction, amplicons

were generated using a nested PCR protocol spanning protease (PR, aa 1-99) and reverse transcriptase (RT, aa 1-335). After purification and quantification of the amplicons, RAMs were detected by Sanger sequencing on the Applied Biosystems 3730xl genetic analyser. In patients who experienced virological rebound (>60 copies/ml), plasma samples underwent UDS. I performed the sample preparation for UDS according to a protocol that I had previously optimised to increase the quality of the reads. Briefly, samples with viral load <10,000 copies/ml were first enriched by ultra-centrifugation. After nucleic acid extraction, a 1300bp amplicon was generated covering PR (aa 1-99) and RT (aa 1-335). Following amplicon purification and quantification, a DNA library was prepared for sequencing using Illumina MiSeq. Reads were analysed applying a 1% interpretative frequency cut-off. Major RAMs were defined according to the Stanford HIV drug resistance algorithm (v8.8). Darunavir RAMs comprised V11I, V32I, L33F, I47V, I50V, I54L/M, T74P, L76V, I84V, and L89V. The genotypic susceptibility score (GSS) of the ART regimen at study entry was calculated by assigning a score of 0 for high-level resistance, 0.25 for intermediate resistance, 0.5 for low level resistance, and 1 for potential low-level resistance or full predicted susceptibility, based on the Stanford algorithm predictions. HIV-1 subtypes were determined by phylogenetic analysis as detailed in Chapter 2. Paired PBMC and plasma sequences and sequences from patients showing the protease RAM D30N were evaluated for phylogenetic linkage as described in Chapter 2. RT sequences were screened for evidence of APOBEC3G (A3G) hypermutation using the Los Alamos Hypermut 2.0 programme. Sequences showing D30N in PR, and E138K, M184I, or M230I in RT were explicitly evaluated for the presence of hypermutation[232–234]. HIV-1 DNA sequences were also analysed for the presence of in-frame stop-codons to indicate defective proviruses[235].

4.2.4 Gag sequencing

In a subset of patients with viraemia, following nucleic acid extraction, a 2200bp amplicon spanning the entire *gag* region was generated by nested PCR followed by Sanger sequencing as detailed in Chapter 2. Sequences

were aligned with the HIV-1 HXB2 as the reference sequence and gag mutations were defined as any change relative to the reference HXB2 sequence. Mutations were classed as cleavage site (CS) mutations if they occurred at one of the 5 gag cleavage sites (p17/p24, p2/p7, p7/p1, p1/p6, p24/p2); other substitutions were classed as non-CS mutations. Based on previous work by Dr Ana Garcia, a PhD student that worked under Professor Geretti's primary supervision, CS mutations considered to be associated with PI exposure comprised: V128I, Y132F, S373T, A374S, T375A, T375N, A431V, K436R, I437V, L449F, S451T, S451R, R452S and P453T; non-CS mutations considered to be associated with PI exposure comprised: L61I, I94V, K103R, K113Q, K114R, D121G, D121A, T122E, N126S, Q127K, T186M, T190I, A210S, E211D, S310T, T371Q, F463L, T469I and P478Q.

4.2.5 Statistical analyses

The characteristics of patients with or without HIV-1 DNA sequencing results were compared using Wilcoxon rank-sum test, *t*-test, or Fisher's exact test. Predictors of virological failure were explored by logistic regression analysis; all available variables were considered for inclusion and those with $p \leq 0.2$ in the univariate analysis were retained in the multivariable model.

4.3 RESULTS

4.3.1 HIV-1 DNA RAMs at study entry

The study population is described in (Table 4.1). There were 76/81 (93.8%) patients with PBMC samples, and of these 60/76 (78.9%) yielded a HIV-1 DNA sequence. Five patients did not have a PBMC sample for sequencing, whereas 16 did not yield a HIV-1 DNA sequence in ≥2 attempts and no further attempts were possible due to limited sample volume. Relative to patients with HIV-1 DNA sequencing results, those without had a lower HIV-1 DNA load ($p=0.0004$) and a higher nadir CD4 cell count ($p=0.05$) (Table 4.2).

Table 4.1. Characteristics of the darunavir/ritonavir arm at study entry (n=81)

Characteristic		
Female, n (%)		61 (75.3)
Age, median years (IQR)		45 (38, 51)
Time since HIV diagnosis, median years (IQR)		8.8 (5.9, 11.1)
CD4 count, median cells/mm^3 (IQR)		466 (341, 615)
Nadir CD4 count, median cells/mm^3 (IQR)		90 (37, 167)
History of AIDS, n (%)		12 (14.8)
HIV-1 DNA, median log_{10} copies/10^6 PBMC (IQR)		2.9 (2.5, 3.3)
Antiretroviral drugs ever experienced, n (%)	Lamivudine	81 (100)
	Tenofovir	73 (90.1)
	Zidovudine	72 (88.9)
	Stavudine	41 (50.6)
	Didanosine	13 (16.0)
	Abacavir	9 (11.1)
	Nevirapine	50 (61.7)
	Efavirenz	43 (53.1)
	Lopinavir/ritonavir	79 (97.5)
	Indinavir/ritonavir	9 (11.1)
	Atazanavir/ritonavir	5 (6.2)
Duration of exposure, median years (IQR)	Total ART	7.6 (5.3, 9.8)
	Tenofovir	2.9 (1.3, 4.6)
	Zidovudine	2.4 (1.5, 4.0)
	Stavudine	2.6 (1.3, 4.0)
	NNRTI	3.1 (1.6, 5.5)
	PI/r	3.2 (1.3, 5.8)
ART regimen at study entry, n (%)	Tenofovir/lamivudine + lopinavir/ritonavir	69 (85.2)
	Tenofovir/lamivudine + atazanavir/ritonavir	2 (2.5)
	Tenofovir + abacavir + lopinavir/ritonavir	1 (1.2)
	Abacavir + didanosine + lopinavir/ritonavir	5 (6.2)
	Zidovudine/lamivudine + lopinavir/ritonavir	4 (4.9)
GSS of ART regimen at study entry, n (%) [a]	3	22 (27.2)
	2	22 (27.2)
	1.5	1 (1.2)
	1.25	10 (12.3)
	1	5 (6.2)
HIV-1 subtype, n (%) [a]	CRF02_AG	35 (43.2)
	A1	10 (12.3)
	G	5 (6.2)
	Other[b]	10 (12.3)

[a]GSS and HIV-1 subtype were determined in 60/81 patients based on HIV-1 DNA sequencing results; [b]Other subtypes comprised CRF11_cpx (n=3), and subtypes D, H, F1, CRF01_AE, CRF37_cpx, CRF06_cpx, and CRF18_cpx (one of each). Abbreviations: AIDS=acquired immune deficiency syndrome; ART=antiretroviral therapy; NNRTI= non-nucleoside reverse transcriptase inhibitor; PI/r=boosted protease inhibitor.

Table 4.2. Characteristics of patients with and without a HIV-1 DNA sequencing result

Characteristic	HIV-1 DNA resistance test result		
	Available	Not available	P-value
Total number (%)	60 (100)	21 (100)	-
Female, n (%)	42 (70.0)	19 (90.5)	0.080
Age, median years (IQR)	46 (42, 51)	42 (38, 51)	0.311
Time since HIV diagnosis, median years (IQR)	8.3 (5.6, 10.5)	9.9 (6.5, 11.3)	0.190
CD4 count, median cells/mm^3 (IQR)	407 (322, 552)	477 (390, 734)	0.070
Nadir CD4 count, median cells/mm^3 (IQR)	76 (34, 161)	134 (77, 198)	0.050
History of AIDS-defining disease, n (%)	10 (16.7)	1 (4.8)	0.273
HIV-1 DNA, median log_{10} copies/10^6 PBMC (IQR)	3.0 (2.7, 3.4)	2.6 (1.2, 2.9)	<0.001
ART duration, median years (IQR)	7.6 (5.3, 9.4)	6.3 (5.3, 10.2)	0.897
Tenofovir duration, median years (IQR)	2.8 (1.7, 4.7)	3.0 (1.8, 4.0)	0.687
Zidovudine duration, median years (IQR)	2.3 (1.7, 4.1)	2.6 (0.9, 3.6)	0.310
Stavudine duration, median years (IQR)	2.3 (1.3, 3.3)	3.4 (1.6, 4.9)	0.316
NNRTI duration, median years (IQR)	4.0 (2.4, 6.0)	4.6 (1.9, 6.9)	0.859
PI/r duration, median years (IQR)	3 (1.2, 5.5)	3.4 (1.8, 6.0)	0.276
ART regimen at study entry, n (%)			
Tenofovir/lamivudine + lopinavir/ritonavir	51 (85.0)	18 (85.7)	0.789
Abacavir + didanosine + lopinavir/ritonavir	4 (6.7)	1 (4.8)	
Zidovudine/lamivudine + lopinavir/ritonavir	2 (3.3)	2 (9.5)	
Tenofovir/lamivudine + atazanavir/ritonavir	2 (3.3)	-	
Tenofovir + abacavir + lopinavir/ritonavir	1 (1.7)	-	

Abbreviations: AIDS=acquired immune deficiency syndrome; ART=antiretroviral therapy; NNRTI= non-nucleoside reverse transcriptase inhibitor; PI/r=boosted protease inhibitor.

Among 60 patients with a successful HIV-1 DNA sequence, NRTI and NNRTI RAMs occurred in 39/60 (65.0%) and 41/60 (68.3%) patients, respectively (Table 4.3); 37/60 (61.6%) samples had dual-class mutations. The most prevalent NRTI RAMs were M184V/I and thymidine-analogue mutations (TAMs) comprising both pattern 1 (codons 41, 210, 215) and pattern 2 (codons 67, 70, 215, 219) TAMs, with a median number of 2 TAMs (range 1-5). There were three patients (subtype G and CRF02_AG) with the D30N mutation in PR. The three patients had received between 1 and 3 years of ritonavir-boosted lopinavir (LPV/r)-based triple ART and had no history of prior exposure to nelfinavir, an older PI that selected for D30N in patients with viraemia. Two of the patients showed evidence of sequence hypermutation. Overall, 6/60 (10%) HIV-1 DNA sequences showed hyper mutation including the two with D30N and one each with M184I, E138K and M230I. Eleven HIV-1 DNA sequences (18.3%) showed the presence of stop codons at RT amino acid positions 24, 42, 48, 71, 88, 120, 153, 212, 219, 229, and 239), suggestive of defective, non-replicative proviruses.

Table 4.3 Resistance-associated mutations (RAMs) in baseline HIV-1 DNA and rebound HIV-1 RNA

Resistance profile		HIV-1 DNA, N (%)	HIV-1 RNA, N (%)
Total tested		60	21
≥ 1 NRTI RAM, n (%)		39 (65.0)	9 (42.9)
≥ 1 TAM[a]		29 (48.3)	5 (23.8)
	1 TAM	13 (21.6)	1 (4.8)
	2 TAMs	5 (8.3)	3 (14.3)
	≥ 3 TAMs	11 (18.3)	1 (4.8)
	TAM-1 profile	10 (16.7)	2 (9.5)
	TAM-2 profile	15 (25.0)	2 (9.5)
	Mixed/other TAM profile	4 (6.7)	1 (4.8)
≥ 1 Discriminatory RAM		36 (60)	5 (23.8)
	K65R	2 (3.3)	-
	L74V	3 (5.0)	-
	L74I	2 (3.3)	-
	M184V	30 (50.0)	5 (23.8)
	M184I	3 (5.0)	-
	M184I/V	2 (3.3)	-
MDR and Miscellaneous RAMs		5 (8.3)	
	Q151M/L	3 (5.0)	-
	T69ins	1 (1.7)	-
	T69D/N	1 (1.7)	-
	T69N	-	1 (4.8)
≥ 1 NNRTI RAM		41 (68.3)	7 (33.3)
	A98G	9 (15.0)	2 (9.5)
	L100I	1 (1.7)	-
	K101E	4 (6.7)	2 (9.5)
	K101H	1 (1.7)	-
	K101P/T	1 (1.7)	-
	K103N	20 (33.3)	3 (14.3)
	V106A	3 (5.0)	-
	V106M	-	1 (4.8)
	V108I	6 (10.0)	-
	E138G	2 (3.3)	-
	E138K	1 (1.7)	-
	Y181C	14 (23.3)	1 (4.8)
	Y188L	2 (3.3)	-
	Y188C	1 (1.7)	-
	Y188H	-	-
	Y188F/H/L	1 (1.7)	-
	G190A	9 (15.0)	2 (9.5)
	H221Y	5 (8.3)	-
	P225H	2 (3.3)	-
	F227L	3 (5.0)	-
	M230I	3 (5.0)	2 (9.5)
	M230L	1 (1.7)	-
	K238T	4 (6.7)	-
	Y318F	1 (1.7)	-
PI RAM	D30N	3 (5.0)	2 (9.5)

[a]TAM-1 profile comprised ≥1 of M41L, L210W, T215Y; TAM-2 profile comprised ≥1 of D67N/G, K70R, T215F (or T215I/V occurring with other TAM-2 RAMs), K219 Q/E/R; mixed/other TAM profile comprised mixutures of TAM-1 and TAM-2 RAMs or occurrence of T215S as the sole TAM. Abbreviations: PBMC= Peripheral blood mononuclear cells; NRTI= Nucleot(s)ide reverse transcriptase inhibitor; TAM= Thymidine analogue mutation; MDR=

Multi-drug resistance; NNRTI= Non-nucleoside reverse transcriptase inhibitor; PI=protease inhibitor.

4.3.2 HIV-1 DNA RAMs as predictors of virological failure

Overall, 16/81 (19.8%) patients in the DRV/r arm met the definition of virological failure, comprising either confirmed (n=9) or last available (n=7) viral load >400 copies/ml. Mean adherence levels were 97.3% (±2.9) by pill count and 97.3% (±3.5) by visual analogue scale (VAS) in the DRV/r monotherapy arm overall. By univariable analysis, the risk of virological failure was lower in older patients and those with a lower HIV-1 DNA load, higher VAS-based adherence, and either detectable HIV-1 DNA RAMs or unknown HIV-1 DNA RAM status (due to missing data) (Table 4.4). After adjustment, higher adherence by VAS and detection of HIV-1 DNA RAMs remained independently predictive of a reduced risk of virological failure.

Table 4.4 Factors associated with virological failure in the darunavir/ritonavir arm (confirmed or last available viral load >400 copies/ml)

		Univariable analysis		Multivariable analysis	
Characteristic	Alteration	OR (95% CI)	*P* value	AOR (95% CI)	*P* value
Gender	female versus male	0.66 (0.20-2.20)	0.500		
Age	per 5 year older	0.75 (0.54-1.04)	0.087	0.94 (0.87-1.01)	0.119
Time since HIV diagnosis	per year longer	0.92 (0.78-1.09)	0.348		
CD4 count	per 50 cells/mm^3 higher	1.04 (0.94-1.14)	0.496		
Nadir CD4 count	per 50 cells/mm^3 higher	0.92 (0.64-1.31)	0.867		
History of AIDS	yes versus no	1.44 (0.34-6.06)	0.622		
HIV-1 DNA	per log_{10} copies/10^6 PBMC higher	1.80 (0.72-4.50)	0.211	2.80 (0.80-9.99)	0.109
ART duration	per year longer	0.91 (0.76-1.09)	0.325		
PI duration	per year longer	0.96 (0.79-1.16)	0.667		
≥1 RAM in HIV-1 DNA	yes versus no	0.31 (0.09-1.09)	0.069	0.15 (0.03-0.82)	0.028
	unknown versus no	0.17 (0.03-0.94)	0.042	0.29 (0.04-2.06)	0.218
Pill count	per 10% increment	0.55 (0.15-2.03)	0.367		
VAS	per 10% increment	0.21 (0.06-0.69)	0.011	0.04 (0.01-0.37)	0.004

Abbreviations: ART= antiretroviral therapy; PI=protease inhibitor; RAM=resistance associated mutations; VAS= visual analogue scale.

4.3.3 RAMs in rebound plasma HIV-1 RNA

In the darunavir/ritonavir arm, UDS was performed retrospectively with plasma samples from 21 patients. The patients had experienced viral load rebound >60 copies/ml between study week 12 and week 48 (median week 30). UDS was undertaken with plasma samples collected between week 12 and week 48 (median week 36), when the viral load ranged between 2.0 and 4.1 $\log_{10}$ copies/ml (median 3.0). There were 7/21 (33.3%) samples with ≥1 NRTI RAM and 8/21 (38.1%) with ≥1 NNRTI RAM (Table 4.2), including 6/21 (28.6%) samples with mutations to both classes. No sample had darunavir RAMs. Most RAMs occurred at frequency >15%; there were 7 RAMs that occurred at a frequency between 1% and 9%. Two patients (CRF02_AE and CRF02_AG) showed the PR RAM D30N at low frequency (3-4%); the sequences had no evidence of hypermutation. The patients had previously experienced LPV/r for >4 years and had no record of exposure to other PI/r including nelfinavir, and neither had D30N in their baseline PBMC. There was no phylogenetic linkage between the five patients showing D30N in PBMC (n=3) or plasma (n=2) to suggest that they were part of a transmission cluster (Figure 4.1).

4.3.4 Comparison of HIV-1 DNA and HIV-1 RNA sequences

There were 16 patients with paired PBMC HIV-1 DNA and plasma HIV-1 RNA sequencing data. For each patient, the phylogenetic analysis confirmed linkage of the paired sequences (Figure 4.2). When comparing rebound HIV-1 RNA with baseline HIV-1 DNA (Table 4.5), the resistance patterns were either fully or partially consistent in 10/16 (62.5%) samples. There were 7/16 (43.7%) patients with ≥1 RAM in PBMC alone, including 3 with in-frame stop codons indicative of a defective genome.

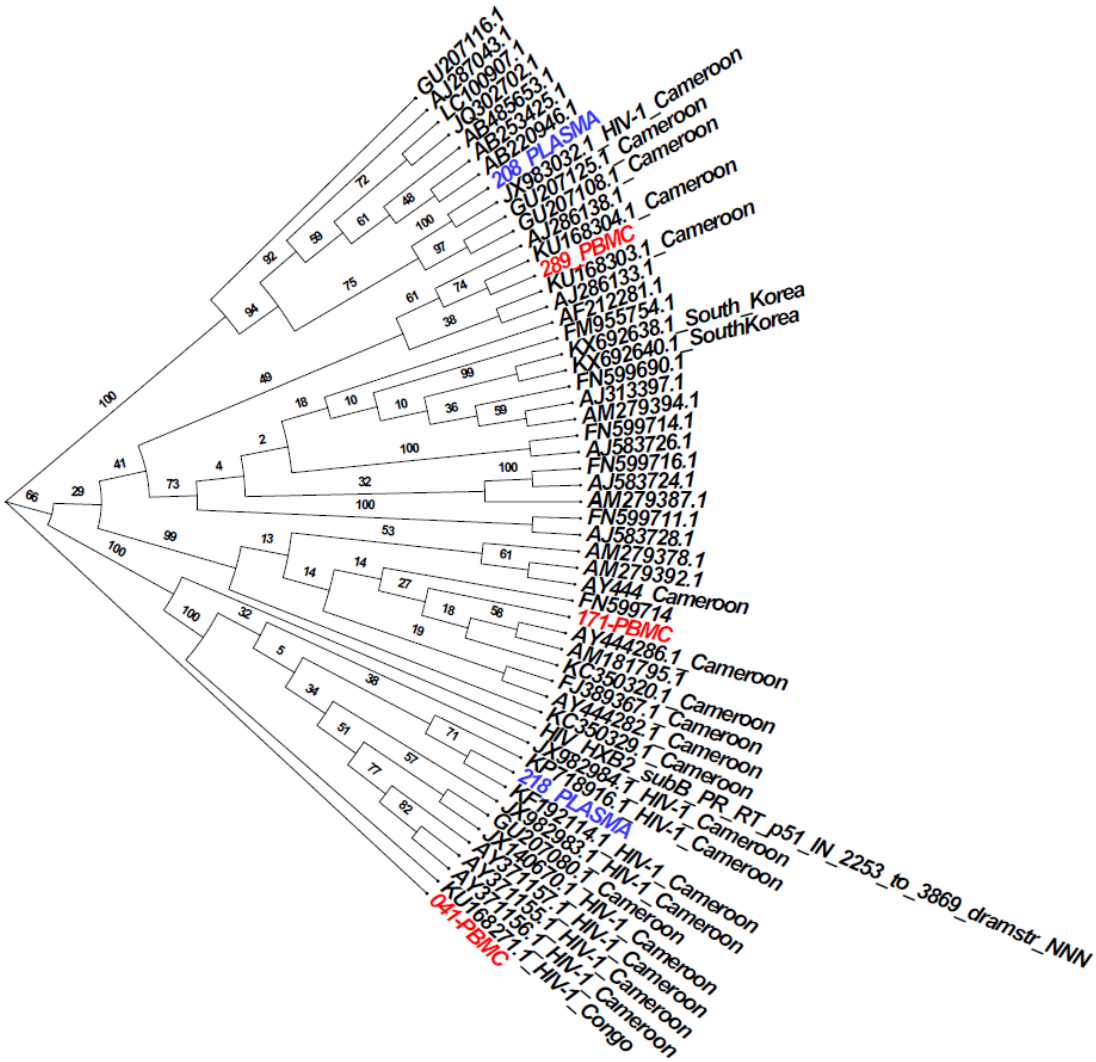


Figure 4.1 Maximum-likelihood phylogenetic tree to evaluate linkage between sequences with the D30N mutation in protease (1000 bootstrap replicates). There were five patients that showed D30N in either peripheral blood mononuclear cell (PBMC)-associated HIV-1 DNA (in red; n=3) or plasma HIV-1 RNA (in blue; n=2).

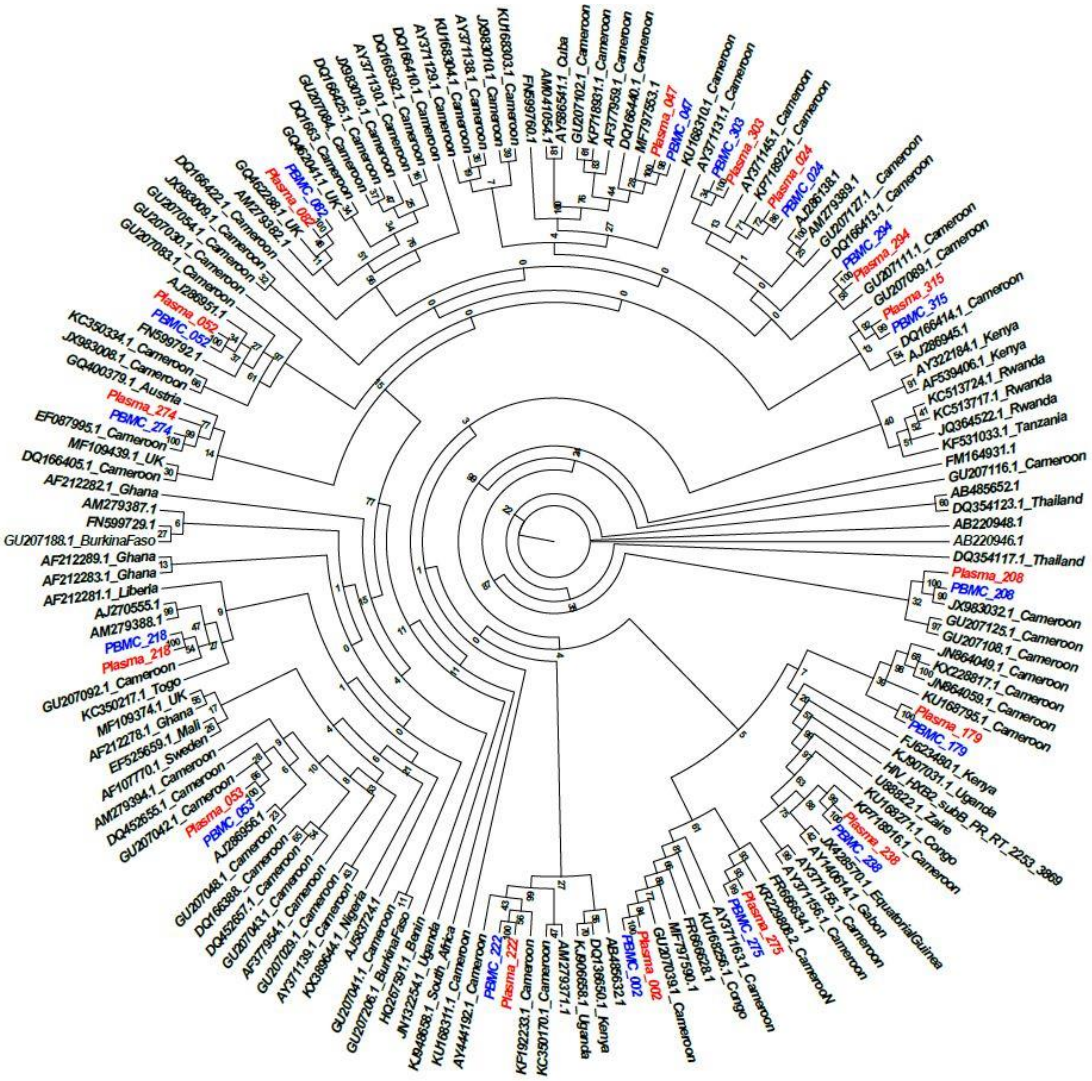


Figure 4.2 Maximum-likelihood phylogenetic tree to evaluate linkage between paired sequences derived in each patient from peripheral blood mononuclear cell (PBMC)-associated HIV-1 DNA and plasma HIV-1 RNA (1000 bootstrap replicates).

Table 4.5: Comparisons of resistance associated mutations (RAMs) in baseline PBMC and plasma rebound

ID / Group		Baseline									Viraemia				Pattern[a]
		Duration of exposure					RAMs in HIV-1 DNA[b]			Week	VL	RAMs in HIV-1 RNA[c]			
	Arm	ART	TDF	TAs	NNRTI	PI	NRTI	NNRTI	PI			NRTI	NNRTI	PI	
024/VF	DRV/r	10.9	4.9	0.0	5.8	10.6	None	None	None	48	410	None	None	None	FC
047/VF	DRV/r	7.6	2.8	4.7	5.8	1.8	None	None	None	48	595	None	None	None	FC
053/VF	DRV/r	1.9	1.7	0.7	0.2	1.9	None	None	None	12	5454	None	None	None	FC
179/VF	DRV/r	3.7	1.0	2.7	2.7	1.0	None	None	None	48	1506	None	None	None	FC
274/VF	DRV/r	8.5	6.4	2.0	2.1	6.4	None	None	None	24	489	None	None	None	FC
082/VF	DRV/r	6.9	1.7	5.8	5.7	1.2	None	K103N	None	24	13024	None	$K103N_{(1)}$	None	FC
002/VF	DRV/r	6.3	1.3	5.0	5.0	1.3	M184V T215F	K101E G190A	None	48	2707	$M41L_{(2)}$ $M184V_{(99)}$ $T215F_{(67)}$	$K101E_{(98)}$ $G190A_{(99)}$	None	PC
315/VF	DRV/r	13.7	5.5	8.1	3.8	9.9	M41L M184V	K101E K103N	None	36	1608	$M41L_{(99)}T215Y_{(95)}$ $T215C_{(4)}$	None	None	PC
238/VF	DRV/r	7.4	5.1	2.3	1.7	5.7	None	None	None	48	1098	None	$M230_{(1)}$	None	PLO
275/VF	DRV/r	5.6	2.7	2.8	4.3	1.3	None	K103N	None	24	13381	None	None	None	PBO
294/VF	DRV/r	1.7	1.7	0.6	0.7	1.0	K70R	None	None	36	621	None	None	None	PBO
052/VF	DRV/r	9.1	4.0	5.1	5.1	4.0	K65R K70R V75I F77L F116Y Q151LM M184I	V108I E138G Y181C G190A H221Y	None	48	3715	None	None	None	PBO/ defective[d]
303/VF	DRV/r	7.7	1.7	4.0	4.0	3.7	M41L D67N K70R L74I M184IV T215F K219R	L100I K103N	None	12	1758	None	None	None	PBO/ defective[d]
208/Blip	DRV/r	9.0	4.4	4.5	4.6	4.4	M184V T215Y	A98G, Y181C	None	48	201	$M184V_{(67)}$ $M41L_{(60)}$ $T215Y_{(24)}$	$A98G_{(33)}$ $Y181C_{(73)}$ $M230I_{(9)}$	$D30N_{(4)}$	PC
218/Blip	DRV/r	9.4	4.6	4.8	4.8	4.6	M41L L74I M184V L210W T215Y	A98G K103N P225H	None	24	105	$M184V_{(53)}$ $T215F_{(38)}$	$A98G_{(21)}$ $K103N_{(32)}$ $Y232H_{(17)}$	$D30N_{(3)}$	PC
222/Blip	DRV/r	7.4	3.5	3.9	4.0	3.4	None	M230I	None	12	823	None	None	None	PBO/ defective[d]

[a]The pattern of RAMs detected in HIV-1 DNA compared to HIV-1 RNA was classed as fully consistent (FC), partially consistent (PC), RAMs in plasma only (PLO), or RAMs in PBMC only (PBO); the detection of defective provirus in indicated. [b]RAMs were detected by Sanger sequencing. [c]RAMs were detected by ultradeep sequencing; the frequency of the mutant is indicated in brackets; [d]Sequences with evidence of hyper mutation (may affect D30N in protease and E138K, M184I, and M230I in reverse transcriptase). Abbreviations: PBMC= peripheral blood mononuclear cells; VL= plasma HIV-1 RNA load in copies/ml; ART=antiretroviral therapy; TDF= Tenofovir; TA= Thymidine analogues (zidovudine or stavudine); NNRTI=non-nucleoside reverse transcriptase inhibitor; PI= protease inhibitor; NRTI= nucleot(s)ide reverse transcriptase inhibitor; VF=virological failure.

4.3.5 Gag mutations at viraemia

Eight patients with virological failure had at least one available plasma sample for gag sequencing, with four patients having samples from ≥2 timepoints; the earliest available timepoint was study week 12 (Table 4.6). The median viral load at time of testing was 3.6 $\log_{10}$ copies/ml (range: 3.1-4.5). Four patients had at ≥1 CS mutation and all had ≥4 non-CS mutations associated with PI exposure; none showed PR RAMs. Of the four patients with CS mutations, two had T375A and one had T375N in p2/p7, and two had K436R in p7/p1; one patient showed both T375A and K436R. All four patients had infection with CRF02_AG and had experienced LPV/r as the sole PI prior to DRV/r. There was one patient with evidence of gag CS evolution during DRV/r monotherapy (subject ID 275, Table 4.6). The patient had received LPV/r-based triple ART for 1.3 years prior to study entry. At week 24 of DRV/r monotherapy (first available time point) T375N was detected in p2/p7. At study week 36, T375N was replaced by K436R in p7/p1. All patients with CS mutations achieved virological suppression after returning to triple PI/r-based ART. There was limited evidence of evolution at gag non-CS sites; one patient (subject ID 243, Table 4.6) showed emergence of two mutations (D121G, P478Q) at study week 36, in the absence of CS mutations; at this time the patient re-introduced triple ART due to virological failure and subsequently achieved virological suppression.

Table 4.6 Gag mutations in rebound HIV-1 RNA during darunavir/ritonavir monotherapy

Subject ID	Study week	Viral load (copies/m)	ART duration (years)	Duration of PI exposure (years)	Protease RAMs	Gag mutations	
						CS	Non-CS
052	48	3715	9.1	4.0	None	T375A, K436R	N126S, T186M, T190I, S310T, T469I
053	12	5454	1.9	1.9	ND	None	D121A, T186M, T371Q, F463L
053	24	15756	2.1	2.1	None	None	D121A, T186M, T190I, F463L
082	24	13024	7.1	1.2	ND	None	K114R, D121A, N126S, T186M, T190I, S310T, T371Q, F463L, T491I
082	36	1137	7.4	1.4	None	None	D121A, N126S, T186M, T190I, S310T, T371Q, F463L, T491I
082	48[a]	3815	7.6	1.7	ND	None	D121A, N126S, T186M, T190I, S310T, T371Q, F463L, T491I
243	24	23933	3.8	1.2	ND	None	K113Q, N126S, T186M, T190I, S310T, T371Q, T469I
243	36	4376	4.0	1.4	None	None	K113Q, D121G, N126S, T186M, T190I, S310T, T371Q, T469I, P478Q
274	36	4005	8.5	6.4	None	T375A	K113Q, T186M, T190I, S310T, T371Q, T469I,
275	24	13381	5.6	1.3	ND	T375N	I94V, K113Q, N126S, T186M, T190I, S310T, T469I, P478Q
275	36	30762	5.8	1.5	None	K436R	I94V, K113Q, N126S, T186M, T190I, S310T, T469I, P478Q
303	12	1758	7.7	3.7	ND	None	N126S, T186M, S310T, T469I
315	12	1156	13.7	9.9	ND	None	D121G, T186M, T190I, S310T, T371Q

[a]Patient re-introduced tenofovir DF/lamivudine at study week 36 due to virological failure. Abbreviations: ART= antiretroviral therapy; PI=protease inhibitor; RAMs= resistance-associated mutations; ND= not done; CSM= cleavage site mutations.

4.4 DISCUSSION

Among patients who switched from suppressive second-line ART with 2 NRTIs + PI/r to DRV/r monotherapy, 19.8% experienced virological failure over 48 weeks. Detection of HIV-1 DNA RAMs at study entry and higher VAS-measured adherence during the study independently predicted reduced odds of virological failure in this population. Thus, the study provides novel insights about the role of RAMs as predictors of virological outcomes in treatment-experienced populations in sub-Saharan Africa. At study entry, the mutational patterns in HIV-1 DNA were overall reflective of prolonged viraemia on first-line NNRTI-based ART. Mutations selected by lamivudine (M184V) and zidovudine or stavudine (TAMs) were common, as were the nevirapine and efavirenz RAMs K103N and Y181C. Further emergence of NRTI RAMs may have occurred during undocumented periods of viraemia on two NRTIs + PI/r. A previous pooled analysis indicated that detecting RAMs at failure of first-line ART with 2 NRTIs + NNRTI predicted improved responses after starting second-line ART with 2 NRTIs + PI/r[142]. Here, detecting NRTI and NNRTI RAMs in HIV-1 DNA of virologically suppressed patients receiving 2 NRTIs + PI/r predicted a reduced risk of virological failure after switching to DRV/r monotherapy. The effect persisted after considering other proposed predictors of responses to PI/r monotherapy, including adherence, HIV-1 DNA load, and nadir CD4 cell counts[236]. Whereas NRTIs are expected to retain partial antiviral activity despite the presence of RAMs, the findings imply a predictive role for pre-existing NRTI RAMs that is partially independent of residual NRTI activity. RAMs may be a proxy for unknown co-variables. It may also be proposed that patients who develop resistance while receiving ART have relatively higher levels of adherence (hence higher drug selective pressure) than patients who experience failure without resistance and maintain higher adherence levels during subsequent treatment lines. Thus, RAMs may act as an additional, sensitive indicator of overall compliance with treatment in these populations.

Previous studies of PI/r monotherapy in Western Europe reported a low risk of emerging protease RAMs in rebound plasma HIV-1 RNA using Sanger

sequencing[231]. Most previous studies employed conventional (Sanger) sequencing for detecting resistance at virological rebound. A recognised limitation of Sanger sequencing is the inability to detect viral variants occurring at low frequency (~ <15%) in a sample. Two studies applied more sensitive testing techniques. One employed single genome sequencing in five patients who had experienced viral load rebound >400 copies/ml during DRV/r monotherapy: darunavir RAMs [V32I, I47V, I50V] were found in one patient lacking the mutations by Sanger sequencing[237]. A second study undertook UDS in 14 patients who had experienced viral load rebound >1000 copies/ml during DRV/r monotherapy: one patient lacking protease RAMs by Sanger sequencing showed I54T, which is not a recognised darunavir RAM[238]. Our study extends these data by undertaking UDS in 21 patients with viral load >60 copies/ml during DRV/r monotherapy. No darunavir RAMs were seen to emerge in the 21 patients. Rather, the resistance profiles in rebound HIV-1 RNA were largely, albeit not fully, reflective of those detected in baseline HIV-1 DNA.

In a subset of patients, additional RT RAMs were found in rebound plasma that had not been seen in HIV-1 DNA. In some cases, the mutations occurred at very low frequency in the patient's sample, casting doubt as to the significance. It should be noted that Sanger sequencing was used to test for RAMs in PBMC and UDS might have increased detection of RAMs in these samples[239]. Conversely, a subset of patients showed additional RT RAMs in baseline PBMC, but three of these had evidence of defective provirus, which is not expected to sustain virus production; although, a recent study has shown defective provirus is capable of transcribing transcripts with replication competent open reading frame which can be detected in peripheral blood cells[240]. Previous studies showed that in patients on ART a large fraction of proviral DNA is defective due to the presence of hypermutation, deletions, and other defects[148,234]. Conversely, little or no hypermutation has been observed in plasma HIV-1 RNA[241]. Two patients had the protease RAM D30N in rebound HIV-1 RNA but not in baseline HIV-1 DNA. D30N is a non-polymorphic substrate-cleft mutation that is selected by and causes high-level resistance to nelfinavir[242]. The aspartate-to-asparagine substitution alters the

hydrogen bond interaction with the aniline NH2 group of darunavir, resulting in some loss of binding affinity[243]. Arguing against selection by DRV/r, D30N was also detected in baseline HIV-1 DNA of three patients prior to exposure to darunavir. Patients with D30N had been exposed to LPV/r for up to 4.6 years and had no recorded exposure to any other PI/r including nelfinavir. There is no evidence that LPV/r would select for D30N[242], leaving the possibility that the mutation was acquired at the time of transmission. The phylogenetic analysis did not show linkage between the five patients, which would suggest multiple sources of infection. HIV-1 subtypes showed no specific association and comprised CRF02_AG in two patients and subtypes A1, G and CRF01_AE in one patient each.

PCR-induced error or viral hypermutation may provide additional explanations for the detection of D30N. Overall, six patients (10%) showed A3G hypermutation in the HIV-1 DNA amplicon. The A3 cytidine deaminases contribute to broad innate immunity by inducing extensive guanidine to adenosine (G to A) mutagenesis in viral progeny[244], which can prevent the formation of functional proviruses[245]. Substitutions that can reflect the effect of hypermutation include D30N in PR[234] and E138K, M184I, G190E, and M230I in RT[148,232,233]. Indeed, we found evidence of hypermutation in two patients with D30N and three patients with a RT mutation; E138K (one patient), M184I (three patients), M230I (three subjects). In addition to hypermutation and in context with the observation of improved virological response during DRV/r monotherapy, it could be further asserted that the high prevalence of RAMs in peripheral cells reduces viral fitness and partially improves virological outcome during DRV/r monotherapy by diminishing virus replication capacity.

Although previous reports have shown low risk of the emergence of PR RAMs during PI/r treatment, PIs can select for mutations in gag, the enzyme natural substrate, which may precede the emergence of mutations in PR[246] and has been proposed to play a role in virological failure[148,241,246]. These mutations can reduce the binding affinity of PIs to the gag-binding cleft and can also improve gag processivity and viral fitness[247–249]. The most common CS

mutation in our study occurred at position 375 of p2/p7. Only in one patient we were able to observe CS evolution during failure of DRV/r monotherapy, whereby the T375N mutation detected at week 24 was replaced by K436R in p7/p1at week 36. There was limited evidence of evolution at gag non-CS sites. T375A/N has been previously observed in both ART-naïve patients and ART-experienced patients from Cameroon[248] and Nigeria[250], and together with the K436R has been implicated in failure of PI-based ART[251]. In a previous report introducing the T375A mutation by site directed mutagenesis in to a wild-type reference strain, replication competence of the wild-type reference strain increased from 100 to 146% indicating improved viral fitness in the presence of this mutation[252]. In our study, all patients with gag CS and non-CS mutations regained virological suppression after returning to triple PI/r-based ART, arguing against a profound resistance effect.

There are limitations to this study. As indicated, due to the need to compare results obtained in the United Kingdom with those generated on site in Cameroon, we used Sanger sequencing for detecting RAMs in HIV-1 DNA. This may have reduced the ability to detect rarer mutations, although any increase in yield would ultimately depend on the size of HIV-1 DNA input, which is often small in ART-suppressed patients. Also, due to the lack of samples, we were not able to perform gag sequencing at study entry or to extend the number of patients undergoing gag sequencing.

Overall, this chapter provides novel insights indicating that detection of NRTI and NNRTI RAMs may act as an additional, sensitive indicator of overall compliance with treatment in populations in sub-Saharan Africa. Further, this study also indicates that the patterns of RAMs archived in HIV-1 DNA during virological suppression are relatively consistent with those that emerge at virological rebound, with discrepancies often reflecting the presence of defective provirus. Finally, the findings confirm that whilst viraemia is common during DRV/r monotherapy, there is no emergence of PR RAMS even by highly sensitive testing. Taking into account the small number of observations, any emergence of gag substitutions does not appear to impact the ability to

regain virological suppression once NRTIs are re-introduced alongside the PI/r.

CHAPTER FIVE: IMPACT OF ANTIRETROVIRAL TREATMENT SIMPLIFICATION ON THE IMMUNE ACTIVATION MARKER SOLUBLE CD27

The work in this chapter addresses the research hypothesis that simplifying treatment within the MANET trial may have resulted in increasing levels of immune activation. Our research group previously published on the relationship between the activation marker soluble CD27 (sCD27) and the detection of residual plasma HIV-1 RNA levels during suppressive antiretroviral therapy (ART). I therefore set up experiments to measure circulating sCD27 in stored samples collected from MANET participants and placed the results in context by also testing stored samples from control populations. I performed the experimental work, analysed as well as interpreted the data.

5.1 INTRODUCTION

Left untreated, HIV-1 infection causes chronic immune activation and progressive damage to the immune system. ART effectively suppresses viral replication and restores immune function, reducing immune-activation and inflammation[253,254]. Despite the immune restorative effects of ART, evidence has shown that recovery of immune function may be incomplete, especially in patients initiating ART in the late stages of disease[255–257].

In September 2015, the World Health Organisation (WHO) recommended that all HIV-1 positive adults should start ART at the time of diagnosis regardless of the CD4 cell count and clinical stage[258]. Prior to this, recommendations indicated that ART should be initiated when the CD4 count declined below 350 cells/mm^3 [259], a threshold later increased to 500 cells/mm^3 [260]. When such thresholds were still being applied, patients typically experienced years of uncontrolled viral replication before commencing ART. The effects of this long-term exposure to chronic immune activation and inflammation are believed to

be long-lasting[261]. Even with early initiation of ART, immune function may not be fully restored, suggesting that some of the persistent, negative effects of uncontrolled HIV replication may be established in the very early stages of HIV infection[262–264]. Furthermore, it is important to highlight that for many patients, a HIV diagnosis is not achieved until the CD4 cell count has already declined below 350 cells/mm^3 [265], precluding early ART initiation.

In a previous study from our research group, HIV-1 positive subjects on stable first-line ART with a fully suppressed viral load (as determined by routine assays) underwent testing for residual plasma HIV-1 RNA using a research-only ultrasensitive assay. Overall, 52/104 (50%) patients showed evidence of residual viraemia. In adjusted analyses, sCD27 levels increased by 1 $\log_{10}$ U/mL for each 0.4 $\log_{10}$ copies/ml increase in HIV-1 RNA. In contrast, no association was found between residual HIV-1 RNA and other inflammatory markers including soluble CD14 and CD30[193].

CD27 is a type-II transmembrane glycoprotein and a member of the 29 molecule Tumor Necrosis Factor (TNF) receptor super-family[266], a group of molecules implicated in lymphocyte survival, differentiation and memory cell formation[266–268]. CD27 is expressed on the surface of B-cells, T-cells, natural killer (NK) cells and hematopoietic progenitor cells[193,267,269]. Its function is to direct the activity of these cells by engaging CD70[270–272]. Cell surface CD27 is also cleaved proteolytically and released as a soluble form[273]. Previous reports have found elevated levels of sCD27 in subjects with dengue fever[274] and lymphatic filariasis[275].

A previous small sized cross-sectional study suggested that people receiving maintenance monotherapy with a boosted protease inhibitor (PI/b) had higher levels of markers of activation and inflammation relative to people on standard ART[276]. Based on this preliminary report, in this chapter, I measured the levels of sCD27 in the MANET population at study entry (whilst on triple ART) and four weeks after the switch to darunavir/ritonavir (DRV/r) monotherapy. Control populations comprised healthy volunteers, untreated and treated

people with HIV-1 infection, and HIV-negative people with chronic hepatitis C virus (HCV) infection.

5.2 METHODS

5.2.1 Study subjects and cohorts

The study comprised MANET study participants, control patient populations whose samples were collected within the ERAS, PROGRESS, EVOCK, HEPIK and OPTIMISE studies, and healthy volunteers recruited within the University of Liverpool. All studies had ethics committee's approval as described in Chapter 2.

The populations represented the following profiles (Table 5.1):

i) MANET study participants sampled at study entry (baseline) and four weeks after treatment simplification to DRV/r monotherapy (chosen due to sample availability)
ii) HIV-1 positive, ART-naïve
iii) HIV-1 positive, virologically suppressed on first-line ART with 2 NRTIs + NNRTI
iv) HIV-1 positive, with plasma HIV-1 RNA load > 60 copies/ml after at least 6 months of triple ART
v) HIV-negative and HCV antibody and RNA positive, untreated
vi) Healthy volunteers

5.2.2 Measurement of soluble CD27

Soluble CD27 was measured using serum (MANET) or plasma (other studies) using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (sCD27, eBiosciences), according to the manufacturer's instructions[277]. The reported detection limit of the sCD27 assay is 0.2 U/mL. Each assay run comprised seven dilutions of a positive control in duplicate to provide the quantification curve; in addition, each run comprised two negative controls (blank wells). All patient samples had been stored in 2mL aliquots at -80^{o}C

and were tested in duplicate. Across each assay, only duplicate values with a coefficient of variation <15% were accepted. Assay results not meeting this requirement were repeated. Raw data from the sCD27 ELISA runs were analysed using the electronic algorithm provided by the manufacturer.

5.2.3 Statistical analyses

The characteristics of the study populations summarised as either categorical or continuous variables were reported as proportions with percentages and medians with interquartile range (IQR), respectively. The characteristics of the study populations were compared by ANOVA with Bonferroni correction. The median levels of sCD27 in different study populations were compared using the non-parametric Mann-Whitney U test. The characteristics of MANET participants showing an increase of >15 U/mL in sCD27 between baseline and study week 4 were compared with the remaining MANET participants using Mann-Whitney U test for continuous variables and chi-squared test for categorical variables. Factors associated with the log-transformed levels of sCD27 in the HIV-1 positive population were analysed using linear regression analysis. Variables showing an association in the univariable analysis ($p < 0.2$) were retained in the adjusted model. Additional analyses were performed to evaluate the effect of HCV infection; this involved i) comparison of expression level of sCD27 in the HCV mono-infected and HIV/HCV co-infected group by Mann-Whitney U test ii) exploring the effect of HCV RNA on sCD27 expression by univariable analysis using linear regression analysis.

5.3 RESULTS

5.3.1 Study populations

The study comprised a total of 546 participants (Table 5.1), including 69 MANET patients with samples available at both study entry (baseline) and week 4 after randomisation to DRV/r monotherapy. When comparing the demographic characteristics of the study populations, the prevalence of women varied with borderline significance ($p=0.072$), from 78.3% in MANET

to 16.8% in HIV-1 positive ART-naïve patients. Median age was comparable between patient groups, whereas healthy volunteers were younger (p=0.005).

Table 5.1: Characteristics of the study populations

		HIV-1 positive				HCV mono-infected	Healthy volunteers
		MANET (Baseline)	ART-Naïve	On ART, suppressed	On ART, viraemic		
Total number		69	89	115	138	90	45
Age, median years (IQR)		43 (38,51)	45 (40, 51)	48 (41, 52)	48 (42, 52)	43 (35,52)	31 (39, 52)
Females, n (%)		54 (78.3)	15 (16.8)	97 (84.3)	49 (35.5)	40 (44.4)	23 (51.1)
CD4 count, median cells/mm^3 (IQR)		423 (327, 604)	519 (436, 629)	390 (220, 629)	546 (382, 702)	n/a	n/a
Nadir CD4 count, median cells/mm^3 (IQR)		90 (36, 167)	450 (364, 562)	169 (70, 256)	194 (125, 296)	n/a	n/a
HIV-1 RNA, median log$_{10}$ copies/ml (IQR)		<60 copies/ml	4.2 (3.8, 4.7)	3.5 (2.2, 4.9)	<60 copies/ml	n/a	n/a
Ethnicity, n(%)	Black	69 (100)	5 (5.7)	40 (34.8)	138 (100)	0 (0)	8 (17.8)
	White	-	84 (93.3)	75 (65.2)	0 (0)	90 (100)	37 (82.2)
ART regimen, n (%)[a]	NNRTI-based	0 (0)	n/a	59 (51.3)	123 (89.1)	n/a	n/a
	PI/r-based	69 (100)	n/a	0 (0)	15 (10.9)	n/a	n/a
	NOR	-	-	56 (48.7)	n/a	n/a	n/a
Time on ART, median years (IQR)		8.1 (5.3, 9.8)	n/a	8.8 (5.4, 11.1)	6.3 (3.5, 9.6)	n/a	n/a
Time since diagnosis, median years (IQR)		9.0 (6.4, 11.3)	1.7 (0.75, 3.25)	9.1 (6.3, 11.6)	6.7 (3.7, 9.9)	n/a	n/a
sCD27 level, median U/mL (IQR)		139.8 (103.7, 177.5)	168.0 (144.6, 232.0)	163.7(123.8, 209.3)	139.2 (99.5, 208.3)	150.3 (116.3, 200.7)	85.5 (67.1, 105.2)

[a]ART regimen not documented for all patients. Abbreviations: ART=Antiretroviral therapy; HCV= hepatitis C virus; sCD27=soluble CD27; n/a= not applicable; NOR=not available on record.

5.3.2 sCD27 levels after treatment simplification in MANET

sCD27 levels measured at baseline were compared with those measured four weeks after treatment simplification to DRV/r monotherapy in 69 patients. Median levels increased overall after simplification and were 139.9 U/mL (IQR) at baseline versus 160.9 U/mL (IQR) at week 4 (p=0.010) (Figure 5.1). There was heterogeneity among individual patients, with levels that tended to increase by ≥15 U/mL in 35/69 (50.7%) patients, decrease in 21 (30.4%), and remain stable in 13 (18.8%) (Figure 5.2). The characteristics of the 35 MANET patients showing an increase in sCD27 levels are summarised in Table 5.2. When comparing this population with the rest of MANET patients, time on ART was significantly longer.

Table 5.2: Characteristic of patients from the MANET trial, stratified according to the change in sCD27 levels observed between study entry and week 4.

Characteristic	sCD27 levels		p-value
	Increased	Not increased	
Total number	35	34	-
Age, median years (IQR)	46.1 (37.9, 51.9)	42.1 (37.9, 51.9)	0.171
Females, n (%)	27 (77.1)	25 (73.4)	0.785
CD4 count, median cells/mm^3	451 (327, 566)	407 (328, 687)	0.084
Nadir CD4 count, median cells/mm^3 (IQR)	127 (56, 178)	69 (36, 129)	0.083
Time on ART, median years (IQR)	9.1 (6.2, 10.5)	6.4 (4.1, 8.8)	0.014
Time on PI/r, median years (IQR)	4.0 (1.8, 6.2)	2.5 (1.2, 5.0)	0.183
Time since diagnosis, median years (IQR)	9.4 (8.2, 11.4)	7.3 (5.3, 10.4)	0.078
Baseline sCD27, median U/mL (IQR)	107.4 (156.5, 237.7)	175.7 (156.5, 237.7)	<0.001

Abbreviations: ART=antiretroviral therapy; PI/r=ritonavir-boosted protease inhibitor.

5.3.3 sCD27 levels across study populations

All patient populations showed higher sCD27 levels than healthy volunteers (p<0.001) (Figure 5.1). These included people with HIV-1 infection and also people with HCV mono-infection. Within the HIV-1 positive populations, the highest levels of sCD27 were observed in ART-naïve patients. At baseline, MANET patients had lower sCD27 levels than ART-naïve patients (p=0.010); however, once levels increased at week 4, the difference was no longer significant.

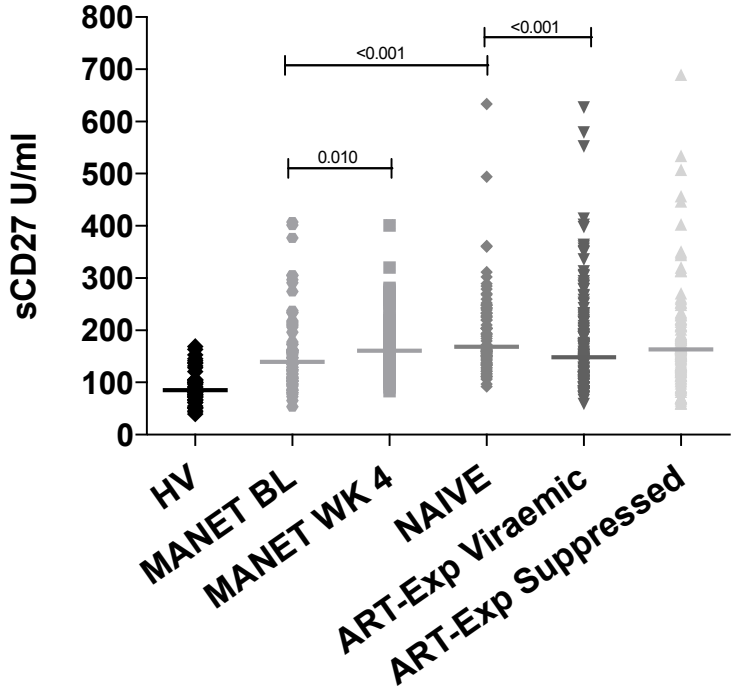


Figure 5.1 Levels of sCD27 across study populations. The middle line indicates medians. Differences in median levels of sCD27 were compared by Mann-U Whitney test. Abbreviations: HV=healthy volunteers; MANET BL= MANET baseline; MANET WK 4= MANET week 4; ART-Exp Suppressed= ART-experienced with suppressed viral load; ART-Exp viraemic= ART- experienced with detectable viral load.

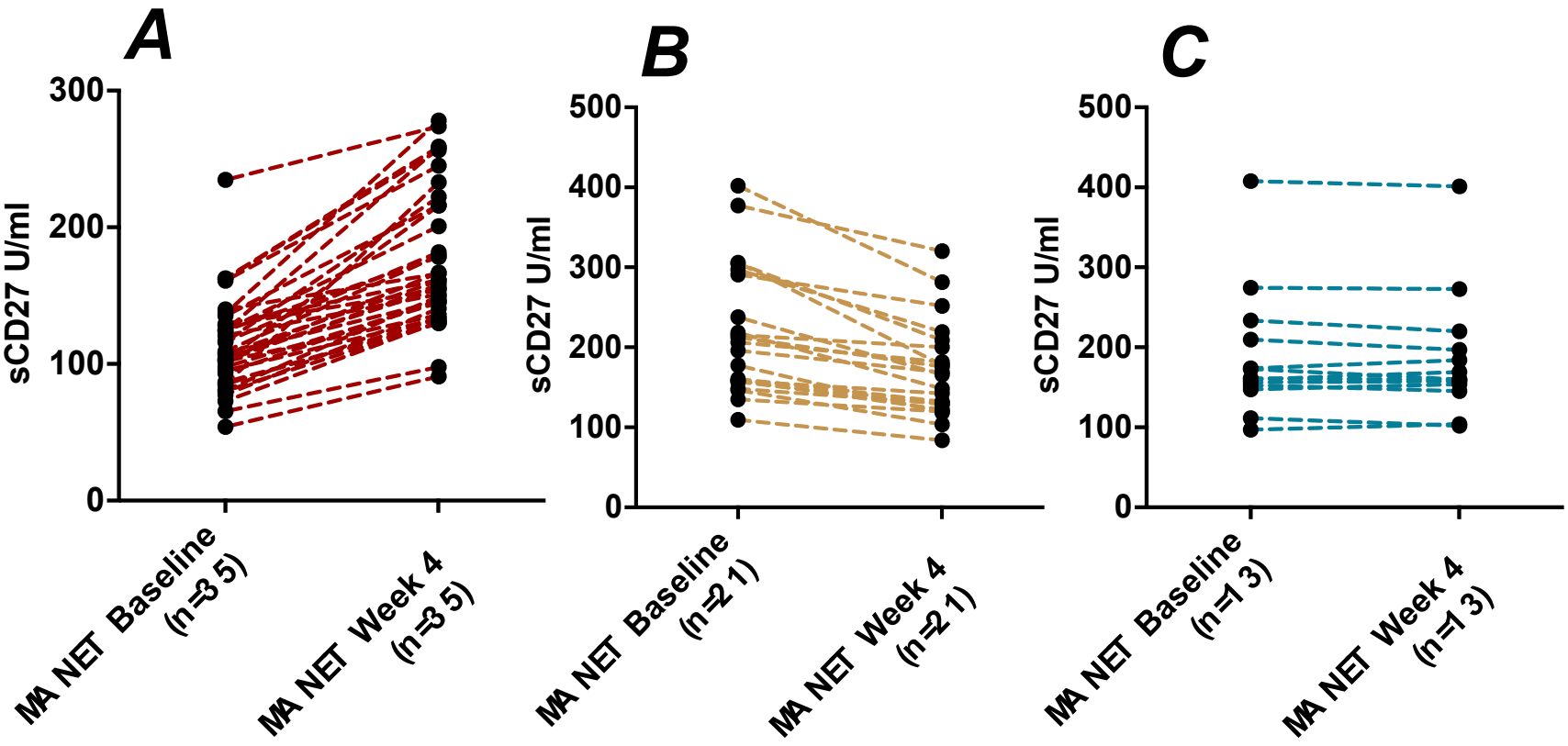


Figure 5.2: Changes in levels of sCD27 in the MANET population sampled at baseline and week 4 after simplification to darunavir/ritonavir monotherapy. In group A and group B, sCD27 levels differed by an arbitrary cut-off of ≥15 U/mL between the two time points. Median expression levels of sCD27 before and after DRV/r was 107.4 vs 160.9 IU/ml for group A; 206.6 vs 166.1 IU/ml for group B and 161.5 vs 160.0 for group C.

5.3.4 Factors associated with sCD27 levels in HIV-1 positive patients

In the univariate linear regression analysis (Table 5.3), factors associated with higher levels of sCD27 comprised male gender, older age, white ethnicity, ART-naïve status and higher viral load. Higher CD4 cell counts and longer time on ART were associated with lower sCD27 levels. Following adjustment in the multivariable model, the viral load remained the strongest predictor of sCD27 levels associated with higher levels of sCD27, with a more marginal effect of CD4 cell counts.

Table 5.3: Factors associated with log-transformed levels of sCD27 in HIV-1 positive patients (n=411)

Characteristics		Univariable analysis			Multivariable analysis		
		Coefficient log_{10} U/mL	95% CI	P-value	Adjusted Coefficient log_{10} U/mL	95% CI	P-value
Gender	Male versus female	+0.12	+0.04, +0.20	**0.004**	+0.01	-0.09, +0.11	0.870
Age	Per 5 years older	+0.32	+0.01, +0.05	**0.005**	+0.02	-0.00, +0.04	0.115
Ethnicity	White versus black	+0.22	+0.11, 0.33	**<0.001**	+0.20	-0.00, +0.40	**0.050**
Time since diagnosis	Per year increase	-0.00	-0.13, +0.01	0.939	ND	ND	ND
CD4 count	Per 50 cell/mm^3 increase	-0.01	-0.02, -0.00	**0.003**	-0.01	-0.02, -0.00	**0.016**
Nadir CD4 count	Per 50 cell/mm^3 increase	+0.01	-0.01, +0.02	0.337	ND	ND	ND
HIV-1 RNA	Per log_{10} copies/ml increase	+4.13	+2.78, +5.49	**<0.001**	+4.09	+2.32, +5.86	**<0.001**
ART status[a]	Naive versus Treated	+0.14	+0.04, +0.24	**0.006**	ND	ND	ND
Time on ART	Per year increase	-0.01	-0.02, +0.00	**0.008**	-0.00	-0.02, +0.01	0.800
ART regimen	NNRTI- versus PI-based	-0.03	-0.12, +0.06	0.521	ND	ND	ND

[a] Excluded from the multivariable analysis due to co-linearity with time on ART. Abbreviations: ART=antiretroviral therapy; ND=not done; NNRTI= non-nucleoside reverse transcription inhibitor; PI=protease inhibitor.

5.3.5 sCD27 levels by HCV RNA status

Median levels of sCD27 were 150.3 U/mL among people with HCV mono-infection, and higher than levels measured in healthy volunteers (p=<0.001). When comparing characteristic between the HCV population stratified by HIV status, there was a significant difference between age (Table 5.4). Expression level of sCD27 was also higher in HIV patients who were also positive for HCV RNA (n=56), in comparison to the HCV mono-infected group (p=0.003). In a separate univariable analyses, HCV RNA positivity was non-significantly associated with higher sCD27 levels (coefficient +0.06; 95% CI -0.02, +0.15; *p*=0.123).

Table 5.4: Characteristic of HCV positive subjects stratified by HIV status

Characteristic			
	HIV/HCV co-infected	HCV mono-infected	p-value
Total number	56	90	-
Age, median years (IQR)	49.0 (46.5, 52.5)	42.5 (35.0, 52.0)	<0.001
Females, n (%)	19 (33.9)	40 (44.4)	0.229
Baseline sCD27, median U/mL (IQR)	180.0 (149.1, 231.7)	150.3 (116.9, 200.4)	0.003

5.4 DISCUSSION

This study prospectively investigated the effect of switching from triple ART with 2 NRTIs + PI/r to maintenance DRV/r monotherapy on the levels of the marker of immune activation sCD27. The findings were placed in the context of a cross-sectional analysis of HIV-1 positive populations with different treatment status, HCV mono-infected patients, and healthy volunteers. Overall, median levels of sCD27 increased after treatment simplification with DRV/r. The effect was driven by a subset of 35 patients that showed ≥15 U/mL increase in sCD27 levels between the two time points. These patients had been established on ART for a significantly longer time than the other MANET patients.

These observations indicate that in at least a subset of patients, switching to DRV/r monotherapy can trigger sCD27 release. As viral load was the strongest predictor of sCD27 levels across the control HIV-1 positive populations we tested, the finding may indicate virus replication in this group. The conclusion is also supported by previous observations from our research group that sCD27 was associated with the detection of residual plasma HIV-1 RNA. In that study, patients had received up to 10 years of suppressive first-line ART with 2 NRTIs + NNRTI without experiencing any detected episode of viral load rebound[193]. Approximately half had detectable HIV-1 RNA (up to 11 copies/mL) and there was a linear correlation between residual viraemia and levels of sCD27.

In a previous cross-sectional study, the markers of immune activation sCD14, sCD163 and IL-6 showed higher levels in 40 patients receiving PI/b monotherapy compared with 20 subjects established on triple ART[276]. However, a cross-sectional study of patients treated for ≥1 year with DRV/r or LPV/r either as monotherapy or with 2 NRTIs found no difference in levels of CRP, IL-6, fibrinogen and D-dimer[278]. A longitudinal study also found that intensification of PI/b monotherapy with raltegravir over 48 weeks reduced levels of residual viraemia but did not affect the levels of the inflammatory markers sCD14, IP-10, IL-6, CRP and D-dimer. Along the same lines, among

HIV-1 positive patients receiving DRV/r monotherapy in the MONET trial, after three years of treatment, levels of the inflammatory markers IL-6 and CRP did not differ from those of the control populations receiving 2 NRTIs + DRV/r[279]. In the MONET study, HCV co-infection was associated with significantly higher IL-6 levels. This is consistent with the known effect of HCV on systemic inflammation[280]. It also noteworthy that we also observed that HCV infection was characterised by increased levels of sCD27. Further to this and in contrast to our observation in this study, a longitudinal study evaluating the role of HCV co-infection in a total of 56 HIV infected subjects on DRV/r monotherapy reported no difference in the expression level of sCD14 that was observed over five timepoints during 24 months of follow-up[281].

ART is expected to drive reconstitution of the immune system and leads to reduction in viral load and increase in the CD4 count which collectively at least drives partial restoration of overall immune function[282]. However, all HIV-1 positive populations in our study showed higher sCD27 levels than healthy volunteers, including the virologically suppressed populations. It will be of interest to determine whether earlier initiation of ART allows an improved control of immune activation, resetting sCD27 levels to those of people without HIV; although, a previous study evaluating CXCL13 as a biomarker of immune activation in HIV patients initiating early ART reported the lack of resetting[283].

One point of note relating to the study population is the heterogenous nature of both the study control and the healthy control population. The study control population, which was selected on the basis of sample availability, included HIV/HCV co-infected patients and ART naïve patient cohorts of different ethnicity and HIV subtypes. Further to this, the role of other co-infection remained unaccounted for. The healthy control population in this study were younger than other groups, and this may have influenced the findings, despite adjustment. The observation of a higher level of expression of sCD27 in relation to other population groups should be interpreted in context. Although data on expression level of sCD27 in HIV-negative

volunteers is limited, published evidence has found similar expression level of scD27 between healthy volunteers and HIV patients suppressed on ART[193]. It would however be expected that ageing is accompanied with immune senescence often related to an inflammatory pathogenesis and the older age observed in the study population could contribute to accelerated progression of HIV disease in HIV-positive ART naïve and treated patients[284] likely been a reflection of diminished immune function.

A further limitation of this study is that we had limited samples from MANET participants, which precluded a number of improvements to the study design. Ideally, we would have i) included all patients across both arms of MANET and over a longer follow-up, ii) performed a viral load measurement at week 4, and iii) measured residual HIV-1 RNA levels at study entry. The data present a research hypothesis – that measuring sCD27 may be a valuable proxy for ongoing virus replication in patients on ART – which warrants further longitudinal studies in patient switching to simplified treatment regimens in other to assess predictive value of sCD27 as a marker for viral load rebound.

CHAPTER SIX: HEPATITIS B VIRUS (HBV) INFECTION AND RE-ACTIVATION DURING NUCLEOS(T)IDE REVERSE TRANSCRIPTASE INHIBITOR-SPARING MAINTAINANCE ANTIRETROVIRAL THERAPY IN THE MANET STUDY

The research hypothesis in the work presented in this chapter was that discontinuation of HBV active drugs in a region of high HBV endemicity such as Cameroon may result in a risk of *de-novo* HBV acquisition or HBV reactivation. Using prospective samples from the MANET trial, I performed quantification and sequencing of HBV DNA. I also organised testing for serological HBV markers at an accredited diagnostic laboratory, analysed the data, and wrote a manuscript that has been published as *Abdullahi A, Fopoussi OM, Torimiro J, Atkins M, Kouanfack C, Geretti AM. Hepatitis B Virus (HBV) infection and re-activation during nucleos(t)ide reverse transcriptase inhibitor-sparing antiretroviral therapy in a high-HBV endemicity setting. Open Forum Infect Dis 2018;5:ofy251.*

6.1 INTRODUCTION

The World Health Organization (WHO) recommends HIV-positive adults in sub-Saharan Africa be treated with two NRTIs in combination with a third agent chosen among a non-nucleoside reverse transcriptase inhibitor (NNRTI), the integrase inhibitor dolutegravir, or a ritonavir-boosted protease inhibitor (PI/r)[158]. Recommended NRTI backbones comprise tenofovir disoproxil fumarate (TDF) or zidovudine (ZDV), together with lamivudine (3TC) or emtricitabine (FTC). TDF, 3TC, and FTC also have antiviral activity against HBV, and treatment guidelines recommend that HIV/HBV co-infected patients receive tenofovir for its potent dual antiviral activity and continue this through their initial and subsequent treatment regimens[158,174]. Outside of sub-Saharan Africa, there is established evidence that TDF, FTC, and 3TC reduce the risk

of HBV reactivation in patients with a resolved HBV infection who receive immune suppressive treatment[285]. In addition, HIV-positive men who have sex with men (MSM) receiving dually active antiretroviral regimens in Japan, Western Europe, and North America showed a reduced risk of HBV acquisition in retrospective cohort analyses[286–289].

Globally, an estimated 257 million people are chronically infected with HBV and an estimated 36.7 million people are living with HIV, with substantial overlap between the two[185,290,291]. Sub-Saharan Africa bears a disproportionate burden with 75 million HBV carriers and 25 million HIV-positive people; while HIV programmes are widely established, policies for HBV remain underdeveloped across most of the region[292]. Universal childhood vaccination programmes are reducing HBV prevalence in some areas, but the impact remains uneven[293,294]. In typical HIV programmatic settings, screening for HBsAg is not implemented systematically and management of HIV-positive patients remains commonly blind to HBV status [292,295–297]. There is also no systematic evaluation of HBV immune status and no systematic adoption of adult catch-up vaccination[298].

The aim of the study was to investigate the evolution of serological markers of HBV infection among patients switching from a programmatic triple ART regimen to a simplified NRTI-sparing regimen with darunavir/ritonavir monotherapy within the MANET trial. Stored samples collected at study entry and at regular follow-up visits over 48 weeks were retrieved and tested retrospectively to investigate *de novo* HBV infection and reactivation.

6.2 METHODS

6.2.1 Study population

Participants were HIV-1 positive adults who took part in the MANET (Monotherapy in Africa, New Evaluations of Treatment) trial (NCT02155101). Patient's eligibility and inclusion criteria are detailed in Chapter 2 and they included HBsAg negativity. A total of 80/81 patients in the monotherapy arm of MANET were included in this sub-analysis based on the availability of

samples stored at regular intervals during the 48 weeks of follow-up. Following randomisation, patients attended scheduled study visits at week 4, 12, 24, 36, and 48, after which they returned to standard of care triple therapy with TDF+3TC and lopinavir/ritonavir (LPV/r). Patients who experienced an adverse event graded as either serious or severe and those with confirmed virological failure (HIV-1 RNA >400 copies/ml) returned to standard of care triple therapy prior to week 48. Serum biochemistry, full blood counts, CD4 cell counts, and plasma HIV-1 RNA load were measured in the diagnostic laboratory of the Centre Pasteur of Cameroon in Yaoundé. The laboratory reference ranges for aspartate transaminase (AST) and alanine transaminase (ALT) were 10-40 IU/L and 8-50 IU/L, respectively.

6.2.2 Laboratory testing

The analysis was performed using serum and plasma samples that had been separated from whole blood within two hours of collection, stored at -80^{o}C at CIRCB, and shipped frozen to the United Kingdom (UK). HBV serological markers were tested at the accredited diagnostic laboratory of Frimley Park Hospital NHS Foundation Trust in the UK. They comprised hepatitis B surface antigen (HBsAg), surface antibody (anti-HBs), total core antibody (anti-HBc), and e-antibody (anti-HBe) and were tested by Architect (Abbott Diagnostics). HBsAg positivity was confirmed by neutralisation as per recommended diagnostic practice[299,300]. Total anti-HBc were confirmed by a second test performed on the Cobas 8000 analyser (Roche Diagnostics). HBV DNA load was quantified at the University of Liverpool using the M2000sp/RealTime assay (Abbott Molecular) with a lower limit of quantification of 10 IU/ml. Samples with HBV DNA >100 IU/ml underwent sequencing of HBV polymerase and surface as described in chapter 2. Following sample extraction and amplification, amplicons were generated using a nested PCR protocol spanning the HBV polymerase region (covering the S and RT genes). After purification and quantification of the amplicons, RAMs were detected by Sanger sequencing on the Applied Biosystems 3730xl genetic analyser. Sequences were aligned with reference genotype A3 and genotype E sequences retrieved from GenBank, edited, and analysed using MEGA 6.0

(Genbank access numbers: MH165306; MH165307). Hepatitis C virus (HCV) RNA was detected with Aptima HCV Quant Dx (Hologic).

6.2.3 Definitions and analysis

All subjects tested negative for HBsAg and HBV DNA at study entry. Their HBV status was classed as non-immune if no other HBV markers were detected; immune if anti-HBs were detected; and past infection if anti-HBc were detected (Figure 6.1). Incident HBV infection was defined as new-onset detection of HBsAg or HBV DNA and total anti-HBc in subjects that tested negative for all HBV markers at study entry[286]. Possible incident HBV infection was defined as new-onset detection of HBsAg or HBV DNA and total anti-HBc in subjects showing anti-HBs as the sole detectable HBV marker at study entry. Among subjects that tested positive for anti-HBc at study entry, HBV reactivation was defined by either new-onset detection of neutralisable HBsAg reactivity, or new-onset detection of HBV DNA in ≥2 separate samples; possible HBV reactivation was defined as new-onset detection of HBV DNA in a single sample. The strength of reactivity of HBV markers was graded to facilitate interpretation (Table 6.1). Standard reporting methods were used to describe the characteristics of the population at study entry, which were expressed as either categorical or continuous variables, and reported as either proportions or medians with interquartile ranges (IQR), respectively. Current and nadir CD4 cell counts of anti-HBc positive patients with or without HBV reactivation were compared by the Wilcoxon-Mann-Whitney test. HBV incidence rates and reactivation rates per 1000-person-years with 95% confidence interval (CI) were calculated by dividing the number of patients with occurrence of incident or possible incident infection and reactivation or possible reactivation respectively, by the total number of person-years of follow-up. Analyses were performed with STATA version 14 (StataCorp).

Table 6.1. HBV markers reactivity keys

HBsAg	
s/c	key
<1	-
1-10	+
10-100	++
>100	+++
>1000	++++
anti-HBc	
s/c	key
<1	-
1-5	+
5-10	++
anti-HBs	
IU/ml	key
<10	-
10-50	+
50-100	++
>100	+++
anti-HBe	
s/c	key
>1	-
<1	+
HBV DNA	
IU/ml	key
<10	-
10-100	+
100-1000	++
1000-10000	+++
10000-100000	++++
>100000	+++++

6.3 RESULTS

6.3.1 Study population

At study entry, the 80 patients switching to maintenance monotherapy had received ART for a median of 7.4 years, including a median of 3.1 years of PI/r-based triple ART (Table 6.2). Most patients were receiving TDF plus 3TC

(70/80, 87.5%), and the predominant PI/r was LPV/r (78/80, 97.5%). The median CD4 count was 466 cells/mm^3, although previous profound immune suppression was evidenced by a median nadir CD4 cell count of 92 cells/mm^3. During follow-up, 10/80 (12.5%) patients returned to standard of care triple ART prior to week 48 due to a severe or serious adverse event or confirmed virological failure.

Table 6.2 Characteristics of the population at study entry, stratified according to the new-onset detection of markers of HBV replication following NRTI discontinuation

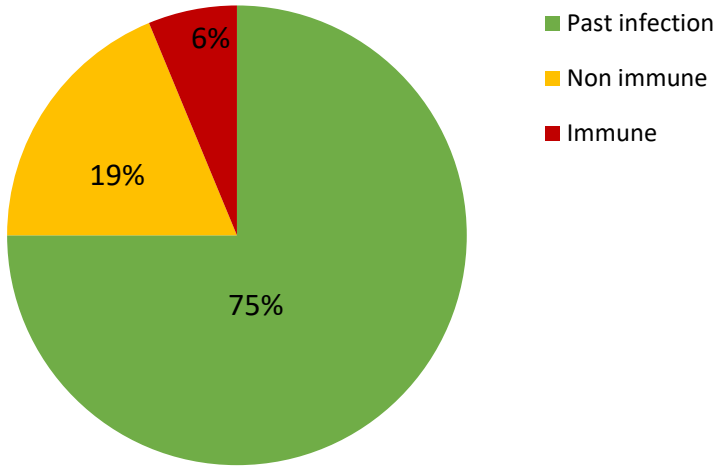
Characteristic		Total	Markers of HBV replication	
			Yes	No
Total number (%)		80 (100)	9 (11.3)	71 (88.7)
Females, n (%)		60 (75.0)	5 (8.3)	55 (91.7)
Age, median years (IQR)		45 (38, 52)	48 (36, 53)	43 (38, 50)
BMI, median kg/m^2 (IQR)		25.5 (21.8, 29.1)	24.6 (23.3, 29.9)	25.7 (21.1, 28.4)
CD4 count, median cells/mm^3 (IQR)		466 (341, 615)	535 (328, 721)	423 (238, 569)
Nadir CD4 count, median cells/mm^3 (IQR)		92 (37, 173)	68 (38, 229)	96 (36, 167)
History of AIDS, n (%)		11 (13.8)	1 (11.1)	10 (14.1)
Haemoglobin, median g/dL (IQR)		12.3 (11.5, 13.2)	12.6 (12.5, 13.8)	12.3 (11.4, 13.1)
Platelets, median cells x10^3 /mm^3 (IQR)		209 (173, 256)	216 (189, 244)	230 (191, 275)
Bilirubin, median mg/L (IQR)		5.3 (3.8, 7.7)	6.0 (4.1, 7.7)	6.6 (4.1 9.0)
Alkaline phosphatase, median U/L (IQR)		95.0 (76.8, 123.3)	144.0 (77.0, 177.0)	116.0 (92, 138)
AST, median U/L (IQR)		23.0 (18.0, 30.0)	27.0 (19.0, 36.0)	29.0 (24.0, 35.0)
ALT, median U/L (IQR)		20.0 (15.0, 28.0)	22.0 (16.0, 32.0)	24.0 (18.0, 33.0)
NRTI backbone, n (%)	TDF + 3TC	70 (87.5)	7 (10.0)	63 (90.0)
	ZDV + 3TC	4 (5.0)	0 (0)	4 (100)
	ABC + TDF	1 (1.3)	0 (0)	1 (100)
	ABC + ddI	5 (6.2)	2 (40.0)	3 (60.0)
ART duration, median years (IQR)		7.4 (5.3, 9.5)	9.1 (7.1, 11.0)	7.3 (5.3, 9.8)
PI/r duration, median years (IQR)		3.1 (1.3, 5.5)	4.0 (2.2, 5.2)	2.6 (1.3, 5.8)
3TC duration, median years (IQR)		6.8 (5.0, 9.3)	8.8 (6.8, 9.3)	6.3 (4.5, 9.1)
TDF duration, median years (IQR)		2.9 (1.5, 4.6)	3.6 (1.6, 4.6)	2.8 (1.2, 4.6)

BMI=Body mass index; AST= Aspartate transaminase; ALT= Alanine transaminases; NRTI= Nucleos(t)ide reverse transcriptase inhibitors; TDF= tenofovir disoproxil fumarate; 3TC=lamivudine; ZDV=zidovudine; ABC= abacavir; ddI= didanosine; ART= antiretroviral therapy; PI/r= ritonavir-boosted protease inhibitor.

6.3.2 HBV status at study entry

All patients were HBsAg and HBV DNA negative at study entry. Overall, 60/80 (75.0%) had evidence of a past HBV infection based on anti-HBc detection (Figure 6.1). Of these, 47/60 (78.3%) also had anti-HBs and/or anti-HBe, whereas 13/60 (21.6%) subjects had isolated anti-HBc. Anti-HBs were detected as the sole HBV marker in 5/60 (8.3%) patients, with median levels of 39 IU/ml (range 12-179). A further 15/80 (18.8%) patients tested negative for all HBV markers.

Figure 6.1: HBV markers among subjects that tested HBsAg and HBV DNA negative at study entry (n=80)



Anti-HBc	Anti-HBs	Anti-HBe	HBV DNA	Interpretation	Total n (%)
Positive	Positive	Positive	Negative	Past infection	25 (31.2)
Positive	Negative	Positive	Negative	Past infection	11 (13.7)
Positive	Positive	Negative	Negative	Past infection	11 (13.7)
Positive	Negative	Negative	Negative	Past infection	13 (16.2)
Negative	Negative	Negative	Negative	Non immune	15 (18.9)
Negative	Positive	Negative	Negative	Immune	5 (6.3)

6.3.3 Evolution of HBV status during follow-up

6.3.3.1 Incident and possible incident HBV infection

Among anti-HBc negative subjects, 3/20 (15.0%) showed profiles indicative or suggestive of incident HBV infection, totalling 163 cases per 1000-person years of follow-up (95% CI 139-190). Among the 15 subjects lacking all HBV makers at study entry, one (6.7%) experienced *de novo* HBV infection, yielding an incidence of 73 cases per 1000 person-years (95% CI 58-91). The patient (CUI-125, Table 6.3) experienced a flu-like illness followed by icterus and grade 4 ALT and AST elevations at week 24. Based on the adverse event being graded as severe, TDF+3TC were reintroduced at week 26, after which the patient returned to standard of care triple ART. The patient was recalled at week 36 and week 105 for post-study follow-up. Retrospectively, the patient was negative for all HBV markers at study entry. Strong HBsAg reactivity and HBV DNA levels of 28,259 IU/ml and 232,569 IU/ml were detected at week 12 and week 24, respectively. HBV DNA sequencing showed genotype E (Figure 6.2), with no resistance mutations in polymerase and no mutations in the major hydrophobic region (MHR, amino acids 99-169) of HBsAg. HBsAg and HBV DNA became undetectable by week 26, and anti-HBc seroconversion was demonstrated at week 105, in the absence of detectable anti-HBs and anti-HBe.

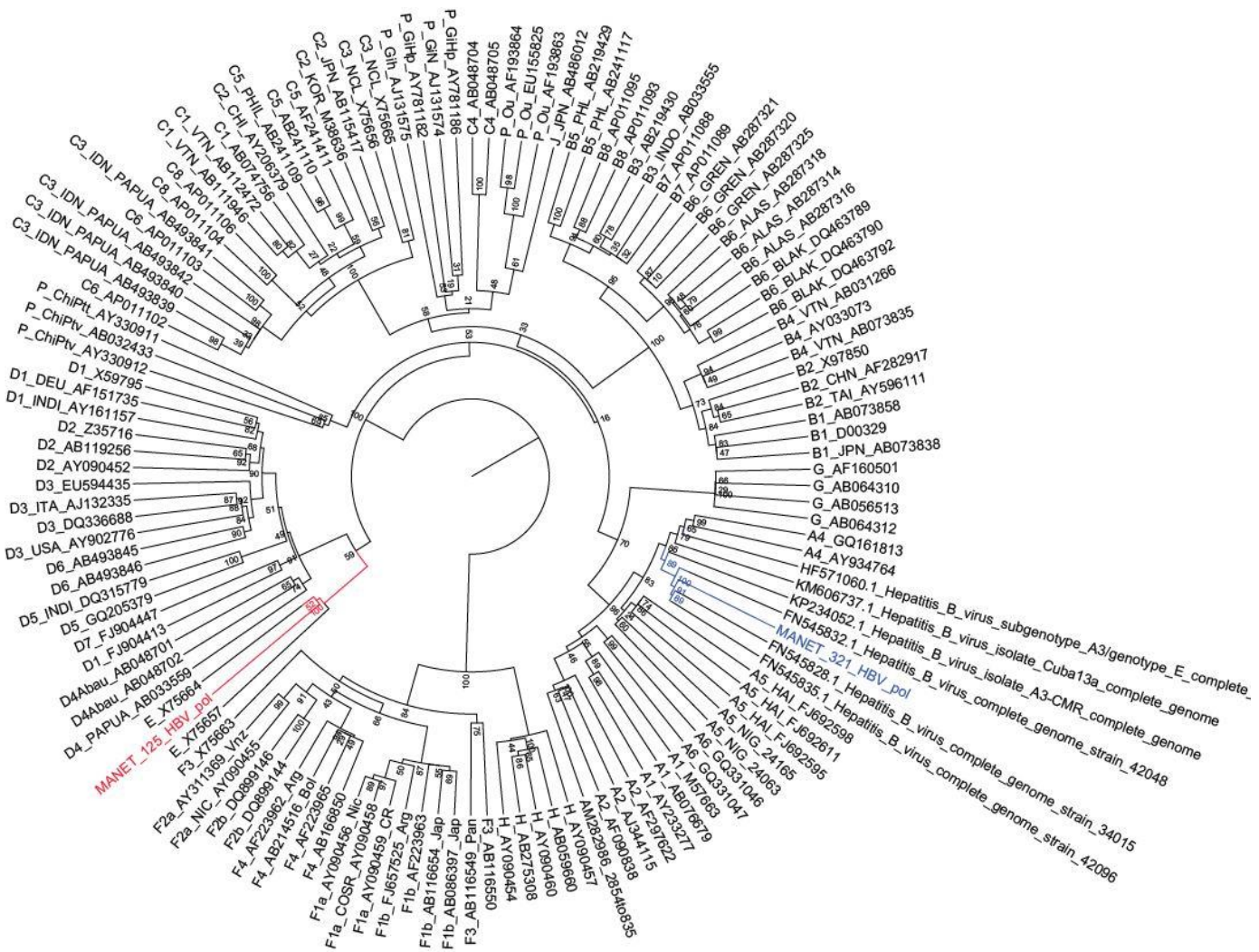


Figure 6.2: Maximum-Likelihood phylogenetic tree showing HBV subtypes

Among the five subjects showing only anti-HBs at study entry, two (40.0%) with anti-HBs levels of 17 IU/ml and 12 IU/ml respectively had evolving HBV markers after treatment simplification, suggesting incident HBV infection (Table 6.3). In patient CUI-156, anti-HBc seroconversion was first detected at week 24, followed by detection of neutralisable HBsAg reactivity at week 36 and week 48. ALT levels increased by up to 2-fold relative to levels measured at study entry. HBV DNA was not detected at multiple sampling points (week 4, 12, 24, and 48) (Table 6.3). In patient CUI-218, HBV DNA was first detected at week 24 (85 IU/ml) and again at week 36 (20 IU/ml). Seroconversion for anti-HBc and anti-HBe was detected at week 36, accompanied by a rise in anti-HBs levels from 12 IU/ml at study entry to 197 IU/ml at week 36. In this patient, no HBsAg was detected at multiple sampling points (week 12, 24, and 26) (Table 6.3). A grade 1 elevation in ALT and AST levels was observed at week 48, with the ALT increasing by >5-fold relative to study entry; HBV markers were not measured at this time due to the unavailability of stored samples. HCV RNA was not detected in this group.

Table 6.3. HBV markers among subjects with incident (patient CUI-125) or possible incident (patients CUI-156 and CUI-218) HBV infection[a]

Marker	CUI-125 (female, aged 29 years)[b] Week							CUI-156 (male, aged 54 years) Week						CUI-218 (female, aged 65 years) Week					
	0	4	12	24	26	36	105	0	4	12	24	36	48	0	4	12	24	36	48
HBsAg	-	-	+++	++++	-	-	-	-	nd	nd	-	++	+	-	nd	-	-	-	nd
anti-HBs	-	nd	nd	nd	nd	nd	-	+	nd	nd	++	nd	+	+	nd	+	nd	+++	nd
anti-HBc	-	nd	-	nd	nd	nd	+	-	-	-	+	nd	+	-	nd	-	-	++	nd
anti-HBe	-	nd	-	-	nd	nd	-	-	nd	nd	-	nd	-	-	nd	-	nd	+	nd
HBV DNA	-	-	++++	+++++	nd	nd	-	-	-	-	-	nd	-	-	nd	-	+	+	nd
AST U/L	19	23	19	661	nd	nd	nd	27	30	41	42	37	31	36	21	21	23	19	102
ALT U/L	28	12	20	824	nd	nd	nd	27	21	35	59	39	44	20	16	18	19	16	109
HIV-1 RNA[c]	UD	nd	UD	UD	nd	nd	nd	UD	nd	UD	UD	UD	UD	UD	nd	UD	105	UD	nd
CD4 count[d]	340	nd	nd	426	nd	nd	nd	338	nd	nd	268	nd	337	535	nd	nd	667	nd	674
NRTIs	TDF 3TC	None	None	None	TDF 3TC	TDF 3TC	TDF 3TC	TDF 3TC	None	None	None	None	None	TDF 3TC	None	None	None	None	None

[a]After study entry, study visits were planned at week 4, 12, 24, 36, and 48; given the retrospective nature of the analysis, stored samples for HBV testing were not available from all study visits; missing time points are indicated as not done (nd). [b]Patient reintroduced TDF+3TC and discontinued the study at week 26 due to an adverse event (acute hepatitis); the patient was recalled at week 36 and week 105 for follow-up. [c]Plasma HIV-1 RNA load in copies/ml; [d]CD4 count in cells/mm^3. HBsAg= Hepatitis B surface antigen; anti-HBs= Hepatitis B surface antibody; anti-HBc= Total hepatitis B core antibody; anti-HBe= Hepatitis B e antibody; AST= Aspartate transaminase; ALT= Alanine transaminases; UD= undetectable (<60 copies/ml); NRTIs=Nucleos(t)ide reverse transcriptase inhibitors; TDF= Tenofovir disoproxil fumarate; 3TC= Lamivudine

6.3.3.2 HBV reactivation and possible HBV reactivation

Among anti-HBc positive subjects, 6/60 (10.0%) showed profiles indicative or suggestive of HBV reactivation, totalling 109 cases per 1000 person-years (95% CI, 90-131). Median CD4 counts at study entry were 508 cells/mm^3 (IQR 274-637) vs. 420 cells/mm^3 (IQR 330-568) in anti-HBc-positive subjects with and without HBV reactivation, respectively (p=0.79); in the same population, median nadir CD4 counts were 59 cells/mm^3 (IQR 29-108) vs. 92 cells/mm^3 (IQR 37-167), respectively (p=0.31). At study entry, patients CUI-030, CUI-143, CUI-238, and CUI-321 had anti-HBs levels ranging between 14 and 191 IU/ml, whereas patients CUI-052 and CUI-213 had no detectable anti-HBs. Profiles indicative of HBV reactivation were observed in three subjects (CUI-030, CUI-052, and CUI-143) (Table 6.4). Patient CUI-030 showed detectable HBV DNA at week 12 (qualitative detection <15 IU/ml), week 24 (49 IU/ml), and week 48 (83 IU/ml); neutralisable HBsAg reactivity was detected at week 48, accompanied by increased anti-HBc reactivity. Weak anti-HBe reactivity was transiently detected at week 24, which was confirmed by repeat testing of the same sample. Anti-HBs were 191 IU/ml at study entry and 179 IU/ml at week 36. Transaminase levels were not increased at any study visit. Patient CUI-052 only had two sampling points available for testing of HBV markers, at week 24 and week 36. HBV DNA was detected at week 24 (84 IU/ml) and week 36 (247 IU/ml). At week 36, HBsAg was not detected although ALT levels showed a marginal increase (~2-fold) relative to levels measured at study entry. Patient CUI-143 showed neutralisable HBsAg reactivity at week 36, following a negative HBsAg test at week 12 and week 24. HBV DNA remained below detection limits at all available sampling points (week 4, 12, 24, and 36). Anti-HBs levels were 169 IU/ml at study entry and 211 IU/ml at weeks 12, but declined to 69 IU/ml at week 36 coinciding with the detection of HBsAg. The patient experienced a grade 1 increase in transaminase levels at week 12 (<2-fold) preceding the detection of HBsAg.

A possible HBV reactivation was observed in three subjects (CUI-213, CUI-238, and CUI-321) based on the new-onset detection of HBV DNA at a single time point, in the absence of HBsAg detection (Table 6.5). HBV DNA levels

ranged between 20 and 60 IU/ml in this group. Transaminase levels remained within the laboratory reference range, although ALT levels increased by around 2-fold relative to study entry. Patient CUI-238 had anti-HBs levels of 14 IU/ml at study entry and these increased to 49 IU/ml at week 36 when HBV DNA was detected. Patient CUI-321 showed detectable HBV DNA at week 48, coinciding with a marked increase in anti-HBs levels from 21 IU/ml at study entry to >1000 IU/ml at week 48. In this patient, sequence data from week 48 showed genotype A3 with no resistance mutations in polymerase and the MHR mutations Y100C and Y161FY in surface; in addition, arginine (R) was present at position 122 instead of lysine (K), as per the consensus sequence for genotype A3. HCV RNA was not detected in this group

Table 6.4. HBV markers among subjects with HBV reactivation[a]

Markers	CUI-030 (female, aged 31 years) Week						CUI-052 (male, aged 41 years) Week						CUI-143 (female, aged 51 years) Week					
	0	4	12	24	36	48	0	4	12	24	36	48	0	4	12	24	36	48
HBsAg	-	nd	nd	-	-	+	-	nd	nd	nd	-	nd	-	nd	-	-	+	nd
anti-HBs	+++	nd	nd	++	+++	nd	-	nd	nd	nd	nd	nd	+++	nd	+++		++	nd
anti-HBc	+	nd	nd	nd	nd	++	+	nd	nd	+	nd	nd	+	nd	nd	nd	nd	nd
anti-HBe	-	nd	nd	+	-	nd	-	nd	nd	nd	nd	nd	-	nd	nd	-	-	nd
HBV DNA	-	nd	+	++	nd	++	-	nd	nd	+	++	nd	-	-	-	-	-	nd
AST U/L	18	24	12	15	15	14	35	35	26	24	46	30	67	62	92	40	55	26
ALT U/L	22	16	17	11	12	13	25	25	25	28	49	30	61	78	102	38	65	26
HIV-1 RNA[b]	UD	nd	UD	UD	UD	UD	UD	nd	UD	UD	UD	3715	UD	nd	UD	UD	UD	nd
CD4 count[c]	317	nd	nd	360	nd	409	734	nd	nd	660	nd	nd	604	nd	nd	657	nd	600
NRTIs	TDF 3TC	None	None	None	None	None	TDF 3TC	None	None	None	None	None	TDF 3TC	None	None	None	None	None

[a]After study entry, study visits were planned at week 4, 12, 24, 36, and 48; given the retrospective nature of the analysis, stored samples for HBV testing were not available from all study visits; missing time points are indicated as not done (nd). [b]Plasma HIV-1 RNA load in copies/ml. [c]CD4 count in cells/mm^3. HBsAg= Hepatitis B surface antigen; anti-HBs= Hepatitis B surface antibody; anti-HBc= Total hepatitis B core antibody; anti-HBe= Hepatitis B e antibody; AST= Aspartate transaminase; ALT= Alanine transaminases; UD= undetectable (<60 copies/ml); NRTIs=Nucleos(t)ide reverse transcriptase inhibitors; TDF= Tenofovir disoproxil fumarate; 3TC= Lamivudine

Table 6.5. HBV markers among subjects with possible HBV reactivation[a]

Markers	CUI-213 (female, aged 51)[b] Week						CUI-238 (female, aged 47) Week						CUI-321 (male, aged 48) Week					
	0	4	12	24	36	48	0	4	12	24	36	48	0	4	12	24	36	48
HBsAg	-	nd	-	nd	nd	nd	-	nd	nd	nd	-	nd	-	nd	nd	nd	nd	-
anti-HBs	-	nd	-	nd	nd	nd	+	nd	nd	nd	+	nd	+	nd	nd	nd	+	++++
anti-HBc	+	nd	nd	nd	nd	nd	++	nd	nd	nd	++	nd	+	nd	nd	nd	+	nd
anti-HBe	+	nd	nd	nd	nd	nd	+	nd	nd	nd	nd	nd	-	nd	nd	nd	nd	nd
HBV DNA	-	-	+	nd	nd	nd	-	nd	-	nd	+	nd	-	nd	-	nd	-	+
AST U/L	19	12	10	23	15	15	33	24	26	31	28	24	14	12	14	16	19	21
ALT U/L	15	13	17	16	14	30	17	14	35	33	10	26	8	11	11	23	17	21
HIV-1 RNA[c]	UD	nd	UD	UD	UD	UD	UD	nd	UD	UD	496	1098	UD	nd	UD	UD	UD	UD
CD4 count[d]	566	nd	nd	728	nd	680	449	nd	nd	495	nd	484	143	nd	nd	154	nd	192
NRTIs	TDF 3TC	None	None	TDF 3TC	TDF 3TC	TDF 3TC	TDF 3TC	None	None	None	None	None	TDF 3TC	None	None	None	None	None

[a]After study entry, study visits were planned at week 4, 12, 24, 36, and 48; given the retrospective nature of the analysis, stored samples for HBV testing were not available from all study visits; missing time points are indicated as not done (nd). [b]Patient re-introduced TDF+3TC and discontinued the study at week 19 due to an adverse event; the patient was recalled at week 24, 36, and 48 for follow-up. [c]Plasma HIV-1 RNA load in copies/ml; [d]CD4 count in cells/mm^3. HBsAg= Hepatitis B surface antigen; anti-HBs= Hepatitis B surface antibody; anti-HBc= Total hepatitis B core antibody; anti-HBe= Hepatitis B e antibody; AST= Aspartate transaminase; ALT= Alanine transaminases; UD= undetectable (<60 copies/ml); NRTIs=Nucleos(t)ide reverse transcriptase inhibitors; TDF= Tenofovir disoproxil fumarate; 3TC= Lamivudine

6.4 DISCUSSION

This study was the first to assess the evolution of markers of HBV infection in HIV-1 positive adults who introduced NRTI-sparing ART in sub-Saharan Africa. The finding that patients were at risk of both clinically manifest acute hepatitis B and more subtle evidence of resumed HBV replication has implications for researchers and policy makers alike. Investigating or rolling out NRTI-sparing regimens in sub-Saharan Africa requires adoption of measures and interventions to address highly prevalent HBV exposure: a) systematic HBV screening including but not limited to HBsAg; b) vaccination of non-immune subjects; and c) monitoring of subjects with a previous HBV infection for evidence of reactivation. Across most of sub-Saharan Africa, such measures and interventions currently have limited to no implementation in routine practice[291–295,298].

WHO recommends that HIV-1 positive patients in sub-Saharan Africa receive ART regimens containing either TDF plus 3TC or FTC, or ZDV plus 3TC if HBsAg negative[158,174]. 3TC alone is expected to exert at least partial prophylactic activity against HBV acquisition[288] and reactivation[285]. Our data, combined with those of the published literature, indicate that NRTI-sparing and other regimens omitting both tenofovir and 3TC or FTC would carry a more substantial risk of *de-novo* HBV infection and reactivation. HBsAg prevalence is 8.8% across sub-Saharan Africa and is highest in Central and West Africa[301]. HBV infection rates are similarly high among HIV-positive people in the region[185]. In Cameroon, whilst data are heterogeneous, HBsAg prevalence is around 10% in the general population[302] and among HIV-positive patients[296], with overall higher rates in rural than in urban populations[302]. HBV transmission occurs early in life across sub-Saharan Africa, commonly through horizontal spread among young children[303]. Consistent with widespread risk of exposure, most HBsAg-negative patients in our study had evidence of a previous HBV infection, and were therefore at risk of reactivation.

Previous studies in high-income settings have reported on incident HBV infection predominantly among MSM. In Amsterdam, the overall incident rate was 11/1000 person-years in this group, ranging from 29/1000 person-years in the absence of HBV-active drugs, to 14/1000 when only lamivudine was used and 1.4/1000 in the presence of tenofovir [288]. A similar protective effect of dually active ART was reported among MSM in Japan [286], and North America[287], and among HIV patients in Switzerland[289]. Previous studies have also reported on HBV reactivation in the context of HIV-induced immune suppression, typically in case reports[304–306]. Evidence indicates that the likelihood of HBV reactivation during immune suppression is effectively reduced by the use of HBV-active antivirals[285]. In the setting of HIV, dually active ART has also been shown to reduce HBV DNA detection among HBsAg negative and anti-HBc positive patients[307]. Clinical trials that have evaluated NRTI-sparing ART strategies in high-income countries, including trials of PI/r monotherapy, have not reported on the risk of HBV infection or reactivation[163,308,309]. CD4 cell counts were overall satisfactory in our cohort, without a significant difference between patients with and without HBV reactivation. Patients however had experienced low CD4 cell counts prior to immune reconstitution on ART, and there was a trend for lower nadir CD4 counts in patients with HBV reactivation relative to those without.

HBV reactivation carries a risk of liver disease ranging from mild to fatal[310]; the risk of liver disease progression is augmented by HIV co-infection[311]. Detection of anti-HBs reduces the risk of HBV reactivation in populations with previous infection [307,312]; yet two subjects in our study showed persuasive evidence of HBV reactivation despite anti-HBs levels >100 IU/ml. HBsAg contains multiple antigenic sites within MHR and mutations in this region can allow escape from both antibody-mediated neutralisation and HBsAg detection in diagnostic assays[304,311,313]. A patient showing anti-HBs levels >1000 IU/ml coinciding with the detection of HBV DNA carried the MHR mutations Y100C and Y161FY. In addition, the patient harboured HBV genotype A3, which circulates in Cameroon and carries arginine (R) rather than lysine (K) at surface position 122. Y100C and K122R have been previously recognised in the context of HBsAg-negative HBV infection (“occult

hepatitis B") [314,315]; Y100C has also been previously described in a patient with co-circulating HBsAg and anti-HBs[316]. When occurring in isolation, neither mutation appears to reduce detection of HBsAg in diagnostic tests [317,318]; a more substantial effect has been proposed for multiple mutations occurring in combination[318]. The impact of HBV genetic evolution and escape in the context of HBV hyperendemicity warrants further investigations.

Universal vaccination programmes have variable coverage across sub-Saharan Africa[188]. Cameroon introduced infant vaccination in 2005 and coverage with three vaccine doses was 85% in 2016[188]. In our study, about one in five patients lacked evidence of HBV immunity. This should be interpreted in light of the lack of an adult catch-up immunisation programme in Cameroon, as also indicated by the reported poor vaccination coverage in healthcare workers[319]. A further five patients had anti-HBs as the only detectable HBV marker at study entry, which may be taken to indicate previous vaccination; their age (38-65 years) made them unlikely recipients of infant vaccination, and the subjects did not report vaccination, although we have previously noted that patients' recall of vaccination history is generally poor[320]. An alternative hypothesis is that anti-HBs may have reflected a previous HBV exposure in these subjects, despite absence of anti-HBc. Although antibodies against HBV core usually appear shortly after infection and remain positive lifelong, anti-HBc negativity has been reported despite evidence of HBV replication, typically in the context of immune suppression, and including cases with detectable anti-HBs[321–323]. This could be explained by mutations or partial deletion of the core protein leading to the decreased levels of HBsAg, HBcAg and HBeAg and their respective antibodies. Despite this, it would be unusual for patients to have lost HBcAb resulting from previous exposure as HBcAb persists longer than any other marker and is regarded as an ubiquitous marker for diagnostic and epidemiological assessments of HBV [324]. Thus, the two patients with possible incident HBV infection might in fact have experienced a reactivation, although it should be noted that anti-HBc did appear during follow-up alongside detection of HBsAg or HBV DNA.

This study has limitations. It was advantageous to have access to samples collected at scheduled visits over 48 weeks within a controlled trial, as this provided good retention into follow-up during the study period. However, the retrospective nature of the study prevented comparison to another treatment group and restricted sample availability. Thus, not all HBV markers were measured in all subjects at all study visits. HBV DNA sequences were recovered in only two subjects; due to the combination of low HBV DNA levels and small sample volumes no sequencing amplicons were obtained in other patients. A further consideration is that follow-up was attempted more than one year after the end of the study; whilst the treating clinician was made aware of the findings, study samples post week 48 could only be collected from one patient. Finally, standard serological measures were used to categorise patients with incident HBV infection or reactivation, and this must be regarded as a simplified approach when considering the complexity of the HBV marker profiles, we observed, the multiple possible phenotypes of HBV infection, and the modulatory effect of HIV co-infection.

Nonetheless and in summary, this is the first study to report on the risk of *de novo* HBV infection and HBV reactivation among HIV-1 positive patients who discontinue HBV-active agents in sub-Saharan Africa. Results clearly indicate that HIV-1 treatment strategies for the region must take into consideration available infrastructure for assessing and appropriately managing HBV status, and must consider level of access to adult immunisation.

CHAPTER SEVEN: DISCUSSION

7.1.1 Discussion

It has been more than four decades since the characterisation of HIV as the causative agent of AIDS and more than two decades since the introduction of antiretroviral therapy (ART) for its treatment. Increasing access to treatment across sub-Saharan Africa (SSA), which account for 25.6 of 36.5 million people globally infected, has improved prognosis and survival rates of infected individuals and led to a global decline in incident infections. Despite the efficacy in suppressing HIV replication and preventing disease progression and HIV transmission, ART the infection. Viral rebound is usually seen within 2 weeks after cessation of therapy[325], making HIV treatment lifelong. This is driven by the latent reservoir, which is established early in the course of the infection and continues to be replenished while HIV replicates. The reservoir resides within latently infected memory CD4 cells[325–329] and persists even while HIV replication is fully suppressed on ART. Treating people with HIV for life poses a significant burden and even more so across the SSA region, which is home to 70% of the poorest people in the world and three-quarter of HIV-infected individuals.

The expansion of therapeutic options since the 1990s, when some HIV regimen comprised up to 20 tablets, has greatly improved efficacy, barrier to resistance, tolerability and convenience of treatment. However, there remains a desire to consider strategies to reduce treatment burden in long- term therapy. In this context, simplification of therapy after virological suppression is achieved remains an attractive option. The aims of treatment simplification are to reduce the pill burden and improve adherence, preserve future treatment options, reduce short and long-term drug related toxicities, and improve cost-effectiveness[214,330–332]. NRTIs are the “common denominator” or “backbone” of preferred ART regimens. Especially some of the older NRTIs had significant risk of toxicity, including renal and bone toxicity[333–336] after prolonged exposure. One of the first attempts at simplification was the use of PI/b maintenance monotherapy for patients already suppressed on triple ART.

PI/b represent an attractive option to reduce treatment cost and NRTI-associated drug toxicities because of high potency and barrier to resistance[163,337–341].

In this context, this thesis evaluated the outcomes of maintenance monotherapy with ritonavir-boosted darunavir within the MANET trial, which was one of the first to explore the strategy in an African population receiving suppressive second-line ART with 2 NRTIs and a PI/b, after failure of first-line NNRTI-based ART. In Cameroon, until recently, treatment failure was defined as two consecutive viral load >5000 copies/ml, clinical deterioration, or a fall in CD4 cell count[259]. ART is free, however until recently viral load testing was not subsidised and was therefore rarely used for patient management. This meant that patients were likely to experience prolonged virological failure and accumulate extensive drug resistance prior to a change to second-line ART[342–344]. This is well reflected in the population of the MANET trial. Patients had been exposed to ART for a median of 7.5 years, including PI/b-based triple ART for a median of 3.1 years, without routine measurement of viral load. When I analysed the HIV-1 DNA in PBMC collected at study entry, when the viral load was suppressed, I detected a high prevalence of resistance-associated mutations (RAMs) affecting the NRTIs and NNRTIs.

One other interesting observation was related to the virological data obtained at screening. Overall, 38% of subjects on second-line ART were viraemic and this was in line with a previous pooled analysis from Professor Geretti's research group indicating that across SSA, 62% patients on second-line ART were virologically suppressed[142]. A further important observation however was that patients with suppressed viral load at the first screening, showed a high rate of viral rebound (14.8%) at the second screening visit, which occurred at a median interval of 7 weeks. Such high rate of rebound during screening should be taken into account when considering the results of the MANET trial. Findings from the virological outcome analysis showed a high risk of both low level and high level viraemia during DRV/r monotherapy, albeit without the emergence of DRV RAMs. This indicated that use of PI/b monotherapy would require frequent virological monitoring and prompt treatment intensification to

prevent potential clinical deterioration and risk of onward transmission[159,221]. This analysis however further provided novel insights into an interesting concept. In previous observations, it was observed that patients with detectable RAMs after failure of first-line NNRTI-based ART have a significantly higher (rather than lower) odds of attaining virological suppression following switch to second-line ART with 2 NRTIs and a PI/b[345–347]. The justification proposed was the effect of the adherence intervention offered at the time of switching to second-line ART, combined with residual NRTI activity despite the presence of RAMs[142]. Findings from MANET however expanded this concept by showing that both adherence and detection of RAMs in HIV-1 DNA at study entry were independent predictors of reduced odds of virological failure after switching to DRV/r monotherapy. This indicated that the predictive effect of NRTI and NNRTI RAMs was at least partially independent of residual NRTI activity and possibly reflective of overall compliance with treatment beyond what can be measured via adherence assessment. In this respect, it should be noted that adherence was measured by visual analogue scale and pill count, with limited concordance between measures, clearly indicating their limitations and adherence assessment using biological measures would have provided better insights.

Another element I explored during this PhD work was the relationship between HIV-1 DNA at study entry and rebound HIV-1 RNA during DRV/r monotherapy. Overall, I found that the archived mutational profile was largely consistent with that detected at rebound, although this was not full, possibly reflecting the presence of defective proviral genomes. There is interest currently in the use of HIV-1 DNA sequencing in patients on a suppressive ART regimen, to guide treatment modifications. The conclusions of the data presented in this thesis are that detection of RAMs in PBMC-derived HIV-1 DNA does not necessarily reflect the full mutational patterns that may re-emerge at rebound. One clear limitation is the small input size, with only a few cells contributing HIV-1 DNA to the sequencing reactions.

Further to the virological and resistance outcome analysis, the work explored another aspect of switching virologically suppressed patients to DRV/r

monotherapy. The research group had previously identified that the levels of sCD27 were associated with persistent detection of plasma HIV-1 RNA (at levels <11 IU/ml) among patients receiving suppressive NNRTI-based triple ART. Here, I asked the question of whether levels of sCD27 in patients entering MANET were comparable to those detected in other patient groups and whether they changed after switching to DRV/r monotherapy. Interesting, levels of sCD27 increased in a subset of patients after the switch, suggesting that sCD27 could provide a marker of virus control and thus guide the implementation of simplification strategies. Further studies are needed to expand on my preliminary observations.

MANET also gave me the opportunity to explore a novel aspect that had previously not been evaluated in the published literature. HIV treatment guidelines have to consider geographical location in terms of both access to drugs and epidemiological dynamics of the highly prevalent infection with the hepatitis B virus (HBV). The dual activity against both HIV and HBV of tenofovir and lamivudine, which form the most common NRTI backbone across SSA, offers prophylactic activity against both HBV infection and HBV reactivation. Treating HIV patients with simplified regimens thus creates concern if not using drugs with prophylactic activity against HBV in regions where HBV prevalence is high. As first documented in this research study, both *de-novo* acquisition of HBV and reactivation of previously resolved infection was observed after simplification to DRV/r monotherapy. It was notable that despite a high prevalence of both current and resolved HBV infections, a subset of patients had no evidence of HBV immunity, bringing into sharp relief the need for adult immunisation catch-up programmes.

7.1.2 Future direction

As we found evidence that some HIV-1 positive patients with detectable surface antibodies remained susceptible to HBV infection, a future study should address the neutralisation activity of anti-HBs and the role of escape HBsAg mutants in a setting where HBV hyperendemicity combines with HIV-1 infection.

I aim at post-doctoral level to evaluate if HIV resistance would compromise the success of dolutegravir-based ART.

7.1.3 General recommendation

Based on the findings from this research work, the following recommendations can be made:

- PI/b maintenance monotherapy should be contraindicated in HIV positive patients in SSA due to the high risk of viraemia, evidence of increasing levels of immune activation after simplification, and risk of HBV reactivation

- Detecting HIV-1 RAMs in PBMC-associated HIV-1 DNA can provide a helpful albeit incomplete assessment of the patient's resistance profile

- A subset of HIV-1 positive patients on long-term ART continue to shed HIV-1 RNA in seminal fluid, requiring a prospective assessment of the role of STIs as well as of infectiveness.

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APPENDIX

APPENDIX 1: Protocol Document

MANET: Monotherapy in Africa, New Evaluations of Therapy

Protocol Number: CUI_001; Design and Phase: Open-label randomised pilot feasibility study (IIIb)**; Study Drug:** TMC114, darunavir

SUMMARY OF STUDY PROTOCOL

The aim of this pilot study is to assess the feasibility, efficacy and safety of Darunavir/ritonavir 800/100 mg once daily (DRV/r) monotherapy as a switch-maintenance strategy for patients receiving second-line ART at Yaoundé Central Hospital (YCH) in Cameroon. HIV-infected adults receiving second-line antiretroviral therapy (ART) for ≥3 months with 2 nucleos(t)ide reverse transcriptase inhibitors (NRTIs) plus either lopinavir/ritonavir (LPV/r) or atazanavir/ritonavir (ATV/r) will undergo plasma HIV-1 RNA ("viral") load testing. Those with a viral load below 50 copies/ml (<50 cps/ml) will undergo a repeat test ideally 4-6 weeks later (allowed up to 16 weeks); if the viral load is confirmed as <50 cps/ml the patient will be invited to join the randomised phase of the study. Patients (n=150) will be randomised 1:2 to either continue the current triple ART regimen (n=50) or switch to DRV/r monotherapy (n=100). The study will assess feasibility by measuring the number of patients on 2NRTIs plus LPV/r (or ATV/r) that have a viral load <50 cps/ml (viral load monitoring is not routinely available at YCH); the number of eligible patients that consent to recruitment (experiences of HIV clinical trials is very limited at YCH); and the number of patients that once recruited are retained into follow-up (loss to follow-up is a problem in HIV services across sub-Saharan Africa). From an efficacy and safety perspective, the primary end-point will be viral load suppression <400 cps/ml at week 24; secondary end-points will be viral load suppression <50 cps/ml at week 12 and week 24, safety, tolerability, and emergence of protease inhibitor (PI) drug-resistance. Patients who discontinue DRV/r before week 24 will be retained in observation until week 24. Patients randomised to the DRV/r arm who are still on DRV/r at week 24 will continue observational follow-up to week 48. Those who discontinue DRV/r after week 24 and before week 48 will be retained in observation until week 48. After week 48, patients will return to local standard of care. In addition to routine samples to measure efficacy and safety, additional blood samples will be collected for a pharmacokinetics (PK) and pharmacogenomics sub-study to correlate plasma concentrations of DRV to outcomes. Furthermore at recruitment, peripheral blood mononuclear cells (PBMC) will be collected for HIV-1 drug resistance testing to detect mutants archived at the time of first-line ART failure and for measuring HIV DNA load. A cost-effectiveness analysis will test the hypothesis that savings can be achieved by switching to DRV/r monotherapy without affecting quality of care. Recruited subjects who at week 12, 24, 36 or 48 show a viral load >50 cps/ml will undergo confirmation of viraemia and managed according to the viral load level: 1) ≤400 cps/ml: re-enforce adherence; continue to monitor; 2) >400 to 1000 cps/ml: re-introduce the NRTIs if in the DRV/r monotherapy arm; continue to monitor if in the triple ART arm (as per standard of care); 3) >1000 cps/ml: change therapy (recommended third-line ART in Cameroon is with 2 NRTIs plus DRV/r 600/100 twice daily). Subjects with a screening viral load

≥50 cps/ml will undergo clinical, medication and adherence review, and management according to local standard of care.

STUDY DRUG
DRV/r (800mg/100mg) will be administered orally once daily with food.

LOCATIONS OF TRIAL
Yaoundé Central Hospital (YCH) and Chantal Biya International Reference Center for HIV/AIDS Research on Prevention and Treatment (CIRCB), Yaoundé, Cameroon.

SPONSOR
University of Liverpool

FINANCIAL SUPPORT
University of Liverpool
Janssen Pharmaceuticals (Providing funding and product [funder])

RATIONALE
DRV/r has shown excellent efficacy in ART-experienced and ART-naïve patients when co-administered with other antiretroviral drugs (Mills 2009, Madruga 2007). In these randomised comparisons, DRV/r showed superior activity to LPV/r. In the Artemis trial, ART-naïve patients were randomised to DRV/r (800/100mg once daily) or LPV/r each in combination with 2 NRTIs (Mills 2009). Over 192 weeks once daily DRV/r was noninferior and statistically superior in virological response to LPV/r, and with a more favourable gastrointestinal profile (Orkin 2012). In the Titan trial of ART-experienced patients randomised to DRV/r (twice daily) or LPV/r plus optimised background therapy, at week 48, significantly more DRV/r than LPV/r treated patients had a viral load <400 cps/ml (77% vs. 68%); there was also a reduced risk of emerging protease and NRTI resistance in the DRV/r arm at the time of virological failure (Madruga 2007). Furthermore, once daily DRV/r (800/100mg) was non-inferior in virological response to twice daily DRV/r (600/100mg) at 48 weeks in ART-experienced patients with no DRV resistance mutations, and with a more favourable lipid profile (Cahn 2011). Similar supporting data have been reported in ART-experienced patients with suppressed HIV-1 RNA while on a twice daily ritonavir-boosted protease (PI/r) regimen who were switched to once daily DRV/r (Goshn 2012). In this study patients had previously experienced a median of 2 (1-5) PIs.

The efficacy of once daily DRV/r monotherapy has been demonstrated in patients suppressed on their previous regimen and without prior failure (Arribas 2010, Katlama 2010). The MONET study demonstrated non-inferior efficacy of once daily DRV/r monotherapy compared with a triple regimen containing DRV/r +2NRTIs at the primary endpoint at 48 weeks (Arribas 2010). Although patients on DRV/r monotherapy experienced more frequent episodes of viral load rebound >50 cps/ml during the 144 weeks of follow-up, these were usually between 50 and 400 cps/ml and often resolved spontaneously without intervention. Importantly, we and others recently demonstrated that treatment simplification to DRV/r monotherapy is not associated with significant increases in cellular HIV-1 DNA load over up to 144

weeks of follow-up compared with continuing triple therapy with DRV/r + 2NRTIs (Geretti 2012; Lambert-Niclot 2012). In routine clinical practice, simplification to once daily DRV/r monotherapy has been shown to be safe and effective in previously treated patients with suppressed HIV-1 RNA (Santos 2012). In the study by Santos et al. patients had previously received a median of 5 (3-9) ART lines. There are no studies of DRV/r monotherapy in sub-Saharan Africa.

Monotherapy with a PI/r is currently not standard-of-care, but has been included in some treatment guidelines, such as the European AIDS Clinical Society (EACS) guidelines, as a treatment option in suppressed patients without prior failure to PIs (EACS 2011; Calza 2012). Data informing the use of PI/r monotherapy as second-line treatment are limited. A recent study evaluated responses to LPV/r monotherapy among 123 adults in resource-limited settings (5 sites in Africa and Asia) (Bartlett 2012). Patients were receiving NNRTI-based first-line therapy with a viral load of 1000-200,000 cps/ml. The primary endpoint was remaining on LPV/r monotherapy without virological failure at week 24. Overall 87% of patients met the primary endpoint. Thus, in this pilot study conducted in diverse settings, LPV/r monotherapy demonstrated promising activity as second-line treatment. In a recent study from Thailand however, patients experiencing virological failure (viral load >1000 cps/ml) on first-line NNRTI-based therapy showed higher response rates with LPV/r + 2NRTIs compared with LPV/r monotherapy (Bunupuradah 2012). Over 48 weeks, the proportions with viral load suppression <50 cps/ml were 83% vs. 61% respectively; baseline viral load was predictive of subsequent treatment failure. These findings are consistent with the observation that LPV/r monotherapy is suboptimal for inducing viral load suppression in patients with detectable viraemia (Calza 2012). It should be noted that here we are proposing a simplification study of patients who are already virologically suppressed on their current triple ART regimen.

Patients in Cameroon currently have access to LPV/r. However given that the World Health Organisation (WHO) has recently recommended ATV/r, the trial will allow patients who may have been receiving ATV/r by the time the trial starts. The vast majority of patients receiving 2 NRTIs + LPV/r at the centre are on second-line therapy, having experienced failure of a first-line regimen consisting of 2 NRTIs plus either nevirapine or efavirenz. Switching to second-line therapy is generally based upon a confirmed viral load >5000 cps/ml (two measurements within 4 weeks), a fall in CD4 cell counts or clinical deterioration. There is no routine virological monitoring subsidized by the government at the centre, although patients able to pay can undergo viral load testing. Thus, prior to changing from first- to second-line ART, patients usually experience prolonged virological failure and may be expected to start second-line therapy with extensive NRTI resistance (Marcelin 2006; Gupta 2008).

In a study from Cameroon, among 75 patients experiencing virological or immunological failure of first- or second-line ART, 80% had resistance to at least one antiretroviral drug, mostly to the NRTIs (80%) and the NNRTIs (76%), which are the recommended components of first-line regimens in the

country (Charpentier 2011). The patients showed multiple mutations affecting all NRTIs, including those included in recommended second-line regimens. Therefore we propose that the activity of the second-line regimen containing 2 NRTIs + LPV/r (or in the future ATV/r) is mostly related to that of the PI/r. The study will investigate the emergence of PI resistance in patients who experience confirmed viral load rebound >50 cps/ml during the trial. The plasma PK of DRV will also be determined, with the aim of gathering data that can be correlated with treatment outcomes.

Objectives

The aim of this pilot study is to assess the feasibility, efficacy and safety of DRV/r monotherapy as a maintenance strategy for patients receiving second-line ART and showing a suppressed HIV viral load. The feasibility assessment is critical to enable the design of a larger multicentre study. The assessment will address: 1) the number of patients receiving second-line therapy at YCH who have a viral load <50 cps/ml (viral load testing is not routinely available at YCH to provide estimates); 2) the number of eligible patients willing to be randomised to monotherapy; 3) retention into follow-up after recruitment. In addition, the study will have the following efficacy and safety objectives:

Primary virological objective

- To evaluate efficacy in terms of the percentage of subjects who have plasma HIV-1 RNA levels <400 cps/ml after 24 weeks of follow-up following a switch to DRV/r monotherapy vs. continuing triple therapy containing 2 NRTIs + LPV/r (or ATV/r) (FDA Snapshot method).

Secondary objectives

1. To compare the percentage of subjects who have plasma HIV-1 RNA levels <50 cps/ml after 12 weeks of follow-up following a switch to DRV/r monotherapy vs. continuing triple therapy containing 2 NRTIs + LPV/r (or ATV/r), using the TLOVR method.
2. To compare the percentage of subjects who have plasma HIV-1 RNA levels <50 cps/ml after 24 weeks of follow-up following a switch to DRV/r monotherapy vs. continuing triple therapy containing 2 NRTIs + LPV/r (or ATV/r), using the TLOVR method.
3. To evaluate and compare safety and tolerability of DRV/r monotherapy versus triple therapy over 24 weeks.
4. To observe the efficacy, safety and tolerability of DRV/r monotherapy from week 24 to week 48.
5. To evaluate and compare the loss of treatment options with DRV/r monotherapy versus triple therapy at week 24 and evaluate the loss of treatment options with DRV/r from week 24 to week 48, as defined by treatment-emergent PI resistance mutations in patients experiencing confirmed viral load rebound >50 cps/ml.
6. To evaluate DRV (and ritonavir) plasma levels in patients receiving DRV/r monotherapy with the aim of correlating findings to treatment outcomes. The primary aim for this analysis is to assess the relationship between DRV exposure and virological responses (including both confirmed and transient episodes of viral load rebound >50 cps/ml) in patients on DRV/r monotherapy. Secondary aims are: i) to assess major covariates of DRV

exposure, in particular weight, gender, liver function; ii) to correlate the PK findings to host genetic predictors of drug metabolism.

7. To investigate the presence of archived HIV drug resistance in HIV DNA present within PBMC collected at the time of recruitment and measure the HIV DNA load in the same sample to determine how it relates to virological responses.
8. To perform a cost-effectiveness analysis of switching to DRV/r monotherapy versus continuing triple therapy.

Hypothesis

We propose that maintenance therapy with DRV/r monotherapy is a feasible, effective and safe treatment option for patients receiving second-line ART in Yaoundé.

Feasibility

We propose that within six months of opening the study to recruitment, 250 patients will be identified at YCH that have a viral load <50 cps/ml (confirmed over two tests 4-6 and up to 16 weeks' apart) while receiving 2 NRTIs + LPV/r (or ATV/r); that 200 will meet the other eligibility criteria; and that 150 will consent to recruitment. Of 150 recruited patients, we hypothesise that 128 (85%) will be retained into follow-up over at least 24 weeks. Such data would confirm the feasibility of running a larger trial.

Efficacy

The primary efficacy hypothesis for this study is that, after 24 weeks of randomised treatment, the percentage of subjects taking DRV/r monotherapy with a viral load <400 cps/ml is similar to the percentage in the triple therapy arm containing 2 NRTIs + LPV/r (or ATV/r). Secondary efficacy hypothesis are that after 12 and 24 weeks of randomised treatment the percentage of subjects taking DRV/r monotherapy with a viral load <50 cps/ml is similar to the percentage in the triple therapy arm containing 2 NRTIs + LPV/r (or ATV/r).

Safety

Secondary hypotheses are that DRV/r monotherapy is safe over 48 weeks, and that it offers a reduced risk of diarrhoea and blood lipid abnormalities than 2 NRTIs plus LPV/r during 24 weeks of randomised observation.

Study design

This is a 24-week, single-centre, open-label, randomised pilot feasibility (Phase III) trial to assess feasibility, safety and efficacy of DRV/r 800/100 mg once daily as monotherapy for patients with a suppressed HIV viral load while receiving second-line ART with 2 NRTIs plus LPV/r (or ATV/r). A total of 150 HIV-infected subjects treated for at least 3 months with 2 NRTIs + LPV/r (or ATV/r) will undergo viral load testing (screening 1); if the viral load is <50 cps/ml the patients will undergo a repeat viral load ideally 4-6 weeks later (screening 2; documented results obtained up to 16 weeks previously will be accepted). Those with confirmed viral load suppression will be invited to join the randomised phase of the study. This will consist of randomisation to 24 weeks treatment with either monotherapy with DRV/r or continuation of triple therapy containing 2 NRTIs + LPV/r (or ATV/r) in a ratio of 2:1. Subjects with

hepatitis B infection or a current CD4 count <100 cell/mm^3 will be excluded. After week 24, patients randomised to DRV/r monotherapy will continue observational follow-up to week 48. Any patient discontinuing DRV/r prior to the end of the study will continue observation until week 48 (for discontinuations prior to week 24) or until week 48 (for discontinuations after week 24).

The trial will include a 4-6 week (up to 16 weeks) screening period to determine and confirm viral load suppression <50 cps/ml, a 24 week treatment period, and either a 12 weeks follow-up visit or phone call for patients randomised to continuing triple therapy, or a 24 week observation period for patients randomised to DRV/r monotherapy, followed by a follow-up visit or phone call 4 weeks later.

An independent Data Monitoring Committee (DMC) has been commissioned for this study. One formal DMC review of the interim analysis results will occur when all subjects have had at least 12 weeks of treatment or discontinued earlier. There are no a priori stopping guidelines defined. The DMC will make recommendations to the Trial Steering Committee regarding the continuation, modification or termination of the trial, based on their review of the efficacy and safety data. The interim report will also be submitted to the Ethics Committees in the UK and in Cameroon.

SUBJECT SELECTION

Inclusion

1. Subjects with documented HIV-1 infection.
2. Male or female aged $\geq$ 21 years old.
3. Subjects receiving ART with 2 NRTIs + LPV/r (or ATV/r) for at least 3 months at the time of screening 1.
4. Current CD4 >100 cells/mm^3 (documented at screening 2)
5. Plasma HIV-1 RNA <50 copies/ml at screening 1 confirmed ideally 4-6 weeks later at screening 2 (two results must be documented; a first result obtained up to 16 weeks earlier will be accepted).
6. Subjects can comply with the protocol requirements. In particular, subjects should be willing to be followed up at least until week 24 (discontinuation prior to week 24) and for the DRV/r arm up to week 48 (discontinuation after week 24) even if they discontinue randomized treatment.
7. Subjects who have voluntarily signed and dated the consent form.

Exclusion

1. Clinical or laboratory evidence of significantly decreased hepatic function or decompensation, irrespective of liver enzyme levels (liver insufficiency).
2. Co-infection with hepatitis B (HBsAg positive).
3. Grade 3 or 4 laboratory abnormality as defined by DAIDS, including haemoglobin ≤8mg/dL; platelets ≤50 000/mm^3; estimated creatinine clearance ≤60mL/ minute, AST; ALT and alkaline phosphatase >3 times the upper limit of normal; and total bilirubin >2.5 times the upper limit of normal; with the following exceptions unless clinical assessment foresees an immediate health risk to the subject:
   - Pre-existing diabetes or asymptomatic glucose grade 3 or 4 elevations.

- Asymptomatic triglyceride or cholesterol elevations of grade 3 or 4.

4. Presence of any currently active AIDS defining illness (Category C conditions according to the CDC Classification System for HIV Infection 1993) with the following exceptions:
   - Stable cutaneous Kaposi's Sarcoma (i.e., no internal organ involvement other than oral lesions) that is unlikely to require any form of systemic therapy during the study.
   - Wasting syndrome due to HIV infection.

   Note: An AIDS defining illness that is not clinically stabilized for at least 30 days will be considered as currently active.
5. Pregnant or breastfeeding women.
6. Active substance abuse, including alcohol or recreational drugs.
7. Any clinically significant disease (e.g., tuberculosis, cardiac dysfunction, pancreatitis, acute viral infections) or life threatening disease in the previous 14 days, or findings during screening of medical history or physical examination that, in the investigator's opinion, would compromise the subject's safety or outcome of the study.
8. Any medical or psychiatric condition which, in the opinion of the investigator, could compromise the subject's safety or adherence to the trial protocol.
9. Previously demonstrated clinically allergy or hypersensitivity to any of the excipients of the investigational medication (DRV).

   Note*:* DRV is a sulfonamide. Subjects who have previously experienced a sulfonamide allergy will be allowed to enter the trial. To date, no potential for cross sensitivity between drugs in the sulfonamide class and DRV has been identified in subjects participating in phase II trials.
10. Participation in any other clinical trials that involve administration of antiretrovirals or other drugs within the last 4 weeks and during the participation in this trial.

Prohibitions and Restrictions

Potential subjects must be willing to adhere to the following prohibitions and restrictions during the course of the study to be eligible for participation.

There are no adequate and well-controlled studies with DRV in pregnant women. Studies in animals showed no teratogenic potential in rats, mice and rabbits. An absence of any effect on fertility, teratogenicity and pre- and post-natal development with DRV treatment can be stated, but only in so far as the relative exposure levels were only comparable to human. Therefore, patients randomized to DRV will be advised to avoid pregnancy while on the drug by using contraceptive measures including the use of condoms to prevent HIV transmission. Furthermore, women should not breast-feed when taking DRV, as the effects to their newborn child are unknown. It is not known if DRV is excreted in human milk but there is indication that DRV is excreted in milk in rats. For details on the existing data with regard to the reproductive toxicity of DRV, please see the current Investigator's Brochure.

Subjects should not take any medications that are contra-indicated with DRV/r (see below and also refer to up to date product label).

Patients receiving 2 NRTIs + LPV/r will not be allowed to switch from LPV/r to ATV/r during the trial.

Dosage and Administration

Eligible patients will be randomised (2:1) to either:

a) DRV/r 800/100mg once daily monotherapy (n=100).
b) Continuation of current triple therapy with 2 NRTIs plus LPV/r (or ATV/r) (n=50).

DRV/r will be administered orally as two 400mg tablets darunavir and one 100mg tablet ritonavir within 30 minutes after completion of a meal. The intake of drugs in the triple therapy arm will be according to the local prescribing information. Full reference to SPC should be made. Description of reasonable foreseeable risks and discomfort from DRV/r are indicated in the risk section. Also refer to unforeseeable risks to subject embryo or foetus.

CONCOMITANT THERAPY

The following medications are disallowed for the duration of this study:

- Investigational agents (from 90 days before screening onwards);
- Experimental vaccines;
  Note: Approved vaccines are allowed if they are given at least 4 weeks before a viral load measurement.
- Systemic dexamethasone (topical formulations are allowed);
- Stimulants: amphetamines, amphetamine derivatives;
- Herbal supplements: all products containing Hypericum perforatum (St John's Wort);
- Antibiotics: rifampin, rifapentine, telithromycin;
- Anticonvulsants: phenobarbital, phenytoin, carbamezepine;
- Antiarrhythmics: bepridil, flecainide, propafenone, systemic lidocaine, quinidine, mexilitine, disopyramide, amiodarone;
- Anticoagulants: warfarin;
- Calcium channel blockers: felodipine, nifedipine, nicardipine;
- Immunosuppressants: cyclosporin, rapamycin, tacrolimus, sirolimus;
- Antihistamines: astemizole, terfenadine;
- Prokinetic: cisapride;
- Antipsychotics: pimozide;
- Ergot derivatives: dihydroergotamine, ergonovine, ergometrine, ergotamine, methylergonovine;
- Benzodiazepines: midazolam, triazolam;
- Narcotic analgesics: meperidine (pethidine);
- Lipid lowering agents and HMG-CoA reductase inhibitors: pravastatin, lovastatin, simvastatin;
- Antifungals: systemic use of ketaconazole, or itraconazole at > 200 mg/day.

Should a patient develop TB while on the study drug, one of two management options will be available: 1) Switch back to standard of care and use rifampicin; 2) continue on study drug using rifabutin (150 mg three times a week) instead

of rifampicin. As rifabutin is now becoming available at low cost across Africa, the second option will be possible.

EVALUATIONS

Feasibility

1. Proportion of patients with viral load <50 copies/ml at screening 1 and screening 2
2. Proportion of eligible patients that consent to enter the study
3. Proportion of recruited patients retained into follow-up for at least 24 weeks and up to 48 weeks

Efficacy

- Plasma HIV-1 RNA load measurement at screening 1, screening 2, study entry (baseline), week 4 (retrospective), week 12, week 24, and for DRV/r monotherapy arm alone week 36 and week 48 (CIRCB).

  Note: Results of viral load testing at screening 1, week 24 for both arms and at week 36 and 48 for the DRV/r monotherapy arm will be returned to the clinic within 4 weeks of sampling. Week 12 viral load results will be retained for study use.

- Drug-resistance testing of plasma HIV-1 RNA on 2nd sample for confirmed viral load rebound >50 cps/ml at any time during the study (CIRCB and UoL).

Pharmacokinetics (PK) and Pharmacogenomics (UoL)

- Drug levels in plasma for DRV and ritonavir at baseline, week 4, week 12, and week 24 will be compared in patients remaining virologically suppressed relative to those who experience transient or confirmed viral load rebound >50 cps/ml during the study. Blood samples will be taken at trough – defined as 20-28 hours post dose. If this is not possible (due to dosing – i.e., at night and patients come to clinic in the morning) sampling will be taken 10-14 hours post dose. Plasma will be obtained and stored frozen.
- Covariate analyses will explore associations between important PK parameters (trough, AUC, CL/F) and covariates such as age, weight, ethnicity, sex, co-morbidities, co-medications.
- Rich ("intensive") PK sampling (7 samples over 24h – at 0, 2, 4, 6, 8, 12, 24 h) will be undertaken in 10 patients who volunteer for (opt-in) this sub-study.
- In a subset of patients willing to provide consent (opt-in), host genetic predictors of antiretroviral drug metabolism will be investigated using plasma-recovered DNA. Specifically, in patients receiving DRV/r monotherapy, identification of subpopulations at particular risk of high or low DRV plasma concentrations is important, and genomic DNA will be analysed for host genetic predictors of antiretroviral drug exposure. These will include polymorphisms in CYP3A5/ ABCB1, ABCC1, 2, 4 and 10, and SLCO family (including SLCO 3A1).

  Any stored blood may be used by the investigators for further exploratory work following approval by the relevant committee bodies.

Archived drug resistance and HIV DNA load

Presence of HIV-1 drug resistance mutations in reverse transcriptase and protease will be assessed in HIV-1 DNA recovered from PBMC collected at recruitment. HIV DNA load will be measured in PBMC by real-time PCR.

Safety

Safety evaluations will include clinical laboratory assessments performed by CIRCB, physical examinations including evaluation of vital signs, and the reporting of adverse events (AEs).

All AEs, whether serious or non-serious, will be recorded. Serious safety events will be reported from the time a signed and dated informed consent form is obtained until completion of the last study-related procedure (may include contact for follow-up of safety). Please refer to the Study Safety Reporting Standard Operating Procedure.

Safety laboratory assessments (e.g. haematology, biochemistry, urinalysis) will be performed at Centre Pasteur du Cameroun. Planned assessments are:

Hematology: Haemoglobin, white blood cell (WBC) with differential, platelet counts.

Biochemistry: Sodium, potassium, creatinine (with EGFR), glucose, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, total bilirubin, cholesterol/LDL/HDL, triglycerides.

Urinalysis: dipstick.

Immunology: CD4 cell count.

Serology: Hepatitis B surface antigen (HBsAg)

Pregnancy test: at baseline and other visits for women of childbearing potential. Women who become pregnant during the trial will be offered the option to continue to DRV/r and re-introduce two NRTIS, or return to standard of care. Pregnancies occurring on DRV/r and their related outcomes will be recorded.

Additional tests will be performed as indicated by clinical or laboratory findings.

END-POINTS

Feasibility end-points: Percentage of screened subjected that have a HIV viral load <50 cps/ml at both screening 1 and screening 2; percentage of patients with a suppressed viral load that meet other eligibility criteria; percentage of eligible patients who consent to recruitment; percentage of recruited patients who remain in follow-up for at least 24 weeks and up to 48 weeks.

Primary efficacy end-point

- Percentage of subjects who have plasma HIV-1 RNA levels <400 cps/ml after 24 weeks of follow-up following a switch to DRV/r monotherapy versus

continuing triple therapy containing 2 NRTIs + LPV/r (or ATV/r) (FDA Snapshot method).

Secondary end-points

- Percentage of subjects who have plasma HIV-1 RNA levels <50 cps/ml after 12 weeks of follow-up following a switch to DRV/r monotherapy versus continuing triple therapy containing 2 NRTIs + LPV/r (or ATV/r), using the TLOVR method.
- Percentage of subjects who have plasma HIV-1 RNA levels <50 cps/ml after 24 weeks of follow-up following a switch to DRV/r monotherapy versus continuing triple therapy containing 2 NRTIs + LPV/r (or ATV/r), using the TLOVR method.
- Safety and tolerability of DRV/r monotherapy versus triple therapy over 24 weeks.
- Observed efficacy, safety and tolerability of DRV/r monotherapy from week 24 to week 48.
- Loss of treatment options with DRV/r monotherapy versus triple therapy at week 24, and loss of treatment options with DRV/r from week 24 to week 48, as defined by treatment-emergent protease drug-resistance mutations in patients experiencing confirmed viral load rebound >50 cps/ml.

Additional analyses

- DRV (and ritonavir) plasma levels in patients receiving DRV/r monotherapy and pharmacogenomics of antiretroviral drug metabolism.
- HIV-1 drug resistance and HIV DNA load in PBMC collected at recruitment.
- Cost-effectiveness analysis of switching to DRV/r monotherapy versus continuing triple therapy.

STATISTICAL METHODS

Sample Size Determination

This is a pilot feasibility study and no formal sample size calculations are included. The number of patients has been calculated as sufficient to give estimates of feasibility that will guide the design of a larger study.

Interim Analysis

There is an interim analysis planned for this study, which will occur after all subjects have completed their 12 week visit after randomisation or discontinued earlier. The second analysis will occur at week 24 after randomisation, after all subjects have either completed the week 24 follow-up visit, or discontinued earlier. A further analysis will be performed after all patients randomised to DRV/r monotherapy have completed the week 48 follow-up visit, or discontinued earlier.

Efficacy Analysis

All statistical tests will be interpreted at the one-sided 2.5% significance level or at the 5% two-sided significance level, unless specified differently. The analysis will be performed by treatment phase. The main focus will be the comparison between DRV/r monotherapy and the triple therapy with 2 NRTIs plus LPV/r (or ATV/r).

A per-protocol and an intent-to-treat population will be defined as:

- The intent-to-treat (ITT) population will be defined as the set of all subjects who were randomized and who have taken at least one dose of trial medication, regardless of their compliance with the protocol.
- The per-protocol population will be defined as the set of all randomized subjects who have taken at least one dose of trial medication, and who did not take other protease inhibitors (both treatment arms) for more than one week before virological failure or NRTIs (DRV/r monotherapy arm only) for more than one week before virological failure.

The primary population will be the ITT population. The per-protocol population will also be analyzed to investigate the impact of exclusion of major protocol violations listed above on the conclusions.

- ❖ The primary efficacy objective is to evaluate the efficacy of DRV/r monotherapy versus continuing triple therapy, with respect to confirmed virological response, defined as plasma HIV-1 RNA <400 cps/ml at 24 weeks.
- ❖ As a secondary efficacy and safety objectives, the following will be evaluated:
  - The efficacy of DRV/r monotherapy versus triple therapy, with respect to confirmed virological response, defined as plasma HIV-1 RNA <50 cps/ml at 12 and 24 weeks using a "switch included" endpoint. For this analysis, all subjects who discontinue randomized medication will be followed up on their subsequent treatment, and their last HIV-1 RNA levels will be included in the analysis.
  - The safety and tolerability of DRV/r monotherapy versus triple therapy over 24 weeks.
  - The number of subjects in the DRV/r monotherapy arm which needed to be re-started on NRTIs and the number of days on monotherapy.
  - The immunological response in terms of change in the CD4 cell count from baseline over 24 weeks of DRV/r monotherapy versus triple therapy.
  - The development of new drug-resistance mutations in the protease gene in patients who experience confirmed HIV-1 RNA rebound >50 cps/ml at any time during the study.
  - To observed the efficacy, safety and tolerability of DRV/r monotherapy from week 24 to week 48.

Criteria for end-points

Primary antiviral activity parameter:

- Virological response: percent of subjects with confirmed plasma viral load <400 cps/ml at week 24.
- Subjects who discontinue randomized study medication will be considered as non-responders after discontinuation. For the primary analysis, discontinuation will mean stopping treatment with DRV/r for at least 30 consecutive days in the monotherapy arm, or stopping all NRTI treatment in the triple therapy arm for at least 30 consecutive days.
- A subject with an intermediate missing viral load measurement will be considered as a responder if the subject's viral load was <400 cps/ml in the preceding and the subsequent visit, in all other cases the subject will be considered as a non-responder.

Pharmacokinetic analysis
Covariate analyses will explore associations between important PK parameters (trough, AUC, CL/F) and covariates such as age, weight, ethnicity, sex, co-morbidities, co- medications.

Resistance analysis
There will be no formal statistical testing of this parameter.

HIV DNA load analysis
Covariate analysis will explore the association between HIV DNA load and other covariates and outcomes.

Safety Analyses
There will be no formal statistical testing of safety parameters in the trial.

Medical Resource Usage and Health Economics
The cost of antiretrovirals will be calculated for each treatment arm, using prices from the Clinton Health Access Initiative. The cost of diagnostic tests (HIV-1 RNA, CD4 cell counts, resistance testing) will also be calculated, plus the number of outpatient and inpatient visits. The combined costs will be used to estimate the total cost of treatment and care in the two treatment arms.

Patient management
Results of viral load testing at screening 1 and week 24 for both arms and at week 36 and 48 for the DRV/r monotherapy arm will be returned to the clinic within 4 weeks of sampling. For study purposes, patients showing a viral load ≥50 cps/ml at week 12, 24, 36 or 48 will undergo confirmation of viraemia within 2-4 weeks and if this is confirmed the recommended management strategies are indicated below:

Confirmed HIV viral load	**DRV/r monotherapy arm**	**Triple therapy arm**
≥50 and ≤400 cps/ml	Adherence review, continue	Adherence review, continue
>400 and <1000 cps/ml	Re-introduce 2 NRTIs, continue DRV/r	Adherence review, continue
≥1000 cps/ml[1]	Return to standard of care[2]	Standard of care[2]

[1]1000 copies/ml is the cut-off for defining virological failure in the Cameroon HIV Treatment Guidelines.
[2]The Cameroon HIV Treatment Guidelines recommend a third-line regimen of 2 NRTIs plus DRV/r dosed as 600/100 twice daily.

	Screen 1	Screen 2	Baseline	Randomised Period						End of study	Observed period						End of study
										Continue arm	Switch arm						
WEEK	**- 4 to -16**	**-1-4**	**0**	**4**	**8**	**12**	**16**	**20**	**24**	**36**	**28**	**31**	**36**	**40**	**44**	**48**	**52**
Date of visit; subject ID	X	X	X	X		X			X				X			X	
Subject contact details	X	X	X	X		X			X				X			X	
Demographics (age, gender)	X	X															
Informed consent		X															
Inclusion/Exclusion criteria	X	X															
Continue to next visit?	X	X	X	X		X			X[a]				X				
Primary reason for discontinuation	X	X	X	X		X			X				X			X	
Medical & Surgical History		X															
History of HIV Disease		X															
ART history		X															
Concomitant Therapy & Procedures		X	X	X		X			X				X			X	
Childbearing potential		X															
Pregnancy test (urine)		X		X		X			X				X			X	
Randomization			X														
Dispense antiretroviral drugs			X	X	X	X	X	X	X[a]		X	X	X				
Drug accountability (pill count)				X	X	X	X	X	X		X	X	X			X	
Physical Examination & Vital Signs		X		X		X			X				X			X	
Subject status[b]										X							X
Well-being questionnaire			X	X		X			X				X			X	
M-MASRI adherence questionnaire			X	X		X			X				X			X	
Sample collection	X	X		X		X			X				X			X	
FBC		X		X		X			X				X			X	
Full biochemistry 1[b]						X											
Full biochemistry 2[c]		X							X							X	
Glucose		X							X							X	
Partial biochemistry[d]				X									X			X	
Urinalysis		X		X		X			X				X			X	
HBV status	X																
HIV viral load	X	X				X			X				X			X	
PK sampling				X		X			X								
CD4 cell count		X							X							X	
Adverse Events		X		X		X			X				X			X	

	Screen 1	Screen 2	Baseline	Randomised Period						End of study	Observed period						End of study
										Continue arm	Switch arm						
WEEK	**- 4 to -16**	**-1-4**	**0**	**4**	**8**	**12**	**16**	**20**	**24**	**36**	**28**	**31**	**36**	**40**	**44**	**48**	**52**
Plasma storage	X	X		X		X			X				X			X	
PBMC storage		X															
Serum storage	X																
Study termination									X[e]							X	
End of study follow-up visit/call										X							X

[a]Switch arm only; [b]Full biochemistry 1: ALT, AST, Total bilirubin, Creatinine, Sodium, Potassium, Calcium, Cholesterol, HDL, LDL, Triglycerides; [b]Full biochemistry 2: As in full biochemistry 1 + Alkaline Phosphatase; [c]Partial biochemistry: ALT, AST, Creatinine; [d]Subject status: Inclusion/Exclusion criteria, Medical and Surgical Events, HIV-related events, AIDS defining diagnosis, antiretroviral therapy intake; [e]Continue arm only.

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Patient Study Number: CUI-_/__/___
CONSENT FORM
Title of Project: MANET STUDY **Name of Researcher: Dr Charles Kouanfack**

Please initial box

1. I have read and understood the information sheet dated 17/12/2014 (version 7) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from Yaoundé Central Hospital, CIRCB, and the University of Liverpool that are part of the study team, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐
4. I agree to take part in the above study.
5. I understand that research on the samples I have given may also include analysis of genetic markers of drug levels. I understand that it is up to me to decide if I wish to volunteer for this part of the research, or decline it while still taking part in the rest of the study. ☐
6. I agree to take part in the above genetic markers study. ☐
7. I understand that the researchers are looking for volunteers for a “rich” drug levels study. I understand that it is up to me to decide if I wish to volunteer for this research, or decline it while still taking part in the rest of the study. ☐
8. I agree to take part in the “rich” samples drug level study. ☐
9. I understand that left over samples from this study will be stored for 5 years and that other tests will be done only for research purposes and only after approval. ☐
10. I agree to my samples being stored for other tests if other research is approved ☐

_____ _____ _____

Name of Patient Date Signature

APPENDIX 2: ADHERENCE FORM; MANET

Pill count:

1. Study ID: CUI-_/__/___ Sex: F ☐ M ☐ First name initial __ Surname initial __
2. Has patient brought bottles from last prescription? Yes ☐ No ☐
Pill count [HIV drugs only]**:**

No.	Drug name	Dosage details	No. of pills given	No. of pills counted
Example	*Darunavir*	*800 mg once daily*	*1 bottle of 60 tablets*	0
Example	*Ritonavir*	*100 mg once daily*	*1 bottle of 30 tablets*	0
1.				
2.				
3.				
4.				

4. Actioned required? Yes ☐ No ☐ If yes inform Study Doctor
5. Person completing the form: _____ 6. Date: __/__/____ *dd/mm/yyyy*
File in MANET ADHERENCE FOLDER

---

1. Study ID: CUI-_/__/___ Sex: F ☐ M ☐ First name initial __ Surname initial __
2. Has patient brought bottles from last prescription? Yes ☐ No ☐
Pill count [HIV drugs only]**:**

No.	Drug name	Dosage details	No. of pills given	No. of pills counted
Example	*Darunavir*	*800 mg once daily*	*1 bottle of 60 tablets*	0
Example	*Ritonavir*	*100 mg once daily*	*1 bottle of 30 tablets*	0
1.				
2.				
3.				
4.				

4. Actioned required? Yes ☐ No ☐ If yes inform Study Doctor
5. Person completing the form: _____ 6. Date: __/__/____ *dd/mm/yyyy*

Adherence Questionnaire

Clinical Team please complete the shadowed sections before handing the questionnaire to the patient

Subject ID: CUI-_/__/___ **Visit date:** __/__/____ (dd/mm/yyyy)

Your most recent prescription was:

Drug	Number of pills for each dose	How many doses each day
[ ]	[ ]	[ ]
[ ]	[ ]	[ ]

Many people find it hard to always remember their pills. For example some people get busy and forget to carry their pills with them; some people find it hard to take their pills according to all the instructions, such as "with food", "always at the same time"; some people decide to skip pills to avoid side effects or to just not take pills that day. We need to understand what people are really doing with their pills. Please tell us what you are actually doing. Don't worry about telling us you don't take all your pills. We need to know what is really happening, not what you think we want to hear. Please answer a few questions:

Which of the following medications have you taken within the last 30 days?

Drug A [ ]	☐		Drug C [ ]	☐
Drug B [ ]	☐		Drug D [ ]	☐

Now please put an "X" on the line below at the point showing your best guess about how much of each drug you have taken in the last three to four weeks. We would be surprised if this was 100% for most people. For example, if you take Darunavir: 0% means you have taken no Darunavir, 50% means you have taken half your doses of Darunavir, and 100% means you have taken every single dose of Darunavir.

A1. Drug A [ ]

0% 10% 20% 30% 40% 50% 60% 70% 80% 90% 100%

B1. How about Drug B? [ ]

0% 10% 20% 30% 40% 50% 60% 70% 80% 90% 100%

APPENDIX 3: ETHICAL CLEARANCE

COMITE NATIONAL D'ETHIQUE DE LA RECHERCHE POUR LA SANTE HUMAINE

Arrêté N° 0977/A/MINSANTE/SESP/SG/DROS/ du 18 avril 2012 portant création, organisation et fonctionnement des comités d'éthique de la recherche pour la santé humaine au sein des structures relevant du Ministère en charge de la santé publique

N° 2013/07/342/L/CNERSH/SP Yaoundé, le 25 juillet 2013

Cnethique_minsante@yahoo.fr

CLAIRANCE ETHIQUE

Le Comité National d'Ethique de la Recherche pour la Santé Humaine (CNERSH), en sa session du 25 juillet 2013, a examiné le dossier de demande de clairance éthique pour le projet de recherche intitulé **«MANET-Monotherapy in Africa : Evaluation of New Therapies)»** soumis par le Professeur **Anna GERETTI**, investigateur Principal (University of Liverpool), et Docteur **Judith NDONGO TORIMIRO** (Centre International de Référence Chantal BIYA pour la Recherche sur la Prévention et la prise en Charge du VIH/SIDA, Yaoundé), Co-investigateur.

Le projet est d'un grand intérêt scientifique et social. La procédure de recherche est bien décrite. Il s'agira dans cette étude d'évaluer l'efficacité la monothérapie par rapport au traitement standard chez les patients VIH positifs. Les évènements indésirables ainsi que leur gestion ont été présentés. La notice d'information et le formulaire de consentement éclairé des patients à l'essai sont bien élaborés, simples et faciles à comprendre. Les échantillons de sang collectés ne pourront être envoyés à l'Université de Liverpool pour les analyses spécialisées qu'après signature d'un accord de transfert du matériel biologique par les parties concernées et présentation au CNERSH. Les mesures prises pour garantir la confidentialité des données sont décrites dans le protocole de recherche. Les CV des Investigateurs les présentent comme des personnes compétentes à la mise en œuvre de ce protocole. Pour toutes ces raisons, le Comité National d'éthique donne un avis favorable pour mener une étude pilote d'une durée de 48 semaines. Au bout de cette période, si les résultats sont satisfaisants, les investigateurs pourront renouveler cette clairance éthique, et dans le cas contraire arrêter les investigations.

Les investigateurs sont responsables du respect scrupuleux du protocole approuvé et ne devraient y apporter aucun amendement aussi mineur soit-il, sans avis favorable du CNERSH. Les investigateurs sont appelés à collaborer pour toute descente du CNERSH pour le suivi de la mise en œuvre du protocole approuvé. Le rapport final du projet devra être soumis au CNERSH et aux autorités sanitaires du Cameroun.

La présente clairance éthique peut être retirée en cas de non respect de la réglementation en vigueur et des recommandations sus-mentionnées.

En foi de quoi, la présente clairance éthique est délivrée pour servir et valoir ce que de droit

Ampliations

- MINSANTE

LE PRESIDENT

Pr Lazare KAPTUE

N.B : cette clairance éthique ne vous dispense pas de l'autorisation administrative de recherche (AAR) exigée pour mener cette étude sur le territoire Camerounais. Cette dernière vous sera délivrée par le Ministère de la Santé Publique.

UNIVERSITY OF LIVERPOOL

Sarah Wright
Research Governance Officer

Legal Risk & Compliance
Waterhouse Building Block C
Brownlow Hill
Liverpool
L69 3GL

T +44 (0) 151 794 8290
E: ethics@liv.ac.uk

Professor Anna Maria Geretti
Institute of Infection and Global Health

Wednesday, 17 July 2013

Dear Professor Geretti

RE: MANET: Monotherapy in Africa, New Evaluations of Therapy (A new strategy for treating HIV in Africa)

The University of Liverpool's Research Ethics Sub-Committee for Physical Intervention (RESPI) has carried out a thorough research ethics review of your MANET study.

It is University regulation that final research ethics approval can only be granted once the host country has expressed a favourable opinion.

I can confirm that the most recent submission (*Protocol v7 - dated 15th May 2013*) of your application has been approved by the University, subject to a letter from Cameroon REC confirming their approval of the project.

Please note that if any changes to the protocol are required by the REC in Cameroon these will need to be submitted to RESPI for approval.

If you have any queries about this approval and the conditions that apply, please do not hesitate to contact me.

Yours Sincerely

Sarah Wright
Research Governance Officer
Secretary to RESPI

KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY
COLLEGE OF HEALTH SCIENCES

SCHOOL OF MEDICAL SCIENCES / KOMFO ANOKYE TEACHING HOSPITAL

COMMITTEE ON HUMAN RESEARCH, PUBLICATION AND ETHICS

Our Ref: CHRPE/AP/112/19

30th January, 2019.

Prof. Richard Odame Phillips
Department of Medicine
Komfo Anokye Teaching Hospital
Post Office Box 1934
KUMASI

Dear Sir,

LETTER OF APPROVAL

Protocol Renewal: ***"A Study of Hepatitis B Co-Infection among HIV-Positive Patients in KATH."***

Proposed Site: ***Department of Medicine, Komfo Anokye Teaching Hospital.***

Sponsor: ***The Leverhulme-Royal Society Africa Award.***

Your submission to the Committee on Human Research Publication and Ethics on renewal to protocol CHRPE/017/18 of 30th January, 2018 refers.

The Committee has considered the ethical merit of your proposed renewal and approved it. The approval is for a fixed period of one year, beginning 30th January, 2019 to 29th January, 2020 renewable thereafter. The Committee may however, suspend or withdraw ethical approval at any time if your study is found to contravene the approved protocol.

Data gathered for the study should be used for the approved purposes only. Permission should be sought from the Committee if any amendment to the protocol or use, other than submitted, is made of your research data.

The Committee expects a report on your study annually or at the close of the project, whichever one comes first. It should also be informed of any publication arising from the study.

Yours faithfully,

Rev. Prof. John Appiah-Poku.
Honorary Secretary
FOR: CHAIRMAN

Room 7 Block J, School of Medical Sciences, KNUST, University Post Office, Kumasi, Ghana
Phone: +233 3220 63248 Mobile: +233 20 5453785 Email: chrpe.knust.kath@gmail.com / chrpe@knust.edu.gh

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Fax: +44(0)151 705 3370
www.lstmed.ac.uk

Monday, 31 July 2017

Dear Dr Kayuni,

Re. Research Protocol (17-018) Multidisciplinary studies on Male Genital Schistosomiasis (MGS): Its prevalence, morbidity and management and interactions with HIV viral shedding among adult fisherman along Lake Malawi shores in Mangochi, Malawi

Thank you for your letter of 28 July 2017 providing the necessary in-country approvals for this project. I can confirm that the protocol now has formal ethical approval from the LSTM Research Ethics Committee.

The approval is for a fixed period of three years and will therefore expire on 30 July 2020. The Committee may suspend or withdraw ethical approval at any time if appropriate.

Approval is conditional upon:

- Continued adherence to all in-country ethical requirements.
- Notification of all amendments to the protocol for approval before implementation.
- Notification of when the project actually starts.
- Provision of an annual update to the Committee.
  Failure to do so could result in suspension of the study without further notice.
- Reporting of new information relevant to patient safety to the Committee
- Provision of Data Monitoring Committee reports (if applicable) to the Committee

Failure to comply with these requirements is a breach of the LSTM Research Code of Conduct and will result in withdrawal of approval and may lead to disciplinary action. The Committee would also like to receive copies of the final report once the study is completed. Please quote your Ethics Reference number with all correspondence.

Yours sincerely

Angela Obasi

Dr Angela Obasi
Chair
LSTM Research Ethics Committee

Telephone: + 265 789 400
Facsimile: + 265 789 431

All Communications should be addressed to:

The Secretary for Health and Population

In reply please quote No.

.....

MINISTRY OF HEALTH AND POPULATION

P.O. BOX 30377
LILONGWE 3
MALAWI

22nd July, 2018

Sekeleghe Kayuni
University of Liverpool
UK

Dear Sir,

Re: Protocol # 17/05/1805: Multidisciplinary Studies On Male Genital Schistosomiasis (Mgs): Its Prevalence, Morbidity And Management And Interactions With HIV Viral Shedding Among Adult Fisherman Along Lake Malawi Shores In Mangochi, Malawi

Thank you for the above titled proposal that you submitted to the National Health Sciences Research Committee (NHSRC) for review. Please be advised that the NHSRC has **reviewed** and **approved** your application for continuation of the above titled study.

- **APPROVAL NUMBER : 1805**
- The above details should be used on all correspondences, consent forms and documents as appropriate.
- **APPROVAL DATE : 23/07/2018**
- **EXPIRATION DATE**
  This approval expires on **22/07/2019**. After this date, this project may only continue upon renewal. For purposes of renewal, a progress report on a standard form obtainable from the NHSRC Secretariat should be submitted one month before the expiration date for continuing review.
- **SERIOUS ADVERSE EVENT REPORTING:** All serious problems having to do with subject safety must be reported to the NHSRC within 10 working days using standard forms obtainable from the NHSRC Secretariat.
- **MODIFICATIONS**: Prior NHSRC approval using forms obtainable from the NHSRC Secretariat is required before implementing any changes in the protocol (including changes in the consent documents). You may not use any other consent documents besides those approved by the NHSRC.
- **TERMINATION OF STUDY**: On termination of a study, a report has to be submitted to the NHSRC using standard forms obtainable from the NHSRC Secretariat.
- **QUESTIONS**: Please contact the NHSRC on phone number *+265 888 344 443* or by email on mohdoccentre@gmail.com.
- **OTHER**: Please be reminded to send in copies of your final research results for our records (Health Research Database).

SECRETARY FOR HEALTH
2018 -07- 22
P.O BOX 30377, CAPITAL
LILONGWE 3

Kind regards from the NHSRC Secretariat.

.....
For: **CHAIRPERSON, NATIONAL HEALTH SCIENCES RESEARCH COMMITTEE**

Promoting Ethical Conduct of Research

NHS
National Research Ethics Service

Moorfields & Whittington Research Ethics Committee

Royal Free Hospital
Pond Street
London
NW3 2QG

Tel: 020 7794 0552

Dr Anna Geretti
Consultant Virologist and Honorary Senior Lecturer
Dept of Virology
Royal Free Hospital Pond Street
London, NW3 2QG

22 June 2009

Dear Dr Geretti

Study title: **Predicting CD4 cell count slopes in Human immunodeficiency virus(HIV) infected patients: the effects of virological and immunological determinants**
REC reference: **08/H0721/3**

Thank you for sending the progress report for the above study dated 15 June 2009. The report will be reviewed by the Chair of the Research Ethics Committee, and I will let you know if any further information is requested.

The favourable ethical opinion for the study continues to apply for the duration of the research until 31 December 2010.

08/H0721/3:	Please quote this number on all correspondence

Yours sincerely

KClarke

Ms Kathy Clark
Committee Co-ordinator

E-mail: katherine.clark@royalfree.nhs.uk

Copy to: Dr Clara Kalu, R&D Department

NHS
National Research Ethics Service
South East London Research Ethics Committee 3
(formerly King's College Hospital Research Ethics Committee)
1st Floor Camberwell Building
King's College Hospital
94 Denmark Hill
London SE5 9RS

Telephone: 020 3299 3923
Facsimile: 020 3299 5085

03 June 2010

Dr Anna Maria Geretti
Lead Virology Consultant
Royal Free Hospital NHS Trust and UCL Medical School
Department of Virology
Pond Street
Hampstead
London NW3 2QG

Dear Dr Geretti

Study Title:	**Kinetics of HIV-1 proviral DNA load during suppressive antiretroviral therapy**
REC reference number:	**10/H0808/75**
Protocol number:	**10/0108**

The Research Ethics Committee reviewed the above application at the meeting held on 19 May 2010. Thank you for attending and confirming the following:

- **The Chief Investigator confirmed that this is a cross-sectional study, and the aim is to see a continued progression.**
- **The Residual bilinear load is seen to be more sensitive than the RA test i.e. 70% of participants will have detectable RA of less than 50 copies.**
- **The Viral DA load will be compared to the residual RA load, where 2 measurements will be taken over a 6 month period.**
- **The study will be able to quantify things more sensitively. The Plasma RA results will not be used or repeated, as these results are not useful for clinical data analysis.**
- **The Chief Investigator confirmed that the results for analysis of repeatability are a comparison of different groups.**
- **It was confirmed that as there is no risk to the participants, they do not need to be covered by insurance.**

Ethical opinion

The members of the Committee present gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of

South East London REC 3

Attendance at Committee meeting on 19 May 2010

Committee Members:

Name	*Profession*	*Present*
Dr Colin Ball	Consultant Paediatrician	Yes
Dr Will Bernal	Consultant Liver Intensivist	Yes
Professor Rebecca Cassidy	Senior Lecturer	Yes
Mr Kai Lok Chan	Assistant Director of Pharmacy	Yes
Ms Madeleine Colvin	Barrister	Yes
Dr Nora Donaldson	Head of Clinical Research Statistics	Yes
John Fowler	Lay member	Yes
Professor John Garrett	Professor Emeritus, Oral Pathology	Yes
Dr Patrick Gordon	Consultant Rheumatologist	Yes
Dr David Jewitt	Consultant Cardiologist	Yes
Mrs Carole Jordan	Senior Nurse	No
Dr Thomas Kabir	MHRN Service Users in Research Coordinator	No
Dr Mike Philpot	Consultant Old Age Psychiatrist	Yes
Ms Catherine Walton	Consultant Midwife	Yes

Also in attendance:

Name	*Position (or reason for attending)*
Miss Juliet Kirk-Buaku	Committee Coordinator

Written comments received from:

Name	*Position*
Dr Colin Ball	Consultant Paediatrician
Ms Madeleine Colvin	Barrister

the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

For NHS research sites only, management permission for research ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk. Where the only involvement of the NHS organisation is as a Participant Identification Centre, management permission for research is not required but the R&D office should be notified of the study. Guidance should be sought from the R&D office where necessary.

Sponsors are not required to notify the Committee of approvals from host organisations.

- **The Chief Investigator to add the following sentence on the PIS: "the results will not be used for clinical management".**
- **The reference to CERES mentioned on study documents to be removed, as this organisation no longer exists.**

It is responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

You should notify the REC in writing once all conditions have been met (except for site approvals from host organisations) and provide copies of any revised documentation with updated version numbers.

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Covering Letter		09 April 2010
REC application		09 April 2010
Protocol Subst. Amend V2. 25/7/2010	~~1~~ ✓	~~12 March 2010~~
Investigator CV		26 March 2010
Participant Information Sheet Subst Amend V3 25/7/2010	~~1~~	17 March 2010
Participant Consent Form	1	17 March 2010
GP/Consultant Information Sheets	1 ✓	12 March 2010
Evidence of insurance or indemnity	7	15 March 2010
Letter from Sponsor		09 April 2010
Referees or other scientific critique report		06 April 2010
Letter from Statistician	✓	06 April 2010

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

10/H0808/75	Please quote this number on all correspondence

With the Committee's best wishes for the success of this project

Yours sincerely

Dr David Jewitt
Chair

Email: juliet.kirk-buaku@nhs.net

Enclosures: *List of names and professions of members who were present at the meeting and those who submitted written comments*

"After ethical review – guidance for researchers" SL-AR2 for other studies

Copy to: *Mr Dave Wilson – R&D, UCL*

NHS
Health Research Authority
London – Dulwich Research Ethics Committee
Health Research Authority
Skipton House
80 London Road
London SE1 6LH

Tel: 020 7104 8113
Email: nrescommittee.london-dulwich@nhs.net

24 February 2016

Dr Anna Maria Geretti
Professor of Virology and ID
University of Liverpool
Institute of Infection and Global Health
APEX Building, 8 West Derby Road
Liverpool L69 7BE

Dear Dr Geretti

Study Title:	**Kinetics of HIV-1 proviral DNA load during suppressive antiretroviral therapy**
REC reference:	**10/H0808/75**
Protocol number:	**10/0108**
IRAS project ID:	**50399**

Thank you for sending the progress report for the above study dated 23 February 2016. The report will be reviewed by the Chair of the Research Ethics Committee, and I will let you know if any further information is requested.

The favourable ethical opinion for the study continues to apply for the duration of the research as agreed by the REC.

Where research involves the use of human tissue in England, Wales or Northern Ireland, legal authority to hold the tissue under the terms of the ethical approval remains in place for the duration of the approved project.

10/H0808/75:	**Please quote this number on all correspondence**

Yours sincerely

Michael Higgs

Michael Higgs
REC Manager

APPENDIX 4: NON-COMMERICAL MATERIAL TRANSFER AGREEMENT

NON-COMMERICAL MATERIAL TRANSFER AGREEMENT

BETWEEN: The University of Liverpool, The Foundation Building, 765 Brownlow Hill, Liverpool, L69 7ZX (the **"University"**);

AND: Liverpool School of Tropical Medicine (the **"Provider"**);

Upon: Professor Anna Maria Geretti (the "**Principal Investigator**"), an employee of the University, requesting plasma/semen for use at the University's premises in the project entitled "**Multidisciplinary studies on Male Genital Schistosomiasis (MGS): Its prevalence, morbidity and management and interactions with HIV viral shedding among adult fisherman along Lake Malawi shores in Mangochi, Malawi"**

The Provider confirms it is willing to supply the Material subject to the following terms.

It is Agreed by the parties as follows:

1. DEFINITIONS AND INTERPRETATIONS

1.1. **"Confidential Information"** means all information including without limitation all ideas, techniques, processes, know-how, routines, specifications, formulae, drawings, methods and other knowledge concerning the Material and the use of the Material in any Replicates or Derivates.

1.2. **"Derivative"** means any material created from the Material that is substantially modified but still represents a non-severable improvement to or amendment of the Material.

1.3. **"Intellectual Property"** shall mean patent applications, patents, trademarks, service marks, registered designs, domain names, applications for any of the foregoing, trade and business names, unregistered trademarks and service marks, know-how, copyrights, rights in designs, rights in databases, rights in inventions, rights in improvements and rights of the same or similar effect or nature, in any part of the world.

1.4. **"Replicate"** means any biological or chemical material representing substantially unmodified copy of all or part of the Material.

2. USE OF THE MATERIALS

2.1. The Material is to be used solely for the Purpose.

2.2. The Provider shall retain all right and title in and to the Material supplied under this Agreement which shall not be distributed or released to any person other than the Principal Investigator and co-workers working under the supervision of the Principal Investigator.

2.3. The Provider agrees that ownership of any Intellectual Property rights arising from use of the Material or any Replicates or Derivatives thereof by the University shall vest in the University. The Provider shall execute all such documents and do all such things as the University may request, at the University's expense, to ensure that such Intellectual Property rights vest in the University.

2.4. The University acknowledges and agrees that the Material or Replicates or Derivatives are not intended for use in humans or animals.

2.5. The University agrees to comply with all restrictions on export from the United Kingdom and re-export from other countries as required by law for the Material, Replicates and Derivatives. Regarding transfers of Material, Replicates, or Derivatives to destinations outside the United Kingdom:

2.5.1. The University assumes all risk and responsibility in connection with complying with applicable foreign law and regulations concerning the import, handling, transportation, storage, use, and misuse or other wrongdoing with respect to the Material, Replicates or Derivatives.

2.6. The Provider may at its discretion provide technical assistance and information with respect to the Material, Replicates or Derivatives as well as other products and procedures associated with use of the Material, Replicates or Derivatives.

3. PUBLICATIONS

3.1. The University will acknowledge the Provider as the source of the Material, Replicates or Derivatives in any and all publications that reference their use.

4. CONFIDENTIALITY

4.1. The University shall not disclose any part of the Confidential Information to any third party without the written permission of the Provider, except where the Confidential Information comes into the public domain without breach of this Agreement by the University; the University was lawfully in possession of the Confidential Information prior to the date of this Agreement; is disclosed with the prior written approval of the Provider; is obtained lawfully from a third party entitled to disclose the same; is required to be disclosed by law or by order of a court of competent jurisdiction.

4.2. The University shall only disclose the Confidential Information to its employees or agents who need to know the same for the Purpose and the University maintains that such employees and agents are obliged by obligations of confidentiality no less stringent than those contained in this Agreement.

4.3. The Provider acknowledges that this Agreement does not intend to prevent publication by the University of the results produced through completion of the Purpose, and the Provider may only reasonably object to publication by the University if the proposed publication contains Confidential Information belonging to it.

4.4. The University shall apply no lesser security or degree of care than that which it applies to its own confidential or proprietary information.

5. INDEMNITY AND LIABILITY

5.1. The University acknowledges that the Material is experimental in nature and the Material and Confidential Information is provided by the Provider without any representation, condition or warranty whatsoever. There is no representation, condition or warranty given by the Provider that the Material or Confidential Information will be fit for the particular purpose required by the Recipient.

5.2. The University shall indemnify the Provider, its directors, employees and representatives from any reasonable loss (with the exception of consequential and/or

indirect losses), claim, damage, injury, expense or other liability resulting from the University's possession, use, storage, transport or disposal of the Material except to the extent that such reasonable loss, claim, damage, injury, expense or other liability is caused or contributed to by the negligence of the Provider.

6. GENERAL

6.1. The Provider warrants that it has received the relevant ethical approval to obtain the Material and that the subjects who have provided the Material did so with informed consent.

6.2. This Agreement shall come into force on the date of the last signature to the Agreement, and shall expire 5 years from that date. Either party may terminate this Agreement upon 30 days written notice to the other.

6.3. The University may terminate this Agreement immediately upon written notice to the Provider if the Provider breaches any of the terms of this Agreement. Failure by the University to immediately terminate the Agreement upon notice of such a breach shall not constitute an acceptance of the breach or a waiver of the University's right to terminate the Agreement for the breach in future.

6.4. If any provision of this Agreement is declared void or unenforceable by a Court of competent jurisdiction it shall be severed from the Agreement and the remaining provisions shall continue to the fullest extent permitted by law.

6.5. Nothing in this Agreement creates a relationship of employment, agency or partnership between the parties.

6.6. The Provider shall not be entitled to assign this Agreement without the express written permission of the University.

6.7. This Agreement contains the entire agreement between the parties. No amendments or modifications to this agreement will be of any effect unless in writing signed by authorised representatives of both parties.

6.8. This Agreement is subject to the laws of England and both parties hereby submit to the exclusive jurisdiction of the English Courts.

Signed by and on behalf of The University of Liverpool acting by a duly authorised signatory:)

Name:

Date: 30/04/2019

Signed by and on behalf of LSTM (provider) acting by a duly authorised signatory)

Name: JRS TOTHARD

Date: 30.4.2019

BETWEEN: The University of Liverpool, The Foundation Building, 765 Brownlow Hill, Liverpool, L69 7ZX (the **"University")**;

AND: The Chantal Biya International Reference Centre for Research on Prevention and Management of HIV/AIDS (CIRCB) (the **"Provider"**);

Upon: Professor Anna Maria Geretti (the "**Principal Investigator**"), an employee of the University, requesting plasma (the "**Material**") for use at the University's premises in the project entitled *The MANET: Monotherapy in Africa – Evaluations of New Therapy* (the "**Purpose**"). The Provider confirms it is willing to supply the Material subject to the following terms.

It is agreed by the parties as follows:

1. DEFINITIONS AND INTERPRETATIONS

1.1. **"Confidential Information"** means all information including without limitation all ideas, techniques, processes, know-how, routines, specifications, formulae, drawings, methods and other knowledge concerning the Material and the use of the Material in any Replicates or Derivates.

1.2. **"Derivative"** means any material created from the Material that is substantially modified but still represents a non-severable improvement to or amendment of the Material.

1.3. **"Intellectual Property"** shall mean patent applications, patents, trademarks, service marks, registered designs, domain names, applications for any of the foregoing, trade and business names, unregistered trademarks and service marks, know-how, copyrights, rights in designs, rights in databases, rights in inventions, rights in improvements and rights of the same or similar effect or nature, in any part of the world.

1.4. **"Replicate"** means any biological or chemical material representing substantially unmodified copy of all or part of the Material.

2. USE OF THE MATERIALS

2.1. The Material is to be used solely for the Purpose.

2.2. The Provider shall retain all right and title in and to the Material supplied under this Agreement which shall not be distributed or released to any person other than the Principal Investigator and co-workers working under the supervision of the Principal Investigator.

2.3. The Provider agrees that ownership of any Intellectual Property rights arising from use of the Material or any Replicates or Derivatives thereof by the University shall vest in the University and the Provider. The Provider shall execute all such documents and do all such things as the University may request, at the University's expense, to ensure that such Intellectual Property rights vest in the University and the Provider.

2.4. The University acknowledges and agrees that the Material or Replicates or Derivatives are not intended for use in humans or animals.

2.5. The University agrees to comply with all restrictions on export from the United Kingdom and re-export from other countries as required by law for the Material, Replicates and Derivatives. Regarding transfers of Material, Replicates, or Derivatives to destinations outside the United Kingdom:

2.5.1. The University assumes all risk and responsibility in connection with complying with applicable foreign law and regulations concerning the import, handling, transportation, storage, use, and misuse or other wrongdoing with respect to the Material, Replicates or Derivatives.

2.6. The Provider may at its discretion provide technical assistance and information with respect to the Material, Replicates or Derivatives as well as other products and procedures associated with use of the Material, Replicates or Derivatives.

3. PUBLICATIONS

3.1. The University will acknowledge the Provider as the source of the Material, Replicates or Derivatives in any and all publications that reference their use.

4. CONFIDENTIALITY

4.1. The University shall not disclose any part of the Confidential Information to any third party without the written permission of the Provider, except where the Confidential Information comes into the public domain without breach of this Agreement by the University; the University was lawfully in possession of the Confidential Information prior to the date of this Agreement; is disclosed with the prior written approval of the Provider; is obtained lawfully from a third party entitled to disclose the same; is required to be disclosed by law or by order of a court of competent jurisdiction.

4.2. The University shall only disclose the Confidential Information to its employees or agents who need to know the same for the Purpose and the University maintains that such employees and agents are obliged by obligations of confidentiality no less stringent than those contained in this Agreement.

4.3. The Provider acknowledges that this Agreement does not intend to prevent publication by the University of the results produced through completion of the Purpose, and the Provider may only reasonably object to publication by the University if the proposed publication contains Confidential Information belonging to it.

4.4. The University shall apply no lesser security or degree of care than that which it applies to its own confidential or proprietary information.

5. INDEMNITY AND LIABILITY

5.1. The University acknowledges that the Material is experimental in nature and the Material and Confidential Information is provided by the Provider without any representation, condition or warranty whatsoever. There is no representation, condition or warranty given by the Provider that the Material or Confidential Information will be fit for the particular purpose required by the Recipient.

5.2. The University shall indemnify the Provider, its directors, employees and representatives from any reasonable loss (with the exception of consequential and/or indirect losses), claim, damage, injury, expense or other liability resulting from the University's possession, use, storage, transport or disposal of the Material except to the extent that such reasonable loss, claim, damage, injury, expense or other liability is caused or contributed to by the negligence of the Provider.

6. GENERAL

6.1. The Provider warrants that it has received the relevant ethical approval to obtain the Material and that the subjects who have provided the Material did so with informed consent.

6.2. This Agreement shall come into force on the date of the last signature to the Agreement, and shall expire 5 years from that date. Either party may terminate this Agreement upon 30 days written notice to the other.

6.3. The University may terminate this Agreement immediately upon written notice to the Provider if the Provider breaches any of the terms of this Agreement. Failure by the University to immediately terminate the Agreement upon notice of such a breach shall not constitute an acceptance of the breach or a waiver of the University's right to terminate the Agreement for the breach in future.

6.4. If any provision of this Agreement is declared void or unenforceable by a Court of competent jurisdiction it shall be severed from the Agreement and the remaining provisions shall continue to the fullest extent permitted by law.

6.5. Nothing in this Agreement creates a relationship of employment, agency or partnership between the parties.

6.6. The Provider shall not be entitled to assign this Agreement without the express written permission of the University.

6.7. This Agreement contains the entire agreement between the parties. No amendments or modifications to this agreement will be of any effect unless in writing signed by authorised representatives of both parties.

6.8. This Agreement is subject to the laws of England and both parties hereby submit to the exclusive jurisdiction of the English Courts.

Signed by and on behalf of **The University of Liverpool** acting by a duly authorised signatory:

Name: Anna Maria Geretti

Date: 21/05/2013

Signed by and on behalf of **The Chantal Biya International Reference Centre (CIRCB)** acting by a duly authorised signatory:

Name: Charles Koufanack

Date: 21/05/2013

NON-COMMERICAL MATERIAL TRANSFER AGREEMENT

BETWEEN: The University of Liverpool, The Foundation Building, 765 Brownlow Hill, Liverpool, L69 7ZX (the **"University"**);

AND: Kwame Nkrumah University of Science and Technology College of Health Sciences (the **"Provider"**);

Upon: Professor Anna Maria Geretti (the "**Principal Investigator**"), an employee of the University, requesting Serum and Plasma (the "**Material**") for use at the University's premises in the project entitled "A study of Hepatitis B Co-infection Among HIV-Positive Patients in KATH" (the "**Purpose**"). The Provider confirms it is willing to supply the Material subject to the following terms.

It is Agreed by the parties as follows:

1. DEFINITIONS AND INTERPRETATIONS

1.1. **"Confidential Information"** means all information including without limitation all ideas, techniques, processes, know-how, routines, specifications, formulae, drawings, methods and other knowledge concerning the Material and the use of the Material in any Replicates or Derivates.

1.2. **"Derivative"** means any material created from the Material that is substantially modified but still represents a non-severable improvement to or amendment of the Material.

1.3. **"Intellectual Property"** shall mean patent applications, patents, trademarks, service marks, registered designs, domain names, applications for any of the foregoing, trade and business names, unregistered trademarks and service marks, know-how, copyrights, rights in designs, rights in databases, rights in inventions, rights in improvements and rights of the same or similar effect or nature, in any part of the world.

1.4. **"Replicate"** means any biological or chemical material representing substantially unmodified copy of all or part of the Material.

2. USE OF THE MATERIALS

2.1. The Material is to be used solely for the Purpose.

2.2. The Provider shall retain all right and title in and to the Material supplied under this Agreement which shall not be distributed or released to any person other than the Principal Investigator and co-workers working under the supervision of the Principal Investigator.

2.3. The Provider agrees that ownership of any Intellectual Property rights arising from use of the Material or any Replicates or Derivatives thereof by the University shall vest in the University. The Provider shall execute all such documents and do all such things as the University may request, at the University's expense, to ensure that such Intellectual Property rights vest in the University.

2.4. The University acknowledges and agrees that the Material or Replicates or Derivatives are not intended for use in humans or animals.

2.5. The University agrees to comply with all restrictions on export from the United Kingdom and re-export from other countries as required by law for the Material, Replicates and Derivatives. Regarding transfers of Material, Replicates, or Derivatives to destinations outside the United Kingdom:

2.5.1. The University assumes all risk and responsibility in connection with complying with applicable foreign law and regulations concerning the import, handling, transportation, storage, use, and misuse or other wrongdoing with respect to the Material, Replicates or Derivatives.

2.6. The Provider may at its discretion provide technical assistance and information with respect to the Material, Replicates or Derivatives as well as other products and procedures associated with use of the Material, Replicates or Derivatives.

3. PUBLICATIONS

3.1. The University will acknowledge the Provider as the source of the Material, Replicates or Derivatives in any and all publications that reference their use.

4. CONFIDENTIALITY

4.1. The University shall not disclose any part of the Confidential Information to any third party without the written permission of the Provider, except where the Confidential Information comes into the public domain without breach of this Agreement by the University; the University was lawfully in possession of the Confidential Information prior to the date of this Agreement; is disclosed with the prior written approval of the Provider; is obtained lawfully from a third party entitled to disclose the same; is required to be disclosed by law or by order of a court of competent jurisdiction.

4.2. The University shall only disclose the Confidential Information to its employees or agents who need to know the same for the Purpose and the University maintains that such employees and agents are obliged by obligations of confidentiality no less stringent than those contained in this Agreement.

4.3. The Provider acknowledges that this Agreement does not intend to prevent publication by the University of the results produced through completion of the Purpose, and the Provider may only reasonably object to publication by the University if the proposed publication contains Confidential Information belonging to it.

4.4. The University shall apply no lesser security or degree of care than that which it applies to its own confidential or proprietary information.

5. INDEMNITY AND LIABILITY

5.1. The University acknowledges that the Material is experimental in nature and the Material and Confidential Information is provided by the Provider without any representation, condition or warranty whatsoever. There is no representation, condition or warranty given by the Provider that the Material or Confidential Information will be fit for the particular purpose required by the Recipient.

5.2. The University shall indemnify the Provider, its directors, employees and representatives from any reasonable loss (with the exception of consequential and/or indirect losses), claim, damage, injury, expense or other liability resulting from the University's possession, use, storage, transport or disposal of the Material except to the

extent that such reasonable loss, claim, damage, injury, expense or other liability is caused or contributed to by the negligence of the Provider.

6. GENERAL

6.1. The Provider warrants that it has received the relevant ethical approval to obtain the Material and that the subjects who have provided the Material did so with informed consent.

6.2. This Agreement shall come into force on the date of the last signature to the Agreement, and shall expire 10 years from that date. Either party may terminate this Agreement upon 30 days written notice to the other.

6.3. The University may terminate this Agreement immediately upon written notice to the Provider if the Provider breaches any of the terms of this Agreement. Failure by the University to immediately terminate the Agreement upon notice of such a breach shall not constitute an acceptance of the breach or a waiver of the University's right to terminate the Agreement for the breach in future.

6.4. If any provision of this Agreement is declared void or unenforceable by a Court of competent jurisdiction it shall be severed from the Agreement and the remaining provisions shall continue to the fullest extent permitted by law.

6.5. Nothing in this Agreement creates a relationship of employment, agency or partnership between the parties.

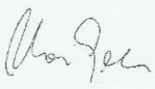
6.6. The Provider shall not be entitled to assign this Agreement without the express written permission of the University.

6.7. This Agreement contains the entire agreement between the parties. No amendments or modifications to this agreement will be of any effect unless in writing signed by authorised representatives of both parties.

6.8. This Agreement is subject to the laws of England and both parties hereby submit to the exclusive jurisdiction of the English Courts.

Signed by and on behalf of The University of Liverpool acting by a duly authorised signatory:)
)
)

Name: Professor Anna Maria Geretti



Date:12/12/2011

Signed by and on behalf of)
.....RBPhillips..... acting by a duly)
authorised signatory)
)

Name: Dr. Richard Odame Phillips

Date: 12.12.2011

Appendix 5: Other relevant training/qualifications

Certificate

Higher Education Academy

This is to certify that

Adam Abdullahi

has achieved the status of

Associate Fellow

of The Higher Education Academy

in recognition of attainment against the
UK Professional Standards Framework for
teaching and learning support in higher education.

Recognition reference:
PR172065
Date of recognition
22/08/2019

Alison Johns
Chief Executive Advance HE

Nigel Carrington
Chair of the Board of Directors Advance HE

JOHNS HOPKINS
UNIVERSITY

COURSE
CERTIFICATE

03/08/2020

Adam Abdullahi

has successfully completed

Design and Interpretation of Clinical Trials

an online non-credit course authorized by Johns Hopkins University and offered through Coursera

Janet Holbook, PhD, MPH
Department of Epidemiology
Bloomberg School of Public Health
Johns Hopkins University

Lea T. Drye, PhD
Department of Epidemiology
Bloomberg School of Public Health
Johns Hopkins University

Verify at coursera.org/verify/822G3TKHRTSS

Coursera has confirmed the identity of this individual and their participation in the course.