

The impact of HIV specific broadly neutralising antibodies on mucosal immunity and the HIV reservoir

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Declaration

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Abstract

HIV-specific broadly neutralising antibodies (bNAbs) have shown promise for maintaining prolonged antiretroviral therapy (ART)-free viral suppression in people living with HIV. However, it remains unclear if longer-acting variants of bNAbs (LS-bNAbs) achieve adequate levels in tissue to impact the gut-associated latent HIV reservoir.

This thesis explores the impact and safety of two LS-bNAbs amongst participants enrolled in the RIO trial, a double-blinded, placebo-randomised controlled trial. LS-bNAbs were associated with prolonged ART-free viral suppression after stopping ART compared with placebo. LS-bNAbs demonstrate an excellent safety profile. In my thesis I completed a meta-analysis of time to viral re-suppression after analytical treatment interruption (ATI) with or without trial interventions such as bNAbs and highlights the importance of reporting post-ATI viral suppression outcomes.

The work in this thesis provides the first data on the pharmacokinetic profiles of two doses of LS-bNAbs in serum and rectal tissue. I demonstrated lower levels of rectal tissue LS-bNAbs exposures compared to serum in people living with HIV. Modelling the pharmacokinetic-pharmacodynamics interactions provides mechanistic insights and estimates the time to loss of viral control following LS-bNAbs administration. Despite the lower tissue LS-bNAbs, there was no substantial changes in the tissue-associated total HIV DNA reservoir or rectal mucosal immune cell populations at 12 weeks after dosing in participants who received LS-bNAbs remained suppressed.

In summary, this thesis provides evidence that LS-bNAbs are safe and were associated with long-term viral suppression for people treated early in HIV. The extended viral control observed beyond the duration of LS-bNAbs is intriguing and is not explained by LS-bNAbs persistence in tissue. However, despite the lower tissue exposures, LS-bNAbs appeared to be protective against early increases in the tissue HIV reservoir and HIV-associated mucosal immune injury. These findings will inform the development of improved immunotherapeutic strategies to better target the tissue-associated HIV reservoir.

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Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
AE	Adverse events
AIC	Aiken information criteria
AIDS	Acquired immunodeficiency syndrome
AIM	Activation induced markers
AML	Acute myeloid leukaemia
ART	Antiretroviral therapy
ATI	Analytical treatment interruption
AUC	Area under the curve
BCL-2	B-cell leukaemia/lymphoma 2 protein
BHIVA	British HIV Association
BIC	Bayesian information criteria
bNAbs	Broadly neutralising antibodies
bPI	Boosted Protease Inhibitor
CAR	Chimeric antigen receptors
CD4bs	CD4 binding site
C _{max}	Maximum concentration
CMV	Cytomegalovirus
COVID-19	Coronavirus disease 2019
CTL	Cytotoxic T lymphocytes
DC	Dendritic cells
DMSO	dimethylsulfoxide
DNA	Deoxyribonucleic acid
DSI	Digital shear index
dPCR	digital PCR
E0	Baseline effect
EIA	Enzyme immunoassay
EC50	Half maximal effective concentration
EDTA	Ethylenediaminetetraacetic acid
FAE	Follicular-associated epithelium
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
Fc	Fragment crystallisable
FcRn	Neonatal Fc receptor
FcγRs	Fc gamma receptors
GALT	Gastrointestinal associated lymphoid tissue
GI	Gastrointestinal
GvHD	Graft versus host disease
HBSS	Hank's balanced salt solution
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSCT	Haematopoietic stem cell transplant
HTI	HIVACAT T cell immunogen vaccines

HTLV	Human T lymphotropic virus
IC50	Half maximal inhibitory concentration
IC80	Eighty percent maximal inhibitory concentration
ILF	Isolated lymphoid follicles
I _{max}	Maximal fraction of inhibition
IMP	Investigational medicinal product
IFN γ	Interferon- γ
INSTI	Integrase strand transfer inhibitors
ISCA	Integrase single-copy assay
IPDA	Intact proviral DNA assay
LOB/LOD	Limit of blank/detection
LRA	Latency reversal agents
LS-bnAbs	Long-acting broadly neutralising antibodies
LTR	Long terminal repeats
MDA	Multidisplacement amplification
MHC	Major histocompatibility complex
MPER	Membrane proximal external region
MR	Mutation rate
MSM	Men who have sex with men
MVA	Modified vaccinia ankara
NHP	Non-human primate
NK	Natural killer
NNRTI	Non-nucleoside reverse transcriptase inhibitors
NRTI	Nucleoside reverse transcriptase inhibitors
NSV	Non-suppressible viraemia
NOS	Newcastle Ottawa scale
OD	Optical density
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered solution
PCR/ qPCR	Polymerase chain reaction/ quantitative polymerase chain reaction
PEPFAR	President's emergency plan for AIDS relief
PHI	Primary HIV infection
PIS	Patient information sheet
PK	Pharmacokinetics
PK/PD	Pharmacokinetics and pharmacodynamics
PredInt	Predicted interval
ROB2	Cochrane risk of bias 2 tool
POCT	Point of care test
PTC	Post treatment control
PTO	Phosphorothioate
Q4PCR	Quadruplex quantitative PCR assay
QVOA	Quantitative viral outgrowth assay
Q ² VOA	Quantitative and qualitative viral outgrowth assay
RNA	Ribonucleic acid
RPM	Rounds per minute
RT	Reverse transcriptase
SA	Specific activity
SAE	Serious adverse events

SAEM	Stochastic approximation of the expectation maximisation
SAMBA	Stochastic approximation for model building algorithm
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SHIV	Simian Human immunodeficiency virus
SIV	Simian immunodeficiency virus
SMAC/SMACm	Second mitochondrial activator of caspases mimics
STING	Stimulator of interferon genes
T _{CM}	Central memory T cells
TDF/FTC	Tenofovir disoproxil fumarate / emtricitabine
T _{EM}	Effector memory T cells
T _{FH}	Follicular T helper cells
T _{RM}	Tissue resident memory T cells
Th	T helper
TLR	Toll-like receptor
TP	Total protein
U=U	Undetectable equals Untransmissable
VL	Viral load

Chapter 1

Introduction

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1.1 Why a cure for HIV still matters

Human Immunodeficiency Virus (HIV) infection remains a global public health concern, with around 39 million people globally living with HIV (1). In most people who acquire HIV, untreated infection eventually progresses to Acquired Immunodeficiency Syndrome (AIDS), a terminal condition if untreated. The epidemic came to light after cases of *Pneumocystis jirovecii* pneumonia were reported in previously healthy gay men in the USA in 1981 (2). Since then, the advancement of highly effective life-long antiretroviral therapy (ART) has transformed survival for people living with HIV. The life expectancy for someone newly diagnosed with HIV with unrestricted access to ART and healthcare services are now comparable to people living without HIV (3,4). Prompt treatment also reduces new HIV infections, with unequivocal evidence supporting zero risk of onward transmission when people living with HIV are taking ART and with an undetectable plasma viral load below 200 copies/mL (5–7), forming the basis of the Undetectable equals Untransmissible (U=U) campaign.

In spite of this, nearly one in four people living with HIV are unable to access ART (1), with 630 000 HIV-related deaths reported annually. 82% of people living with HIV are from Sub-Saharan Africa and the Asia-Pacific regions (1), where access to HIV treatment and healthcare are often highly dependent on external funders such as the President's Emergency Plan for AIDS Relief (PEPFAR), the global fund, and other government and public donors (8). Continued support from these funders should not be taken for granted, any interruption of the precarious funding pipeline may leave local providers vulnerable and unable to meet the demands of an ageing population living with HIV, particularly in low resource settings (9,10).

Even with increased accessibility of medications through generic formulations and free health services, there are still drawbacks to current ART regimens: adherence requirements to oral medication, uncertain long term safety profiles, treatment fatigue, drug resistance and expense (11). Moreover, people living with HIV report facing stigma associated with their HIV-status regardless of medication adherence, from within their healthcare settings, education, workplace, communities, and self-stigma (12,13).

Recently, long-acting injectable ART options such as cabotegravir and rilpivirine have become available (14,15) but concerns of drug resistance, and virological failure despite complete adherence remain (16). They are not available for all people living with HIV; barriers include pre-existing drug resistance, costs, and their monthly or two-monthly dosing requirements impose additional burden to existing clinic infrastructure, particularly in resource-limited settings (17).

For these reasons, the development of a treatment of HIV that results in a cure or long-term remission without the need for sustained supply of daily oral medications still remains an urgent priority for people living with HIV and health care systems. A cure would be able to reduce stigmatisation and improve quality of life for people diagnosed with HIV, reduce new infections through treatment as prevention strategies, and maintain a sustainable global HIV response for all (18).

1.2 HIV and the latent HIV reservoir

1.2.1 The natural history of HIV

HIV is a blood-borne virus and the most common route of transmission is sexual contact (19–22). Other routes of transmissions may include exposure through any breaches in the mucosal surfaces, blood transfusion procedures, needlestick injuries or sharing injecting equipment, as well as vertical transmission between mother to child (19–27). It is now clear that the risk of transmission of HIV is directly related to the viral load and that virological suppression with ART prevents sexual transmission of HIV (5,6,28,29). In particular, zero phylogenetically linked transmissions were found in 888 serodifferent couples (both heterosexual and men who have sex with men (MSM) couples) in the PARTNER1 study (5), and 782 MSM couples in the PARTNER2 study (6). These findings provided definitive evidence that the risk of HIV acquisition through sexual transmission is effectively zero, and have directly informed the global U=U campaign.

Our understanding of the natural history of HIV infection, particularly around the time of HIV acquisition, has developed through non-human primates (NHP) studies (30) as well as longitudinal studies of people at risk of HIV such as the Females rising through Education, Support, and Health (FRESH) study in South Africa (31) and RV217 and RV254 studies in Thailand (32,33), and the HEATHER study, a cohort study of people diagnosed during acute or primary HIV infection (PHI) (34). PHI describes the early period (commonly defined as the first six months) after HIV acquisition (35), and is often characterised by high levels of HIV viraemia and transiently low CD4 T cell counts (36). Clinically, people diagnosed during PHI may report non-specific symptoms such as fevers, lethargy, myalgia, lymphadenopathy, or a maculopapular rash, and these constellation of symptoms may be termed as ‘seroconversion illness’. (35). During PHI, the establishment of the HIV reservoir occurs rapidly within days of HIV acquisition (37). PHI may be categorised using laboratory biomarkers into different Fiebig stages (38), as summarised by Figure 1.2.1. These stages start with the

hyper-acute stage I where none of the biomarkers apart from HIV RNA is present, followed by the detection of p24 antigen, IgM-sensitive third generation antibodies by enzyme immunoassay (EIA), and second-generation antibodies by EIA, and finally antibodies detected on with a full western blot pattern with the loss of detectable P24 antigen from stage IV onwards. From stage VI onwards, HIV infection could be further subdivided into the end of the early infection phase, and the chronic infection stage. A combination of clinical data and avidity testing of the HIV antibodies is available under a recent HIV test algorithm (RITA), to determine recent HIV infection at the time of diagnosis in the UK (39).

Figure 1.2.1. Fiebig stages of HIV infection

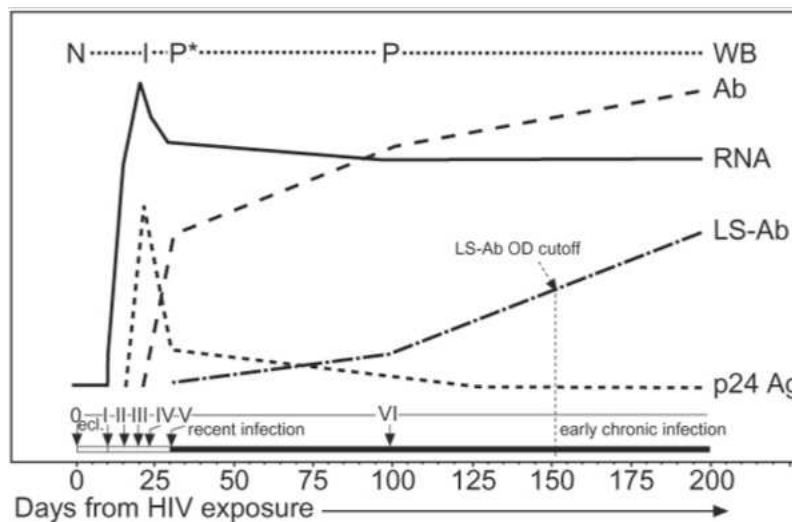


Figure demonstrates progression of HIV laboratory markers. WB, Western blot; Ab, HIV antibody; RNA, HIV RNA; LA-Ab, HIV antibody determined by enzyme immunoassay testing strategy; p24 Ag, HIV p24 antigen; N, Negative western blot; I, indeterminate western blot; P*, positive western blot without p31 band; P, positive western blot with p31 band. Reproduced with permission from Fiebig et al. AIDS (2003) (38).

PHI represents a unique stage of HIV infection during which the interplay of viral dynamics and the initial host antiviral immune responses determine the viral setpoint during the subsequent stages of HIV infection. For example, HIV-specific CD8 T cell expansion and activation following peak plasma viraemia have been

associated with slower disease progression (40,41). However, the early adaptive and pro-inflammatory immune responses during PHI are often dysfunctional (30), including exhaustion and phenotypic skewing of cytotoxic T lymphocytes (CTLs), further impaired by loss of CD4 T helper cells support. Early ART initiation during PHI appears to mitigate the dysfunctional shift of the adaptive immune profile, lowering peripheral CD8 T cell counts to healthy levels compared to people in chronic infection, preventing apoptosis of CTLs and downregulating cytotoxic and activation markers (30,42,43). Thus, while there is widespread consensus to starting ART regardless of CD4 count at the time of HIV diagnosis, there may be additional benefits to consider for rapid or immediate ART initiation for people diagnosed during PHI, and many guidelines recommend discussing and coming to a person-centred decision to consider early ART initiation for people diagnosed during PHI (44,45).

If left untreated, HIV progresses to a chronic asymptomatic stage, with ongoing decline in peripheral CD4 T cells, averaging a drop of 50 – 100 cells/ μ L per year, depending on multiple viral and host factors, such as co-infection with hepatitis C virus, viral strains with tropism to the CXCR4 receptor (46). Once CD4 T cell counts approaches or fall below 200 cells/ μ L, people with HIV become increasingly susceptible to opportunistic infections, such as *Pneumocystis jirovecii*, *Toxoplasmosis gondii*, *Cryptococcus neoformans* (47) to name a few. These infections, and other conditions such as malignancies associated with a severely immunocompromised states, are considered AIDS-defining conditions, and are associated with poor prognosis and eventual fatal progression if left untreated. However, with prompt and sustained treatment with ART, people living with HIV can maintain life expectancies and quality of life comparable to people without HIV (3,4).

1.2.2 HIV replication and the limits of antiretroviral therapy

HIV is a single-stranded ribonucleic acid (RNA) retrovirus, capable of life-long infection and persistence through integration into the genomic material of host cells, and is highly adept at immune evasion. Mature

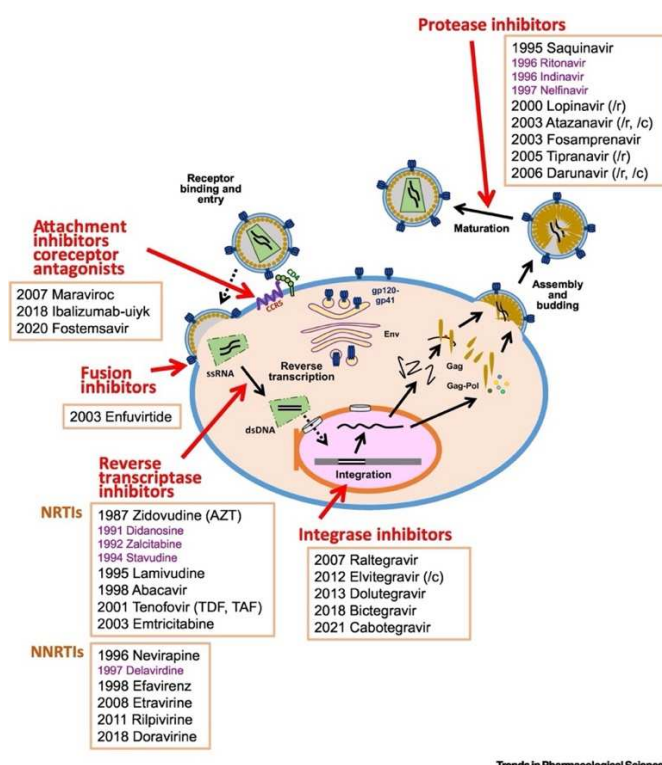
virions are spherical in shape, with a size of 100-120nm, and have an envelope lipid bilayer which includes around 7 - 14 Env glycoprotein spikes (48,49), each made up of a Gp41 and a Gp120 subunit. In addition to the small number of envelope spikes each virion displays, there is also a 'shield' of high density, rapidly shifting glycans that protects sites of potential neutralisation to aid immune evasion (50,51). The viral capsid is made up of the P24 protein, and within the capsid two single-stranded RNA copies are contained along with nucleocapsid (52). Within the 9.2 kilobase HIV genome, the main regions include the Long Terminal Repeats (*LTR*), *gag*, *pol* and *env* genes, along with the HIV accessory genes: *tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu* (HIV-1) or *vpx* (HIV-2). Apart from the *LTR* regions, the nine HIV genes can be broadly classified according to their functions, encoding structural, enzymatic, and accessory proteins.

HIV infects host cells, preferentially CD4 T cells, through interactions with cell-surface makers and the Envelope glycoprotein, made up of two subunits, Gp120 and Gp41 in an heterodimer configuration. Gp120 initially binds to CD4 molecules on the host cell surface, and the interaction allows Gp120 to subsequently bind to either CCR5 or CXCR4 chemokine co-receptors on the cell surface (53). This prompts the structural changes of the HR1 and HR2 domains of Gp41 leading to fusion of viral and cellular membranes, followed by entry into the cell and the uncoating of the virus core after (54). The released viral RNA genome in the cytoplasm, is retrotranscribed into double-stranded complementary deoxyribonucleic acid (DNA) or cDNA by viral reverse transcriptase (RT) (55). The cDNA pre-integration complex enters the nucleus and is subsequently integrated into the host chromosome by viral integrase (56). Viral RNA is then transcribed from this integrated viral DNA, also known as the proviral DNA, using cellular transcription processes (52).

Various ART drug classes target different stages of the HIV life cycle (Figure 1.2.2) to inhibit viral replication or maturation (57). Nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) inhibit the viral enzyme RT, and integrase strand transfer inhibitors (INSTIs) similarly inhibit viral integrase, arresting viral replication prior to proviral integration. Protease inhibitors (PIs) prevent maturation of viral virions by inhibiting proteases necessary for cleaving precursor proteins into

mature virion proteins. Finally, cell attachment and entry are blocked by attachment inhibitors, co-receptor antagonists, or fusion inhibitors. INSTI-containing ART regimens are widely used and second generation INSTIs such as dolutegravir have been associated with genetic barriers and rapid viral suppression (58), therefore are now considered the benchmark drugs against which novel antiretroviral therapies are compared against in modern clinical trials.

Figure 1.2.2: Life cycle of HIV and ART drug classes targets.



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ART alone cannot cure HIV, due to the presence of this long-lived latently infected pool of primarily CD4-positive cells which contain HIV DNA integrated into their nuclear genome. This reservoir is established within blood, gastrointestinal (GI) associated lymphoid tissue (GALT), lymph nodes and genital tract (37,59), but has been described in other tissue compartments throughout the body (60). This reservoir is established rapidly, as early as 10 days after initial acquisition of HIV (37), and even individuals diagnosed and treated

during Fiebig stage I (HIV DNA detected in the absence of p24 antigen and HIV seroconversion, usually corresponding to within two weeks of HIV acquisition) experienced rapid viral load rebound following treatment interruption (61). It has been demonstrated in a NHP model that by starting ART no later than day 3 after viral challenge, seeding of the viral reservoir was attenuated following intrarectal Simian Immunodeficiency Virus (SIV) infection, although even these monkeys eventually experienced a median delayed viral rebound of 21 days after treatment interruption, if they were treated on day 3, compared to a rebound time of 7 days in macaques who were treated later on day 14 (62). In the absence of viral antigen expression and/or targetting, these reservoir cells are effectively invisible to HIV-specific immune responses and persist in a dormant state in the presence of ART.

1.2.3 Characteristics and mechanisms of latent HIV reservoir persistence

Memory CD4 T cells comprise the bulk of the latent HIV reservoir (63). HIV-specific cells have been shown to be preferential targets for HIV infection (64,65). Along with other cells with specific affinities to pathogens such as cytomegalovirus (CMV), *Mycobacterium tuberculosis*, these cells may play an important role in persistence of latently infected reservoir cells due to their risk of antigen exposure, reactivation and clonal expansion during chronic infection (66). HIV latency is established due to the shift from an effector to an inactive memory phenotype, with transient CCR5 up-regulation and inactivation of HIV gene transcription, although the HIV genome remains integrated within the cellular host genome (67). The latent reservoir persists through clonal proliferation of these reservoir memory cells when they are stimulated following antigen exposure and reactivation (68,69). These HIV-containing reservoir cells may be adept at avoiding cell death signals from cytotoxic T lymphocytes (CTL); latently-infected cells that survive ART initiation may as a result have a greater ability to persist after peak viraemia (70). At a cellular level, the up-regulation of B-cell leukaemia/lymphoma 2 protein (BCL-2), an inhibitor of apoptosis (71), and the upregulation of Fas Ligand on HIV-containing cells leading to apoptosis of uninfected CTLs (72) may provide the mechanisms for resisting cell death. Defective virion 'decoys' are also produced by cells containing non-replication competent provirus

which may distract the CTL responses (73). The latter may lead to low-level non-suppressible viraemia (NSV), one study demonstrated all proviruses causing NSV were defective with 5' leader defects (74). The vast majority of HIV proviral DNA within infected reservoir cells contain fatal defects such as deletions or hypermutations in their sequences, rendering them defective and incapable of viral replication (75). Although these defective proviruses are unlikely to generate functional viruses capable of cellular infection, they may be transcriptionally active, generating antigens that may distract and reduce immune responses towards replication-competent virus and the cells hosting them (73).

HIV arises from diverse tissue reservoir sites, with blood and lymphoid tissue within spleen, thymus, lymph nodes, GALT being the predominant sources of viraemia on cessation of ART (60,76,77). However, HIV-containing cells have been found in various other sites, including the central nervous system, lungs, kidneys, liver, adipose tissue, genitourinary tract, and bone marrow (60,76,77). In a peri-mortem study of 6 individuals who provided blood samples before death, and donated whole bodies for post-mortem analysis after death, intact HIV envelope sequences were found throughout 28 anatomical compartments (60). However, tissue sites are challenging to sample, and most analyses on the latent HIV reservoir and the impact of any therapeutics on the size of the reservoir has been focused on blood.

1.2.4 Measuring the latent HIV reservoir

A reliable measurement of intact (or replication-competent) virus amongst the vast amount of defective proviral genome is a key outcome when evaluating cure or remission strategies. In this chapter I will summarise the advantages and disadvantages of existing assays, however there is no consensus on the optimal validated assay to predict clinical outcomes in cure-related studies. Therefore the use of closely monitored analytical treatment interruption (ATI) of ART remains necessary for evaluating novel cure or remission interventions.

There are several existing assays to measure the latent HIV reservoir (78). These assays are summarised in Table 1.2.4. The majority of these assays may be broadly classified as those that quantify replication-competent virus or polymerase-chain reaction (PCR) based techniques. The former usually involves ex-vivo stimulation followed by expansion of cells, and were considered the gold standard, such as the quantitative viral outgrowth assay (QVOA) (63) and its derivatives (78). However, these assays are often costly and labour intensive and underestimate the true replication-competent viral reservoir as not all HIV-containing latent cells are able to be stimulated to produce virus (79). Recent studies have shown people with prolonged viral control following bNAb administration demonstrated a relative reduction in the intact proviral reservoir (80) and may suggest the usefulness of intact proviral reservoir measurements at predicting viral rebound outcomes if accurately and reliably quantified.

Novel PCR based techniques have been developed to measure intact HIV DNA, namely the intact proviral DNA assay (IPDA) (81) and the quadruplex quantitative PCR assay (Q4PCR) (82), with advantages being less labour and cost-intensive, while maintaining sensitivity compared to the previously mentioned QVOA. The IPDA uses digital PCR (dPCR) technology, with probes specific for two highly conserved regions in the HIV proviral sequence to exclude deleted and hypermutated provirus, to screen out 97% of defective provirus. This high throughput, low-cost and rapid platform does not require stimulation or expansion to measure the intact HIV proviral reservoir. However, the assay is unable to perform downstream analyses of the intact

proviral sequences, and may be limited by the highly polymorphic nature of the HIV genome (83). The Q4PCR involves limiting dilution to single genome sequences, followed by using four-probes targeting the conserved regions of packaging site ψ , *gag*, *pol*, *env*, to screen for wells containing two or more probes which are deemed likely intact sequences and sequencing of the single near full length genome (82). The assay is lower throughput, and more costly, time and labour intensive compared to the IPDA, but it allows for downstream phylogenetic interrogation of the intact reservoir. The IPDA and Q4PCR outputs are positively correlated, however the IPDA may overestimate the amount of intact provirus due to amplification bias towards smaller products or probe mismatch, and inefficient amplification within the Q4PCR, along with different cell normalisation denominators, leading to a median 19-fold higher intact provirus reservoir estimates with the IPDA than the Q4PCR (84). Increasing the number of HIV-specific probes to in a multiplex assay may improve accuracy of the IPDA (85,86).

In summary, existing technologies to measure the intact HIV proviral reservoir remain limited by prohibitive technical or time requirements, or inability to provide downstream sequencing of proviral sequences. There remains a need for a high throughput, scaleable assay without requiring excessively large cell numbers, ideally with the capacity for downstream analyses of the HIV and cellular genome and transcriptome at a single-cell level to define the latent HIV reservoir as a target for therapeutic interventions.

Table 1.2.4. List of assays and strategies to estimate HIV reservoir size

Assay	Target measured	Description	Advantages	Disadvantages	Stimulation and/or expansion
Viral outgrowth assays					
Quantitative Viral Outgrowth Assay (QVOA) (87,88)	Frequency of cells induced to produce virus.	Serial dilutions of HIV infected T cells, followed by stimulation and amplification in allogenic T-cell blasts from healthy donors, and measurement of virus produced.	Considered gold-standard to measure the size of the replication-competent HIV reservoir. Clade independent.	Underestimates the replication-competent HIV reservoir size. Large cell numbers required. Time, labour and cost intensive.	Involves ex-vivo stimulation and expansion.
Quantitative and qualitative viral outgrowth assay (Q ² VOA(89)	Frequency of cells induced to produce virus.	Similar to QVOA but also allows sequencing and phenotypic of viruses.	Allows qualitative interrogation in addition to quantification of the HIV reservoir.	Underestimates the replication-competent HIV reservoir size. Large cell numbers required. Time, labour and cost intensive. Possible clade dependence.	Involves ex-vivo stimulation and expansion.
Murine Viral Outgrowth Assay (mVOA) (90)	Presence or absence of replication-competent virus in cells or tissues.	Injection of cells from virally suppressed individuals into immunodeficient mice, and measuring in vivo replication.	Detection of low levels of latent infection. Clade independence.	Multiple animals per sample required for reproducibility. Large cell numbers requirement. Cost intensive.	In vivo expansion.
PCR or Sequencing-based assays measuring intact HIV reservoir					
Intact Proviral DNA Assay (IPDA) (81,85,91,92) (Including traditional two-probe and three or more probes multiplex assays)	Frequency of intact and defective HIV DNA sequences.	Intact HIV proviral DNA detected using two or more probes to conserved regions, using digital PCR.	High throughput Overestimation of the reservoir by ~1.5 fold using two probes, improved by additional probes to increase specificity. No stimulation or expansion required.	Clade dependent Polymorphisms may impact amplification and require alternative primers or probes. Inability to characterise the reservoir sequences or inducibility.	No stimulation or expansion.
Q4PCR(82)	Frequency of intact and defective HIV	Limiting dilution assay where intact HIV proviral DNA is	Highly specific, ability to characterise sequences.	Lower throughput, more time and labour intensive than IPDA.	No stimulation or expansion.

	DNA using near full length amplification.	detected using four probes to conserved regions qPCR and confirmed by sequencing of reactions positive for two or more probes.		Clade dependent Lack of absolute quantification	
Full-length integrated proviral single genome sequencing (FLIP-seq) (79,93)	Frequency of intact and defective HIV DNA using near full length amplification.	Limiting-dilution cell cultures followed by near-full length proviral sequencing.	Highly specific, ability to characterise sequences.	Greater time, labour and cost intensiveness than Q4PCR. May require custom primer design.	No stimulation or expansion.
Proviral ultradeep sequencing (94)	Levels of genetically intact provirus.	The assay uses differences in viral sequences to identify the number of distinct viruses induced to replicate in a culture supernatant from p24+ wells.	Greater cost effectiveness. Does not require limiting dilution.	Potential template resampling and PCR errors. Cost intensive. Clade dependence.	No stimulation or expansion.
Matched integration site and proviral sequencing (MIP-Seq) (95)	Individual proviral sequences and corresponding chromosomal-integration sites.	Near full length HIV sequencing and corresponding matched chromosomal integration site analysis.	Allows interrogation of integration sites and chromosomal positioning of intact proviruses.	Technically complex Cost intensive.	No stimulation or expansion.
Pooled CRISPR inverse PCR sequencing (PCIP-Seq) (96)	Individual long-read proviral sequences and corresponding chromosomal-integration sites.	DNA fragments including the integration sites are sheared by CRISPR guide RNA and primers and circularised. Following PCR the products are combined for long-read sequencing of the proviral DNA and integration sites.	Allows interrogation of integration sites and chromosomal positioning of proviruses.	Partial sequencing of the HIV proviruses captured by this assay. Technically complex Cost intensive.	No stimulation or expansion.
Simultaneous T-Cell Receptor (TCR), integration site, and provirus	Individual proviral sequences, matched integration site and TCR β sequence analysis.	HIV-flow assay followed by near-full length proviral sequencing, integration site analysis, TCR β sequence	Allows in-depth assessment of TCR, integration site and proviral genome.	Unable to assess replication competence. Technically complex Cost-intensive	Stimulation but no expansion.

sequencing (STIP-Seq) (97)		analysis, and phenotyping of the host cells.		Underestimates total inducible HIV reservoir	
Simultaneous Integration site and Provirus sequencing (SIP-Seq) (98)	Individual proviral sequences and matched integration sites.	Whole genome amplification in microfluidic droplets, and tagging of PCR products for full length genome with host cell junctions sequencing.	Allows detection of provirus not recoverable with nested PCR. Allows simultaneous proviral and integration site analysis. More rapid, cost-effective and scalable compared to traditional PCR assays.	Clade dependent. Does not provide information about host cell phenotype and transcriptional activity.	No stimulation or expansion.
HIV proviral unique molecular identifiers (UMI)-mediated long-read sequencing (99)	Frequency of intact and defective HIV DNA using near full-length sequences.	Proviral DNA templates are tagged with dual UMI combinations, followed by long-range PCR amplification and long-read sequencing. Binning of UMI reads allows for high-accuracy consensus sequences to overcome limited single-read accuracy.	High throughput Lower cost compared to FLIP-Seq.	Enrichment of HIV proviral templates by PCR leads to potential amplification bias. Clade dependence. Unable to accurately quantify reservoir clonality.	No stimulation or expansion.
Phenotypic and proviral sequencing (PheP-Seq) (100)	Frequency of cells containing intact and defective provirus, and their matched integration sites.	Antibodies to T cell surface markers are used to tag cells, and a multiplex PCR of genomic HIV DNA and antibody oligonucleotide tags performed. Further non-overlapping regions are amplified along with virus-host chromosomal integration site junctions of large, sequence-identified proviral clones.	Allows single-cell proteogenomic profiling.	Does not provide information on transcriptional activity. Clade dependence. Incomplete proviral sequence coverage.	No stimulation or expansion.

Parallel interrogation of HIV RNA, integration site, and proviral sequencing (PRIP-Seq) (101)	Frequency of cells containing transcriptionally active provirus	Limiting-dilution of cells followed by reverse-transcription of viral RNA and multiple displacement amplification of proviral DNA and chromosomal integration site.	Allows simultaneous analysis of HIV proviral DNA, integration site, and transcriptional activity.	Low throughput, labour intensive, cost-intensive.	No stimulation or expansion.
Assays measuring total HIV DNA or cell-associated HIV RNA					
HIV DNA by qPCR or digital PCR (102–104)	Total levels of HIV DNA	Assays measuring levels of HIV DNA, both intact and defective, using primers and probes targeting a relatively conserved region (e.g. LTR) of the HIV genome, by qPCR or digital PCR.	Rapid, cost-effective, sensitive, cell-sparing, cross-clade applicability.	Excessively overestimates the HIV reservoir, unable to measure translational competence or assess replication competence.	No stimulation or expansion.
Cell-associated HIV RNA by qPCR or digital PCR (105–107)	Levels of HIV RNA transcripts.	Assays measuring levels of HIV RNA, both intact and defective, using primers and probes targeting a relatively conserved region of the HIV genome, by qPCR or digital PCR.	Rapid, cost-effective, sensitive, cell-sparing, cross-clade applicability.	Excessively overestimates the HIV reservoir, unable to measure translational competence or assess replication competence.	No stimulation or expansion.
Focused interrogation of cells by nucleic acid detection and sequencing (FIND-Seq) (108)	Frequency of cells containing HIV DNA	Cells are encapsulated using microfluidics droplets technology for lysis, whole transcriptome RNA sequence recovery, and sorted based on HIV DNA detection.	Allows whole transcriptome profiling.	Not single cell. Does not differentiate between intact and defective provirus.	No stimulation or expansion.
Single-cell assay for transposase accessible chromatin with select antigen	Frequency of cells containing HIV DNA	Single cell Assay of transposon-accessible chromatin sequencing (ATAC-Seq) with simultaneous measurement of cell surface	Phenotyping of cells using cell surface and intracellular protein markers.	Short read sequencing only detects provirus in accessible chromatin. Does not directly measure intactness of proviral sequences,	Stimulated but no expansion.

profiling by sequencing (ASAPseq) (100)		and intracellular protein markers		inferred with alignment towards multiple proviral genes.	
In-situ hybridisation based assays such as DNAscope or RNAscope (109,110)	Spatial cellular location and quantification of HIV.	In situ hybridisation of cells containing total HIV DNA or RNA using immunofluorescent probes.	Allows visualisation, phenotyping, and spatial analyses of tissue-associated HIV reservoir, cost effective, sensitive.	Clade dependence, unable to assess replication competence.	No stimulation or expansion.
Assays measuring inducible transcriptionally or translationally active virus,					
Modified QVOAs (including QVOA p24 SIMOA, QVOA cfRNA) (111,112)	Frequency of cells that can be induced to produce transcriptionally and translationally competent virus.	Detects transcriptionally and translationally active virus.	Requires fewer cells than QVOA.	May overestimate results due to the detection of translationally and transcriptionally active but replication-incompetent virus. Labour and cost intensive. Requires stimulation and expansion.	Involves ex-vivo stimulation and expansion.
Cell-associated p24 protein quantification(113–115)	HIV p24 antigen expression with or without stimulation	Protein-based measurements using either single-molecule immunoassays, or flow cytometry to detect p24-producing cells.	Sensitive, simplicity, time and cost-effective. Low cell requirements. Biologically relevant target for immune response.	May overestimate reservoir size due to the detection of translationally competent but replication-incompetent virus.	Stimulation not necessary, but may be performed without expansion.
Induction-based RNA reactivation assays e.g. TILDA (111,116)	Frequency of cells stimulated to produce HIV Tat/Rev multispliced RNA	Limiting-dilution of cell cultures followed by short stimulation, and two-step qPCR measuring the numbers of cells with HIV (101,117)spliced RNA.	Less complex and more time-effective than the QVOA. Lower cell requirements.	Unable to assess replication competent virus. Cost intensive.	Short ex vivo stimulation without expansion.
Single-cell analysis of inducible virus e.g. FISH-Flow (118)	Frequency of inducible cells with HIV transcription and/or translation.	In situ hybridisation-flow cytometry assay using PrimeFlow RNA probes to detect single cells expressing	Single-cell interrogation of HIV transcription and/or translation, allowing phenotyping of	Unable to measure replication competence.	Stimulation not necessary, but may be performed

		viral transcripts and proteins with or without ex vivo stimulation.	individual cells, clade independence, relative cost-effectiveness		without expansion.
Single cell Sort-Seq (119)	Frequency of inducible cells with HIV transcription.	Stimulation of cells followed by sorting of cells containing HIV RNA using probes.	Allows single-cell transcriptome analysis and paired integration site analysis.	Unable to measure replication-competent reservoir or latent reservoir. Loss of RNA during fixation and permeabilisation.	Stimulation but no expansion.
Other assays					
Ultrasensitive residual viraemia (120)	Low-copy numbers of HIV antigen	Ultrasensitive single-copy real-time reverse transcriptase-initiated PCR for total HIV RNA	Quantification of low-level viral release even in suppressed individuals, cost effective.	Large volumes of plasma or body fluid required, limited dynamic range, clade dependence.	No stimulation or expansion.

1.3 Clinical approaches for HIV cure or remission

1.3.1 Definitions of HIV cure and post-treatment control

A cure for HIV may be either an 'eradication cure', or 'functional cure'. An 'eradication cure' is defined by the eradication of all replication-competent provirus from the cellular and tissue reservoirs of infected cells. To date, seven cases of eradication cure have been described involving haematopoietic stem cell transplants (HSCT) for the treatment of malignancies and will be (121)discussed in detail in section 1.3.3.

The second type of cure may be a 'functional cure' or long-term remission, where the viral reservoir remains but is locked or attenuated to the extent that viral recrudescence is substantially delayed or absent after stopping ART. This may include people who demonstrate post-treatment control (PTC), first described in a cohort by Rosenberg et al. (121) where eight people who were treated during acute HIV demonstrated increased virus-specific T-cell responses during a supervised treatment interruption, with five participants having low-level HIV viraemia under 500 copies/mL after a median of 6.5 months. Subsequently, in the VISCONTI cohort (122), where 14 people living with HIV who started ART during PHI sustained viral control and low or diminishing viral reservoirs after interrupting ART. Since then, a number of small observational and interventional studies (123–130) have described people who experience PTC, however definitions differ across studies with rebound thresholds ranging from 50 to 1000 copies/mL and differing durations of viral control from 6 months to more than 2 years. It remains uncertain how frequently people develop PTC, because current guidelines do not recommend stopping ART. In the CHAMP study 13% of people treated early in HIV infection showed PTC (129), but frequencies differ depending on definitions of PTC used, ranging between 6 – 19% even from the same cohort (131). For the CHAMP study, the definition of PTC is a plasma HIV viral load <50 copies/mL for at least 2/3rd of the time over at least 6 months after stopping ART.

In cure-related clinical trials, participants have demonstrated prolonged viral suppression after stopping ART following administration of immunotherapeutic interventions such as broadly neutralising antibodies

(bNAbs) (80,132). To define and identify these people demonstrating post-interventional drugs control is challenging, due to the difficulty of attributing the prolonged viral control to ART or the subsequent study intervention. In the case of long-acting bNAbs, is even more challenging due to their extended half lives, reported of up to 80 days (133).

1.3.2 Community expectations of HIV cure and cure-related studies

Research towards a HIV cure must involve and be centred around the people most affected by HIV, and address the ethical, social and behavioural issues surrounding the development of a cure and conduct of cure-related studies. People are often motivated to join HIV cure-related studies for altruistic reasons, advancing treatments for the community living with HIV (134,135). Therefore, understanding and incorporating the perspectives of the community as key stake holders from the earliest stages of study design is essential, to inform the feasibility and acceptability (including acceptable risk thresholds) of cure-related studies (136).

Most people living with HIV consider a cure as complete removal of the virus from the body, and often do not see a functional cure as being a true cure, although a welcome advance towards one (137,138). People who were diagnosed and treated during PHI are likely to have smaller and less diverse HIV reservoirs (124) and are often included in cure-related studies due to the possibly lower threshold towards achieving PTC. However, people diagnosed during PHI may have different concerns or perspectives to others living with chronic HIV infection when considering cure-related studies (137,138).

A multi-stakeholder consensus consultation for what criteria the ideal cure target product profile should meet has been published (139). Some optimal features include: a cure that is suitable for all people with HIV as the target population, with a high clinical efficacy where the viral load remains below limits of detection (<50 copies HIV RNA/mL); clinical effectiveness of $\geq 90\%$ of people administered, with below 2% average relapse rate, and a remission duration of more than 3 years or complete eradication of virus; safe with no

grade 3 or 4 adverse events, and exceedingly low frequencies of discontinuation or significant irreversible adverse events. It should ideally be given as a single dose, preferably oral route of administration, and easily portable with no cold chain requirement, and no monitoring required.

1.3.3 Cases of eradication cure

The first person who experienced an eradication cure was Timothy Ray Brown, the Berlin patient (140). He underwent a HSCT for the treatment of acute myeloid leukaemia (AML), with a donor who had a homozygous CCR5 Δ 32 gene mutation conferring resistance to HIV-1 infection. After developing complete chimerism, Mr Brown subsequently stopped ART and remained virus-free for the remainder of his life until his death following a recurrence of AML 12 years later (141,142). Since then, eight further cases of cure or at least long-term remission following HSCT have been described (Table 1.3.3) (143–152).

In all but two cases, these individuals who had predominant R5-tropic virus received stem cells from donors with the CCR5 Δ 32/ Δ 32 mutation, with complete replacement of the host cells with donor cells rendering the recipient resistant to HIV due to the absence of expression of the CCR5 coreceptor. Due to the anecdotal observational nature of these individual cases, as detailed as they have been described, it remains uncertain which aspects are most important towards contributing to a long term cure or remission. For example, six of the seven individuals experienced graft versus host disease (GvHD), which may be crucial to the clearance of the residual latent HIV reservoir (145). However, the life-threatening potential of GvHD and rarity of the CCR5 Δ 32/ Δ 32 mutation means this approach to inducing HIV cure or remission remains challenging to be scalable and generalisable to the wider population living with HIV.

Table 1.3.3: Cases of people who experienced eradication cure or long-term remission of HIV through stem cell transplant

	Timothy Ray Brown – The Berlin patient (140,148)	Adam Castillejo – The London patient (143,149)	Paul Edmonds - The City of Hope patient (147)	The New York patient (144)	Marc Franke - The Dusseldorf patient (145)	The Geneva patient (146)	The 2nd Berlin patient (150)	The Chicago patient (151)	The Oslo patient (152)
Demographics	40yo Caucasian man	40yo Latino man	63yo Caucasian man	Middle-aged, mixed-race woman	53yo Caucasian man	Middle aged Caucasian man	60yo Caucasian man	67yo man	58 yo man
HIV characteristics	HIV diagnosed 1995 ART since 1995	HIV diagnosed 2003 Nadir CD4 290cells/ μ L ART since 2012	HIV diagnosed 1988 Nadir CD4 <100cells/ μ L, no OIs	Diagnosed and treated during Primary HIV infection	HIV diagnosed 2008 Nadir CD4 964cells/ μ L ART since 2010	HIV diagnosed 1990 ART since 2005	HIV diagnosed 2009 ART since 2015	HIV diagnosed 14 years prior to AML diagnosis	HIV diagnosed 2006 ART since 2010
Indication for HSCT	Acute Myeloid Leukaemia	Hodgkin's Lymphoma	Acute Myeloid Leukaemia	Acute Myeloid Leukaemia	Relapse of Acute Myeloid Leukaemia	Biphenotypic sarcoma	Acute Myeloid Leukaemia	Acute Myeloid Leukaemia	Myelodysplastic syndrome
Donor CCR5	CCR5 Δ 32/ Δ 32	CCR5 Δ 32/ Δ 32	CCR5 Δ 32/ Δ 32	CCR5 Δ 32/ Δ 32	CCR5 Δ 32/ Δ 32	CCR5 WT/ WT	CCR5 WT / Δ 32	CCR5 Δ 32/ Δ 32	CCR5 Δ 32/ Δ 32
Intervention	Full-dose chemoradiotherapy Two Allo-HSCT 13-months apart from from same matched unrelated donor due to relapse of AML	Full dose chemotherapy + alemtuzumab (anti-CD52) Allo-HSCT from 9/10 matched unrelated donor.	Reduced-intensity fludarabine/melphalan chemotherapy Allo-HSCT from 10/10 matched unrelated donor	Full dose chemoradiotherapy + Anti-Thymocyte globulin Haplo-Cord HSCT with CD34-selected cells from haploidentical adult donor, followed by 5/8 HLA-matched cord blood units	Reduced-intensity chemotherapy A-HAM chemotherapy and second cycle of HiDAC, followed by 10/10 matched unrelated donor.	Full dose chemotherapy Allo HSCT from 9/10 matched unrelated donor.	Full dose chemotherapy Allo stem cell transplant from 10/10 matched unrelated donor.	Reduced-intensity chemotherapy	
Presence of Graft versus Host Disease	Yes	Yes	Yes	No	Yes	Yes Treated with JAK inhibitor	Yes (mild Grade 1 skin disease)	Yes Grade 2 GVHD, gut	Yes Treated with JAK inhibitor

Evidence of eradication cure	Complete chimerism in peripheral blood No evidence of HIV RNA or DNA in either tissue or blood up to 45 months after transplantation. Complete loss of HIV core-directed antibodies and diminished envelope-specific antibodies on western blot.	Complete chimerism in peripheral blood. No evidence of HIV RNA and only low-levels of 'fossilised' DNA in tissue or blood 30 months after transplantation. Loss of multiple bands of HIV antibodies except for persistent presence of gp160 and gp110/120 antibodies on western blot.	Complete chimerism in peripheral blood. No evidence of HIV RNA, and sporadic, low-level detectable HIV DNA, RNA in PBMC and gut tissue up to 37 months post HSCT Declining humoral responses and no detectable HIV-specific cellular immune response.	Complete chimerism in peripheral blood. No replication-competent latent viral reservoir, and transient trace HIV-1 DNA at 176 weeks post transplant. All other timepoints were negative for HIV DNA and RNA Complete loss of HIV-specific antibodies to all 10 proteins tested by 52 weeks post transplant.	Complete chimerism in peripheral blood. Undetectable HIV proviral DNA and RNA in clinical routine assays, intact proviral, or viral outgrowth assay, but sporadic residual HIV DNA and RNA detected in both blood and tissue up to 77 month after transplant. Progressive loss of humoral and cellular HIV responses.	Complete chimerism in peripheral blood. No detectable HIV RNA, and sporadic detection of proviral DNA. HIV antibodies slightly declined and no HIV-specific T cell responses were detected post treatment interruption.	Complete chimerism in peripheral blood No detectable HIV RNA or DNA in either tissue or blood 5.5 years after treatment interruption. HIV-specific antibodies decreasing and no detectable HIV-specific T cell responses following transplantation.	Complete chimerism in peripheral blood HIV plasma RNA 780copies/ml 2 months after ATI, ART resumed. 2 nd ATI 2 years later, resulted in 10-month and ongoing HIV remission. HIV RNA, DNA remains undetectable in donor cells, and no increase in HIV specific T cell responses.	Complete chimerism in peripheral blood HIV-specific T cell responses were absent Traces of HIV DNA in GALT, no intact HIV DNA in blood or GALT. No replication competent virus on qVOA Waning HIV-specific antibody responses Serum HIV avidity showed weak functional affinity
Duration off ART	2007 till death 2020	Since Sep 2017	Since Jan 2021	3-years post transplant	Since Nov 2018	Since Nov 2021	Since Sep 2018	Since May 2024	Since Nov 2022

1.3.4 Broadly neutralising antibodies

Antibodies targeting the HIV envelope may be able to block viral cell entry in vitro, also known as ‘neutralising’ antibodies. However, these antibodies are often strain-specific, with only 10-30% of people living with HIV who have serum demonstrating broad neutralisation properties, and even fewer (1-10%) develop highly-potent autologous bNAbs (153). Synthetic and exogenously administered HIV-specific bNAbs may present a promising strategy towards long-term remission, and potentially enhance HIV-specific host immunity and reservoir clearance.

BNABs are often IgG antibodies with greater neutralisation breadth and potency towards a variety of HIV strains, as the IgG antibody class have longer half-lives and the capacity to induce Fc effector functions compared to non-IgG options (154). They target major epitopes on the HIV envelope, including the CD4 binding site (CD4bs), N160 glycan-dependent sites associated with V1, V2 loops, the N332 glycan-dependent site on the V3 loop base, and the membrane proximal external region (MPER) on Gp41 (155). Blocking these sites which are required for fusion and entry into host cells results in neutralisation of the virus preventing further cell infection. BNABs often contain mutations in the framework regions of antibodies which increases their potency, by enhancing their flexibility and improving contact with the antigen (156). Another common feature is a long complementarity determining region 3 of the heavy chain, which allows penetration of the HIV glycan shield (157). Finally, approximately 70% of HIV-specific antibodies are polyreactive and able to bind to different antigens with low affinity, a feature not commonly retained during somatic hypermutation and affinity maturation as B cells develop in germinal centres (158).

While most antibodies towards HIV that develop in people living with HIV are strain-specific and are non-neutralising or weakly neutralising (50,159), antibodies with increased breadth and potency towards the HIV envelope spike protein were identified and isolated from people living with long histories of untreated chronic HIV prior to starting ART (160,161). However, there was limited evidence of clinical efficacy of these

first generation neutralisation antibodies towards delaying or preventing viral rebound when used in clinical trials for treatment in people living with HIV despite their neutralising breadth. A combination of three neutralising antibodies (2G12, 2F5, 4E10) displayed only moderate viral suppression, and rapid emergence of resistance to 2G12 (162,163). Single cell antibody cloning methods have allowed the development and purification of a second generation of highly potent bNAbs isolated from people with chronic HIV infection (164).

The highly potent second generation bNAbs were able to demonstrate long-acting viral suppression in animal models when used in combination (165–167). In particular, the combination of 10-1074 and 3BNC117 used in macaques who acquired SHIV_{AD8}, resulted in prolonged viral control between 3-5 weeks after a single dose without evidence of viral escape from both bNAbs in contrast to monotherapy alone (167). These findings were similarly replicated in human clinical trials, which will be discussed below. Animal models also provided the first evidence that bNAbs may induce long-term HIV remission and the possibility of cure. When a triple combination of bNAbs 3BNC117, 10-1074 and PG16 were given to humanised mice early after HIV infection, prolonged viral suppression was observed, with some mice failing to demonstrate any viral rebound and no detectable viral reservoir on completion of the study. This effect was shown to be mediated by the fragment crystallisable (Fc) component of the antibodies rather than through neutralisation alone (168). In macaques who were given combination bNAbs three days after SHIV_{AD8} infection, 6 of 13 remained undetectable after a year, and four demonstrated partial viral control with low-level viraemia. This viral control was shown to be CD8 T cell mediated when viral control was lost following administration of anti-CD8 monoclonal antibodies (169).

In humans, early trials of monoclonal neutralising antibodies, both polyclonal and monoclonal did not achieve substantial viral control (170,171). The promising results of the highly-potent 2nd generation bNAbs used in animal models reignited the use of bNAbs in humans. A spectrum of bNAbs targeting different epitopes on the HIV envelope protein has been used in humans, of which the combination of the two bNAbs

3BNC117 and 10-1074 have been most extensively studied and have progressed to phase two clinical trials with promising results (172). 3BNC117 and 10-1074 are bNAbs that target the CD4bs and V3 glycan supersite respectively on the HIV envelope protein. While trials of monotherapy of 3BNC117 or 10-1074 have demonstrated transient viral suppression during ATI until bNAb serum concentrations fell (173,174) with selection for bNAb resistant variants, the use of multiple bNAbs in combination have proven more robust.

Mendoza et al. demonstrated that when 3BNC117 and 10-1074 were given together to people living with HIV who were virally suppressed on ART, and then subsequently stopped ART, the dual combination was associated with prolonged viral suppression (median 21 weeks) in 7 of the 9 participants who had virus sensitive to both bNAbs at baseline during the ATI and the remaining two did not experience viral rebound beyond the 30 weeks observation period (132). There appeared to be no evidence of dual bNAb resistance from the rebounding viral sequences using the TZM-bl neutralisation assay to investigate rebounding viruses. Viral rebound did not occur when both the serum concentration of bNAbs remained above 10µg/mL. This was the first human study that suggested combination bNAbs targeting different epitopes could induce prolonged viral suppression off ART in people with antibody-sensitive virus.

Subsequent clinical trials have shown similar results with the same dual bNAbs combination (80,175–177), where bNAbs were also associated with prolonged viral suppression during an ATI in people with predicted sensitive virus from proviral *env* sequences or validated neutralisation TZM-bl assay. In these studies a small number participants demonstrated long term viral suppression after stopping ART up to 4 years after receiving the bNAbs 10-1074 and 3BNC117 (177–180). The prolonged control may be mediated by antibody induced HIV-specific T-cell responses and enhanced antibody-dependent cellular cytotoxic (ADCC) effects following bNAb infusion as this effect persisted after plasma antibody levels have declined. This enhanced viral control may be described as an ‘vaccinal’ effect’, mediated by either the bNAbs or their immune complexes (178). The induction of adaptive immune responses by monoclonal antibodies have been described in cancer immunotherapies (181) and other pathogens (182), and larger studies of these bNAbs

may shed further light on the ‘vaccinal effect’ hypothesis. A summary of published or presented therapeutic trials involving bNAbs are presented in table 1.3.4.

Table 1.3.4. List of published or presented bNAb studies

Title	Lead author	Location(s)	Intervention	Number of ATI participants	Acute or chronic HIV	Trial Registry Identifier(s) and citation	ATI Duration	Restart criteria	Results
HIV-1 antibody 3BNC117 suppresses viral rebound in humans during treatment interruption.	Scheid JF. et al.	USA	3BNC117	13	Chronic HIV	NCT02446847 (183)	Until ART restart criteria met (study duration 36 weeks)	ART was resumed when viral load increased to ≥ 200 copies/mL in two consecutive weekly measurements and/or CD4 T cell counts decreased to < 350 cells/ μ L and the result was confirmed with a repeat measurement.	Median time to rebound was 6.7 weeks after 2 doses, and 9.9 weeks after 4 doses.
Relationship between latent and rebound viruses in a clinical trial of anti-HIV-1 antibody 3BNC117.	Cohen YZ. et al.	USA	3BNC117	15	Chronic HIV	NCT02588586 (184)	Until ART restart criteria met (study duration 60 weeks)	"ART was reinitiated after two consecutive plasma viral load measurements exceeded 200 copies/mL or CD4 counts fell below 350/ μ L."	Median time to rebound was 5.5 weeks (range 2 to 17 weeks) after 3-4 doses.
Effect of 3BNC117 and romidepsin on the HIV-1 reservoir in people taking suppressive antiretroviral therapy (ROADMAP): a randomised, open-label, phase 2A trial.	Gruell H. et al.	Denmark, Germany, USA	ROADMAP: romidepsin + 3BNC117	17	Chronic HIV	NCT02850016 (185)	24 weeks	Two consecutive viral load measurements ≥ 200 copies/mL or CD4 T cell count < 350 cells/ μ L.	Median time to viral rebound during ATI was 18 days (IQR 14-28 days) in 3BNC117 + Romidepsin arm, and 28 days (IQR 21 - 35 days) in the romidepsin arm.
Early intervention with 3BNC117 and romidepsin at antiretroviral treatment initiation in people with HIV-1: a phase 1b/2a, randomized trial.	Gunst JD. et al.	Denmark, UK	eCLEAR: romidepsin + 3BNC117	20	Newly diagnosed acute and chronic HIV	NCT03041012 (80)	12 weeks	Two consecutive plasma HIV-1 RNA measurements of $> 5,000$ copies/mL or CD4+ T cell count of < 350 cells.	7/20 did not meet criteria for viral rebound at week 12 after treatment interruption.
Combination therapy with anti-HIV-1 antibodies maintains viral suppression.	Mendoza et al.	Germany, USA	3BNC117 + 10-1074	11	Chronic HIV	NCT02825797 (132)	30 weeks (end of study)	"ART was reinitiated and antibody infusions were discontinued if viremia of > 200 copies/mL was confirmed."	Median time to rebound was 21 weeks (range 5 to > 30 weeks)
Combination anti-HIV antibodies provide sustained virological suppression.	Sneller MC. et al.	USA	3BNC117 + 10-1074	14	Acute HIV	NCT03571204 (177)	28 weeks	"The study participants in group 1 discontinued antibody infusions or placebo and reinitiated	6 of 7 participants met viral rebound in placebo arm compared with none of the 7 participants in the treatment

								ART if they met one of more of the following criteria during the ATI phase: (1) a confirmed >30% decline in baseline CD4+ T cell count; (2) an absolute CD4+ T cell count of <350 cells per µl; or (3) sustained plasma viraemia of >1,000 copies per mL for greater than 4 weeks."	arm after 8 doses during the ATI. Median time off ART was 39.6 weeks (range 9.9 to 49.6 weeks) and 9.4 weeks (range 5.4 to 26 weeks) respectively for the bNAb and placebo arm.
Prolonged viral suppression with anti-HIV-1 antibody therapy.	Gaebler C. et al.	USA	3BNC117 + 10-1074	26	Chronic HIV	NCT03526848 (176)	Group One: 48 weeks; Group Two: 22 weeks	"ART was resumed if there was a confirmed >30% decline in CD4+ T cell count or confirmed CD4+ T cell count decrease to < 350 cells per µl from baseline (day 0). Plasma HIV-1 viral RNA levels were monitored weekly through study week 38 and virologic criteria for ART re-initiation accommodated different phases of the follow-up period: (part A) week 0 to week 26, 2 consecutive plasma HIV-1 RNA levels > 200 copies per mL measured weekly, (part B) week 26 to week 38, sustained (>4 weeks) HIV-1 RNA levels > 1,000 copies per mL measured weekly; and (part C) week 38 to week 48, 2 consecutive plasma HIV-1 RNA levels > 1,000 copies per mL measured	For the 16 group 1 participants, median time to rebound was 28.5 weeks. For individuals who received all seven bNAb infusions, median time to rebound was 32 weeks (range 21 to >48 weeks). But time from last infusion was similar to those who received three infusions. Two remained undetectable past week 48. Group 2 participants maintained viral suppression for a median of 7 weeks (range 4-9 weeks) after ART was discontinued 13 weeks (range 10-15 weeks) after last bNAb infusion.

								every other week. Other ART restart criteria included pregnancy and symptoms consistent with acute retroviral syndrome."	
Impact of a TLR9 agonist and broadly neutralizing antibodies on HIV-1 persistence: the randomized phase 2a TITAN trial.	Gunst JD. et al.	Australia, Denmark, Norway, USA	TITAN: lefitolimod + 3BNC117 + 10-1074	43	Chronic HIV	NCT03837756 (175)	25 weeks	4 weeks with sustained plasma HIV-1 RNA $\geq 1,000$ copies per milliliter or two consecutive measurements $>100,000$ copies per milliliter	Median time to loss of virologic control: placebo/bNAb 17weeks (IQR 12-25), Leftolimod/bNAb 14 weeks (IQR 10-17). Significant delay in loss of virologic control between placebo/bNAb (median delay 12.5 weeks) and lefitolimod/bNAb groups (median delay 9.5 weeks) compared to placebo/placebo group.
Peg-IFN- $\alpha 2b$ + 3BNC117 and 10-1074 keeps HIV at <20 c/ μ L during a 26-week ATI.	Tebas P. et al.	USA	BEAT-2: peginterferon alfa-2b + 3BNC117 + 10-1074	14	Chronic HIV	NCT03588715 (186)	26 weeks	Confirmed return of viral load $>1,000$ copies for six consecutive weeks OR a confirmed CD4+ T-cell count <300 cells/ μ L (or CD4 % decrease greater than 50%).	Greater proportion of participants maintained viral suppression during 26 week ATI compared to non-NNRTI-treated historic controls (HR 16.7, 95%CI 9.9 - 27.9)
Safety and antiviral effect of a triple combination of HIV-1 broadly neutralizing antibodies: a phase 1/2a trial	Julg B. et al.	USA	PGT121 + VRC07-523LS +/- PGDM1400	12	Chronic HIV	NCT03721510 (187)	Open ended with some participants followed off ART until end of the study (week 44)	Viral load ≥ 1000 copies/mL, CD4+ T cell count < 300 cells/ μ L in two consecutive measurements.	10 of 12 (83%) participants maintained virologic suppression <200 copies/mL for duration of bNAb dosing period (6 doses) for at least 28 weeks during ATI
Effect of HIV Antibody VRC01 on Viral Rebound after Treatment Interruption. - NIH 15-I-0140 study	Bar KJ. et al.	USA	VRC01	10	Chronic HIV	NCT02471326 (188)	28 weeks	1) a greater than 30% decline in baseline CD4 T-cell count or an absolute CD4+ T-cell count below 350 cells/mm ³ 2) a sustained (≥ 4 weeks) HIV plasma viremia greater than 5,000 copies/mL	In the NIH trial, the median time to rebound was 5.6 weeks (IQR 4.1 - 5.6 weeks)

								3) any HIV-related symptoms; or 4) pregnancy.	
Effect of HIV Antibody VRC01 on Viral Rebound after Treatment Interruption. - A5340 study	Bar KJ. et al.	USA	VRC01	14	Chronic HIV	NCT02463227 (188)	Open ended but participants not meeting restart criteria after 16 weeks "will be strongly encouraged to resume taking ART"	Confirmed HIV-1 viral load ≥ 1000 copies/mL, or CD4+ T-cell count $<350 \mu\text{L/mL}$ "	In the A5340 trial, 12 of 13 participants had a median time to rebound of 4 weeks (IQR 3-5).
Analytical treatment interruption (ATI) in Peru among MSM, trans & gender non-conforming (GNC) individuals with early ART initiation +/- VRC01 proximate to HIV acquisition: Stakeholder engagement & early clinical data.	Karuna S. et al.	Brazil, Peru, USA	VRC01 (ATI in HVTN 704/HPTN 085 AMP trial participants)	18	Acute HIV	NCT04801758 (189)	Until ART restart criteria are met	CD4 > 250 cells/uL, VL >1000 copies/mL for 4 weeks without 0.5 log decline, HIV-related syndrome, or participant/clinician request.	Median time to meeting ART re-initiation criteria was 7.9 weeks (range 2.7 - 23). These did not differ by VRC01 vs placebo.
Safety and efficacy of VRC01 broadly neutralising antibodies in adults with acutely treated HIV (RV397): a phase 2, randomised, double-blind, placebo-controlled trial.	Crowell TA. et al.	Thailand	VRC01	18	Acute HIV	NCT02664415 (190)	24 weeks, option for additional 24 weeks if ART restart criteria not met	ART was resumed in cases of confirmed HIV-1 RNA $>1,000$ copies/mL or other pre-specified virologic, immunologic, or clinical evidence of disease progression.	Median time to rebound was 33 days (IQR 27-36) in the VRC01 group compared to 14 days (IQR 14-21 days) in the placebo group

The pharmacokinetics, mechanisms of actions, and screening of bNAbs sensitivity will be expanded upon when discussing bNAbs in detail in section 1.4.

1.3.5 Other strategies for cure or remission

Therapeutic vaccination

The greatest challenge for reservoir eradication is the mutable nature of HIV's genetic variability allowing viral escape and immune evasion. Therapeutic vaccine strategies for HIV aim to boost the breadth or magnitude of antigen specificity of HIV-specific T-cell responses in people already living with HIV, with the aim of targeting the latent HIV reservoir. Various vaccine strategies include inactivated virus, viral vectors (modified vaccinia Ankara (MVA), adenovirus, vesicular stomatitis virus, canary pox virus), subunit vaccines with recombinant envelope glycoprotein rgp160, lentiviral vectors, dendritic cell vehicles, DNA and RNA vaccines (191). Although shown to be safe approaches, these vaccines have demonstrated only limited efficacy on viral control. (191,192). The novel HIVACAT T cell immunogen vaccines (HTI) design approach uses T cell response data from more than 1000 individuals with HIV, to direct the T cell response to the most vulnerable sites of HIV (193), eliciting safe and highly immunogenic responses. A combination of DNA.HTI, MVA.HTI, and ChAdOx1.HTI vaccines in early treated people living with HIV, have shown promising results. 40% of vaccine recipients compared to 8% of placebo-recipients remained off ART for 22 weeks without restarting ART (194). The development of in-silico algorithms to design combined mosaic and conserved HIV regions antigens, may provide a better coverage of HIV variants than previous T-cell vaccines. One such vaccine is the tHIVconsvX, which was highly immunogenic in humanised mice (195).

'Kick and Kill' approaches using latency reversal agents (LRAs)

Latently infected cells provide a challenge for the elimination of the HIV reservoir by the immune system due to the lack of expression of target antigens. The 'kick and kill' strategy uses LRAs to stimulate latent HIV-containing reservoir cells to become transcriptionally active, triggering CTL or Natural Killer (NK) cells to

eradicate these antigen-producing cells. Early LRAs were limited by severe toxicities associated with global T-cell activators such as OKT3 and interleukin 2 (196,197). The next generation of LRA classes now include epigenetic histone deacetylase inhibitors (HDACi) (vorinostat, panobinostat and romidepsin); activators of protein kinase C (PKCa), Bryostatins-1; IL-15 superagonists (e.g. N803); toll-like receptor agonists; molecules which mimic second mitochondrial activator of caspases (SMAC mimetics or SMACm) and stimulator of interferon genes (STING) agonists.

The RIVER study, a randomised trial of vorinostat, a histone deacetylase inhibitor LRA, and a therapeutic HIV vaccine (ChAdV63. HIVconsv and MVA.HIVconsv) did not show any difference in measures of the HIV reservoir despite evidence of augmented HIV-specific CD4 and CD8 T cell responses (198). As the participants did not interrupt ART it is unclear if there would have been a clinically relevant impact on post-intervention viral control rates, however the apparent lack of an impact on their HIV reservoir as measured by the total HIV DNA quantity may suggest otherwise. Similarly, trials of other LRAs have not shown substantial changes in the size of the viral reservoir (199).

Current LRAs may not be potent enough to activate more than a fraction of the latent reservoir in *ex vivo* experiments (111). In clinical trials, LRAs are often used at lower doses to avoid off-target toxicities and their associated systemic inflammatory adverse events (200). It is also unclear if the effect of LRAs to activate the latent reservoir penetrates across different anatomical compartments adequately to achieve its desired outcome. NK and CTL functions may be inhibited by LRAs (201), and latent reservoir cells may be inherently resistant to CD8-mediated killing (202). Novel and more potent LRAs are being tested, such as STING agonists which were able to increase SIV RNA and decrease SIV DNA levels *ex vivo* in macaque peripheral blood mononuclear cells (PBMCs) (203). SMACm may be another encouraging avenue, with the SMACm AZD5582 showing minimal side effects and better HIV-specificity in animal models (204). Depletion of CD8-mediated viral suppression may increase LRA effectiveness, as demonstrated when CD8 lymphocyte depletion in SIV-infected rhesus macaques augmented the efficacy of the SMACm AZD5582 (205).

Enhancing T-cell function

CD8 T cells, or CTLs, are a key defence mechanism in control of HIV infection, through the recognition of infected cells via 8-10 amino acid peptides presented on major histocompatibility complex class 1 (MHC class 1) molecules on the cell surface, which triggers the death of these infected cells (206,207). Cellular markers such as PD-1, TIGIT, CTLA-4, Tim-3, or LAG-3, are associated with CD8 T cell exhaustion and immune dysregulation associated with HIV (208). Targeting these inhibitory markers on CD4 T cells may achieve latency reversal. Administration of anti-PD-1 and anti-CTLA-4 antibodies in rhesus macaques appeared to induce reactivation and subsequent reduction of intact HIV proviral reservoir (209) and an increase in the frequency of cells with inducible virus along with a decrease in HIV DNA containing CD4 T cells were observed 24 hours after pembrolizumab, an anti-PD-1 agent, in people living with HIV and cancer (210). However, the use of these agents may be limited by their autoimmune adverse effects (211) and possible irreversibility of immune exhaustion based on epigenetic approaches (212,213).

Engineering enhanced T-cell responses

Although cases of cure or prolonged remission have been achieved following HSCT from donors with CCR5 Δ 32 mutations, the high mortality (41-44%) and complication rates (214) suggest HSCT is not a viable treatment option for most people living with HIV who do not have an existing clinical indication for HSCT. But they raise the possibility of engineering T-cells through gene therapy to achieve HIV remission or cure. These include engineering CD4 CAR-T cells to be resistant to HIV cell entry through the removal of CCR5 receptors, or enhancing HIV-specific CTL responses by modifying chimeric antigen receptors (CAR) in CAR-T cells. The use of CD8 CAR-T cells instead of CD4 CAR-T cells may also avoid infection of the modified CAR T cells, and the HIV-specific CD8 responses may be augmented.

CAR-T cell development strategies often aim to overcome viral antigen escape and increase the potency of these engineered cells. BNAb-based chimeric antigen receptors may broadly recognise over 95% of HIV

strains through targeting conserved sites in the Env protein (215), and the use of bispecific chimeric antigen receptors may recognise conserved epitopes by simultaneous binding CD4 and Gp120 . Although there has been limited progress of CAR-T cells in clinical trials, they have demonstrated the feasibility as a promising area of development (216). Although most studies have demonstrated low numbers of adverse events associated with HIV-specific CAR-T cells particularly if given in people with no or low-level HIV viraemia, cytokine release syndrome remains a concern (216).

Combination approaches

It is likely a successful approach to a cure or remission strategy will require a combination approach, using therapeutic vaccines, latency reversal agents or immune modulators to boost the effector functions of bNAbs or engineered T-cells.

1.3.6 Concerns around ATIs and viral re-suppression

Due to the lack of a validated assay to predict immunological and viral outcomes, clinical trials remain dependent on ATIs to assess the efficacy of cure and remission strategies. Compared to structured treatment interruptions used in earlier studies which involved longer interruptions and were associated with increased morbidity, mortality, disease progression and greater onward HIV transmission risks (217,218), ATIs are more intensively monitored, usually involving a shorter interval of treatment interruption, with more conservative viral and immunological thresholds to restart ART. Key recommendations for conducting studies involving an ATI from a consensus meeting were published in 2019 (219), include initial weekly plasma HIV RNA and clinical symptoms monitoring, fortnightly CD4 count monitoring, and clear and comprehensive ART restart criteria: if requested by the participant or their HIV healthcare provider, pregnancy, symptomatic HIV disease, absolute CD4 count $<350\text{cells}/\mu\text{L}$, HIV RNA $\geq 1000/\text{mL}$ for 4 weeks or more, and absolute HIV RNA $>1000\text{copies}/\text{mL}$. More recently a group of expert, community and key

stakeholders met in Nairobi to update the ATI recommendations to ensure equity diversity and safety for all people with HIV (220).

A systematic review and meta-analysis of 22 studies with ATIs reported an overall proportion of adverse events (AEs) were low (3%, 95%CI 0-7%) (221), with viral ART drug resistance and disease progression being the most common AEs during ATI. Studies with more frequent viral load monitoring (<14days intervals), high baseline CD4 counts and undetectable viral loads (VL) at the time of ATI are safe with negligible risk of adverse events (0%, 95%CI 0-1%) or development of ART drug resistance (0%, 95%CI 0-1%). This was consistent with a previous systematic review by Lau et al. which found fewer AEs associated with more recent studies, where the duration of treatment interruption was shorter (222). Studies investigating the impact of ATIs on the viral reservoir have uncovered mixed results, with some studies suggesting ATIs are not associated with an expansion of HIV reservoirs or immunological abnormalities (223,224), however, a recent study suggest that although the levels of intact proviral DNA normalised to baseline by week 24 after restarting ART in people treated in acute/early HIV infection, the levels of intact HIV DNA remained elevated up to a year post-ATI (225). However, data on safety and viral resuppression rates following ART reinitiation after ATI remains limited.

1.4 Using broadly neutralising antibodies to target the HIV reservoir

1.4.1 Pharmacokinetics of long-acting bNAbs

The prolonged effects of bNAbs may be further extended by modifications to the Fc region to potentially allow 4-6 month dosing strategies. Most bNAbs are IgG monoclonal antibodies, which are cleared from the circulation via endocytosis by endothelial cells and degraded intracellularly. Binding of IgG through their Fc region to the neonatal Fc receptor (FcRn) allows recycling of the endocytosed antibodies into the serum (226). Modification in the Fc region such as the substitutions of Met428Leu and Asn434Ser, also known as LS variants, may increase FcRn binding affinity without affecting other Fc effector functions. This was first demonstrated in bNAbs with VRC01, where the half-life of VRC01LS was shown to be two to four-fold greater than the parental antibody VRC01 when given in both primates and humans (227).

Subsequent trials with 10-1074LS and 3BNC117LS have demonstrated similar extended half-lives. In primates, the median half-life of 3BNC117LS was 2.8 weeks (range 1.5 – 3.2 weeks) compared to a median of 1.4 weeks (range 0.7 – 1.7 weeks) for the non-LS 3BNC117; the median half-life of 10-1074LS was 3.8 weeks (range 3.0 – 4.9 weeks) compared to 1.0 weeks (range 0.6 – 3.2 weeks) for the non-LS 10-1074 (228). Neutralisation activity was similar between LS and non-LS antibodies (228). In previous phase 1 monotherapy studies, 3BNC117LS and 10-1074LS demonstrated half-lives of 62 and 80 days respectively, compared to 17.6 and 24 days for their respective non-LS parental antibodies (133). When given in combination in viraemic individuals, both bNAbs demonstrated lower concentrations compared to those on ART, although the sample size was small (n=6) (133). The long-acting LS-variants brings the possibility of long-acting HIV therapeutics that only require dosing every 4-6 monthly, an advancement over the currently available 2-monthly injectable antiretroviral therapies.

The availability of pharmacokinetics data for bNAbs in tissue are limited, and there is a particular research gap for LS-variant bNAbs when used in people living with HIV. In HIV-negative individuals receiving a shorter

acting non-LS variant bNAb VRC01, vaginal and rectal tissue concentrations were observed to be 10-fold lower than in paired serum samples. Although neutralising bNAb activity within the tissue was seen, breakthrough infections occurred when the tissue was challenged with high dose HIV ex-vivo after day 8, reflecting a decay of functional antibody levels in tissue to cross sub-protective levels more rapidly than in blood (229). It remains a key question if bNAbs achieve therapeutic levels in tissue, particularly the GALT, the largest site of the latent HIV reservoir in the body.

1.4.2 To screen or not to screen: methods for assessing viral sensitivity to bNAbs

Studies of 10-1074 and 3BNC117 have shown that when given as monotherapy, transient reduction with recrudescence of viraemia was observed, and bNAb resistant HIV clones were selected for when the rebound virus were sequenced (230,231). However, when individuals were given the dual bNAbs 10-1074 and 3BNC117 in combination, those with sensitive virus as determined by the TZM-bl neutralisation assay, demonstrated prolonged viral suppression during ATI for a median of 21 weeks (132), with no evidence of selection of dual-resistant variants on rebound. Subsequent studies have consistently demonstrated that people who have virus sensitive to bNAbs were more likely to experience a longer time off ART after receiving bNAbs compared to those who had evidence of resistance to at least one bNAb (80,176). These findings suggest the need for screening for pre-existing resistance to reduce the risk of viral escape through selection for pre-existing resistant variants.

However, there are limitations to existing bNAb screening assays. The current gold standard for assessing bNAb resistance is the in-vitro pseudovirus neutralisation assay also known as the TZM-bl neutralisation assay (232), however this is resource and time intensive, and the results have not correlated consistently with clinical response (132,183). Using molecular methods to sequence Env and identify resistance mutations through epitope mapping for 10-1074 and 3BNC117 may provide a more scalable approach to bNAb sensitivity screening (233,234), particularly with developments in next-generation sequencing and

bioinformatics methods available (235,236). However, current in-silico bNAb sensitivity prediction algorithms using pre-bNAb proviral single genome sequences still poorly correlated with time to rebound responses or resistant clones from rebound viral sequences (176). In particular, the in-silico 3BNC117 prediction algorithm has been suggested to be less reliable at predicting resistance (237).

Apart from the intrinsic accuracy issues with current bNAb sensitivity prediction algorithms, the diversity of HIV variants within individuals living with HIV remains a challenge. While this may be more feasible in people diagnosed and treated during early/acute HIV infection, due to the smaller and less diverse reservoir (238), the diversity of viral quasiespecies increases over time particularly if ART is not started early and is reflected in the integrated proviral reservoir. For people living with chronic HIV, the increased HIV diversity remains a challenge for adequately sampling the breadth of sequences which may harbour resistance. The degree of sampling may also affect screening, with greater quasiespecies diversity, a greater number of individual sequences from one individual to detect minority quasiespecies with bNAb resistance may be required, which may affect the scalability or feasibility of pre-screening for bNAb resistance prior to routine use of bNAbs.

There is evidence that over time, the evolution of HIV strains under humoral pressures may increase resistance to neutralisation by bNAbs on a population level. An analysis of all B clade *env* sequences from the Los Alamos database since 1986 have shown an increasing frequency of predicted 10-1074 and 3BNC117 resistance (239). These findings highlight the urgent need to develop better screening strategies for the therapeutic use of bNAbs, and understanding the relationship between bNAb resistance and pre-screening samples, both in blood and other body sites such as the GI tract which may harbour compartmentalised resistant provirus may be crucial to the success of bNAbs therapy.

1.4.3 Existing evidence for their mechanisms of action and the 'vaccinal' effect

bNAbs are thought to work through two main and a hypothesised third mechanisms (Figure 1.4.3). Firstly, they achieve direct neutralisation of the pathogen through binding of the fragment antigen-binding (Fab)

region to the HIV envelope protein, preventing virus from attaching and infecting host cells. They also have antibody-dependent cell-mediated effects, via the Fc region interactions with Fc gamma receptors (FcγRs) on immune cells. The opsonisation of viruses or viral antigens on infected cells by bNAbs, triggers activation of other immune components, such as natural killer cells, macrophages, and complement protein to kill the infected cell and prevent new virus production (240–242). However, a third mechanism of the bNAbs has been hypothesised to induce long-lasting cell-mediated responses, which may persist even after the clearance of bNAbs from circulation (243). The formation of immune complexes between the viral antigens and bNAbs, enhances antigen uptake and presentation by binding to FcγR on dendritic cells, resulting in enhanced HIV-specific CD8 cellular response.

Figure 1.4.3. Established and proposed mechanisms of actions of broadly neutralising antibodies for the treatment of HIV

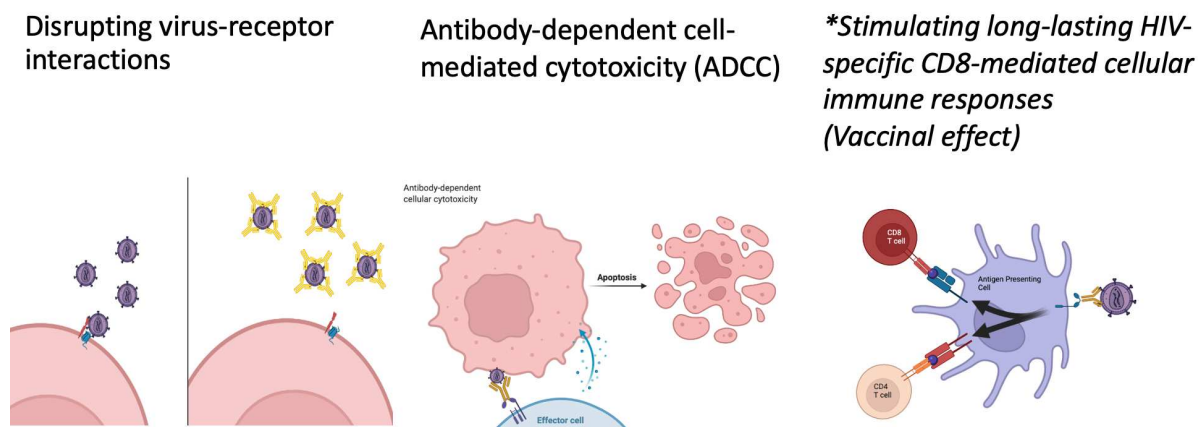


Image made with Biorender

In humanised mice models, treatment with 3BNC117 and 10-1074LS resulted in rapid reduction of HIV-infected cells compared to treatment with an isotype control, in an FcγR-mediated process. This was not seen in when bNAbs with mutations Fc mutations negating FcγR binding, nor in mice who receive FcγR-blocking antibodies (244). When thirteen macaques were given 3BNC117 and 10-1074 three days following SHIV acquisition, six of the thirteen demonstrated post-treatment control, initially experiencing rebound

viraemia before suppressing to undetectable levels with maintenance of CD4 counts (169). A further three demonstrated late viral suppression after four and five years after infection respectively, the macaques maintained control up to six years after infection (179). This was shown to be mediated by CD8 T-cell responses, when CD8 T cell depletion using both an anti-CD8 α and subsequently an anti-CD8 β antibody was used, which resulted in transient viral recrudescence before achieving resuppression upon recovery of cellular responses (169). As bNAbs given at such an hyperacute stage of infection is not considered feasible in human trials, the experiment was repeated in macaques who were given bNAbs two weeks after SHIV infection. Sustained viral suppression was also seen in seven of twelve macaques after a period of viraemia, whether bNAbs were given in combination with ART or without, however the patterns of durable control were different, with slower times to viral suppression up to three years in those macaques given bNAbs at two weeks. The control was similarly demonstrated to be CD8-mediated when anti-CD8 therapy was administered resulting in transient loss of viral control (179). These findings provide further convincing evidence for the use of bNAbs to enhance HIV-specific CD8 responses in vivo corresponding to successful clinical outcomes of durable viral suppression in macaques.

There are encouraging reports that increased HIV-specific T cell responses may be seen in people who receive bNAbs. Niessl et al. (178) reported increased HIV Gag-specific T cell responses on the Activation Induced Markers (AIM) assay 12 weeks after bNAbs infusion compared to baseline, both with CD8 and CD4 cells, in seven and six of nine individuals respectively. These responses were directed to a mix of both pre-existing and new Gag epitopes not seen at baseline. In a randomised 4-arm phase 1b/2a eCLEAR trial of 3BNC117 and romidepsin given to people with HIV who were initiating ART, those who received 3BNC117 demonstrated increased frequencies of Pol- and Gag- specific CD8 cells and Gag-induced interferon- γ (IFN γ) responses up to 12 months after starting ART. Both increased Gag-specific CD8 T cell responses and high-IFN γ responses were associated with viral control over a 12-week ATI (245). In the phase 2a randomised controlled trial of Leftolimod (a toll-like receptor (TLR) 9 agonist) and dual bNAbs 3BNC117 and 10-1074 in

people with HIV screened for bNAbs sensitivity, bNAbs were associated with a longer time to viral rebound during an ATI, with or without leflomimod. However there was no significant increases in HIV-specific T cell responses in individuals who remained suppressed at week 13 to suggest a bNAb-mediated vaccinal effect (175). The presence of viraemia when participants were given bNAbs appears to be able to induce strong HIV-specific T cell responses, as seen in the eCLEAR trial where Gag-specific CD8 T cell responses were significantly higher in participants receiving 3BNC117 compared to the ART-control group up to 1 year after the first dose of bNAb, irrespective of romidepsin administration (245). Greater CD8 T cell responses and higher IFN γ production were associated with a greater proportion of people who had virological control during the treatment interruption, compared to participants who experienced viral rebound (71% vs 23%), although numbers were small (n=20). The RIO study is a two-stage, randomised placebo controlled trial of bNAbs in people treated in acute/early HIV infection which is ongoing (246). RIO may shed further light on the mechanisms of the hypothesised vaccinal effect by interrogating T cell responses in participants who are virally suppressed on ART compared to those who are viraemic when receiving bNAbs. The study methodology and outcomes will be discussed in chapter 2.

1.4.4 BNABs and their impact on the HIV reservoir

The goal of a successful immunotherapeutic cure or remission strategy would be the arrest or elimination of the latent HIV reservoir, to prevent recrudescence of viral replication following cessation of ART. Although bNAbs had shown tantalising progress in animal models, results from early human studies have been mixed. bNAbs have been associated with reductions in SHIV proviral DNA in blood, mucosa and lymph nodes, likely mediated by improved Gag-specific T cell responses (247), and similarly in humanised mice (168). This may be mediated by identification and destruction of cells containing HIV through Fc γ R-binding and ADCC.

In humans, Gaebler et al. (176) reported that prolonged bNAb therapy was associated with a modest reduction in both the absolute and relative proportion of intact provirus reservoir measured using the Q4PCR

assay 6 months after dual bNAbs (10-1074 and 3BNC117) therapy. In contrast, other studies have not shown a difference in the latent reservoir measured by the Q²VOA assay 6 months after VRC01 or 3BNC117 monotherapy (184,188), or with the IPDA such as with the TITAN study (175), although in the TITAN study the intact proviral reservoir expanded after ATI in participants in the non-bNAb arms but was not significantly different in the bNAb arms, suggesting bNAbs restricted reservoir expansion during early ATI. One explanation may be that for bNAbs to exert a substantial impact on the latent reservoir involves a multifactorial process, both in restricting expansion viral reservoir through blocking infection of host cells as well as targeting infected cells through ADCC and subsequent enhanced HIV cellular responses. These factors are likely to be influenced by a host of factors including bNAb sensitivity, presence of viral antigen, host-immune interactions such as Human leucocyte antigen (HLA) subtypes, the role of ART and other adjuvant immunotherapies, and appropriate reservoir measures, all of which have yet to be sufficiently characterised to determine the effect of bNAbs on the intact reservoir.

1.4.5 Safety of exogenous bNAbs therapy

Exogenous BNABs have been shown to be safe and to date there have been no reported cases of anaphylaxis or delayed drug-related reactions associated with either the short acting or long-acting LS-variants of 3BNC117 and 10-1074 given intravenously (M Caskey, personal communication). There was one case of a severe infusion-related reaction related to the 2nd 3BNC117 infusion in the TITAN study, which resolved with intravenous fluid, antihistamine and corticosteroid therapy without any sequelae. A systematic review of ATI studies which included three trials (n = 37 participants) involving bNAbs did not identify safety concerns during the ATI (221). Serious adverse events associated with bNAbs were only reported in one phase one study of subcutaneous 10E8VLS injections, where seven of eight participants experienced erythema and induration at the injection site (248) related to the mode of administration.

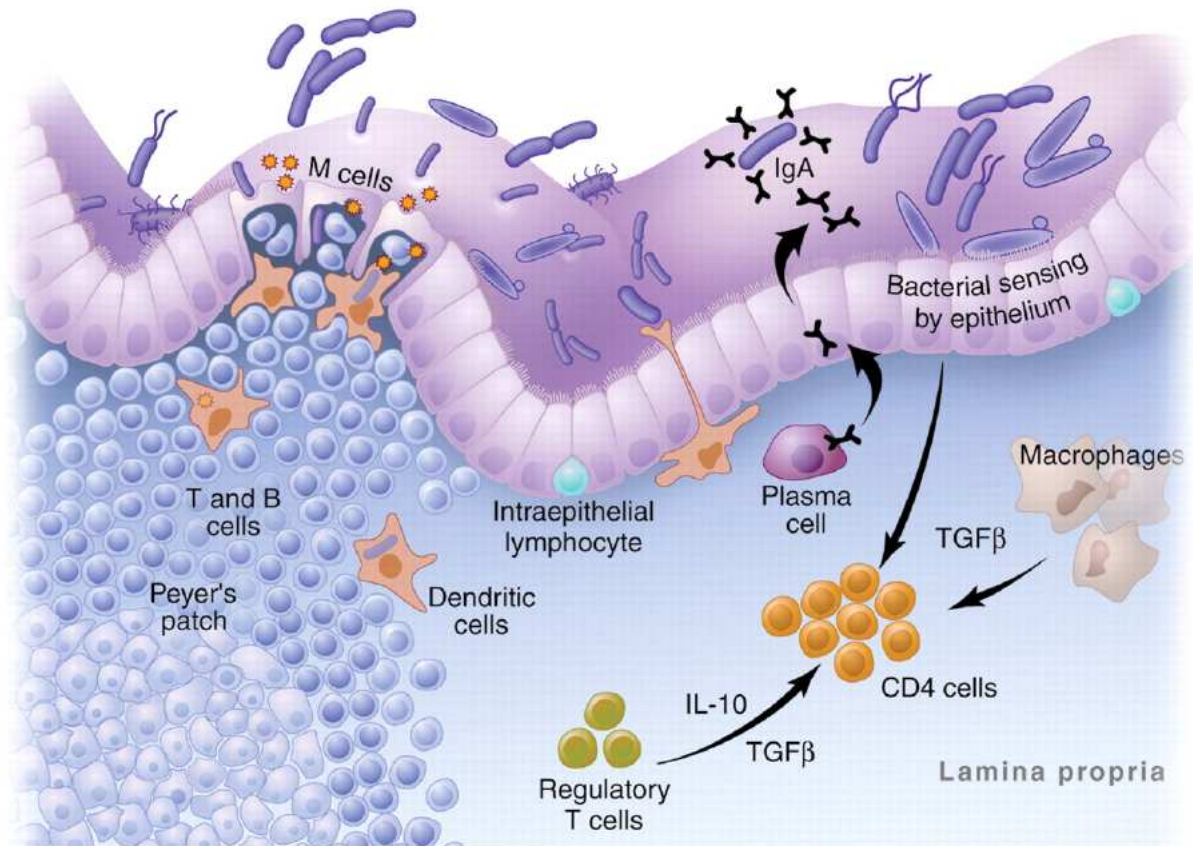
Regarding concerns around autoreactivity in earlier-generation bNAbs, in people with HIV who received infusions of the autoreactive bNAbs 4E10 and 2F5 were well tolerated without thrombotic complications despite high serum titres and low-level cross-reactivity with cardiolipin (249). In vaccination studies, the metabolic enzyme kynureninase is closely mimicked by the HIV Gp41 MPER antigen and is cross-reactive with the bNAb 2F5, however neither vaccination to elicit 2F5 or passive immunisation with 2F5 affected kynureninase nor tissue tryptophan metabolism (250,251). With modern bNAbs being less autoreactive compared to their predecessors, there may be less risk for autoreactive adverse events, although ongoing pharmacovigilance should be undertaken during clinical trials to identify safety concerns associated with any therapeutic bNAbs strategies.

1.5 BNABs and the GI tract

1.5.1 Gastrointestinal mucosal immunology

The GI tract is one of the key complex components of the immune system, and contains most of the lymphoid tissue mass in the body (252). Beyond the barrier role that the GI epithelium provides, GI mucosal immune anatomy can be divided into inductive and effector sites. Inductive sites include mesenteric lymph nodes, Peyer's patches in the small intestine, and isolated lymphoid follicular aggregates in the large intestine, and are the primary sites where antigen is presented to effector immune cells (253). Peyer's patches, which are both present in small intestine but most prominently in the terminal ileum, initiate antigen-specific mucosal immune responses through specialised antigen-sampling cells, or microfold (M) cells (254). These M cells are situated in specific epithelial layers known as the follicular-associated epithelium (FAE) (Figure 1.5.1.1). On the basal membrane, M cells form a pocket which contains T helper (Th) 1 and Th2 cells, B cells, macrophages and dendritic cells (DCs), highlighting how these specialised regions are adept for inducing antigen-specific responses. Germinal centres, where B-cell affinity maturation, isotype switching, and division occurs, are located directly below the FAE regions (253,255). Isolated lymphoid follicles (ILF) are similar to Peyer's patches where they contain B cell clusters embedded in a meshwork of follicular DCs, but lack T cell enriched regions compared to Peyer's patches. ILFs are found throughout the GI tract but are concentrated in the distal ileum, colon and rectum (253). Dispersed throughout the GI mucosa are the GALT effector immune sites: T cells making up most of the lymphocytes in the epithelium, the majority of which are CD8 α/β T cells, and the lamina propria containing a mix of dendritic cells, macrophages, B cells and T cells (256–258).

Figure 1.5.1.1. Gut mucosal immunology



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Science (2005) (259).

There are marked regional immune differences along the GI tract, such as the differential distribution of Peyer's patches in the small intestines compared to predominance of ILF in the large colon and rectum (Figure 1.5.1.2). In addition, there are differences in the activation and memory status of T and B cells between the mesenteric and colon lymph nodes (260). In the colon, CD4 T cell were more likely to have effector phenotypes, expressing high levels of tissue residency markers. Even along the large colon, Th17 and Th1 CD4 T cells exhibit an inverted gradient, and Th1 cells being predominant in the sigmoid and distal colon, and Th17 cells mostly in the proximal caecum. There are region-specific transcriptional profiles of Th and B cells along the length of the colon, particularly with activated IgA producing plasma B cells in the

sigmoid colon compared to the proximal colon. The colon also harbours a higher frequency of macrophages, which are important in maintaining GI homeostasis in addition to antigen presentation and clearance (256,261).

Figure 1.5.1.2. Regional differences in mucosal immunology along the gastrointestinal tract

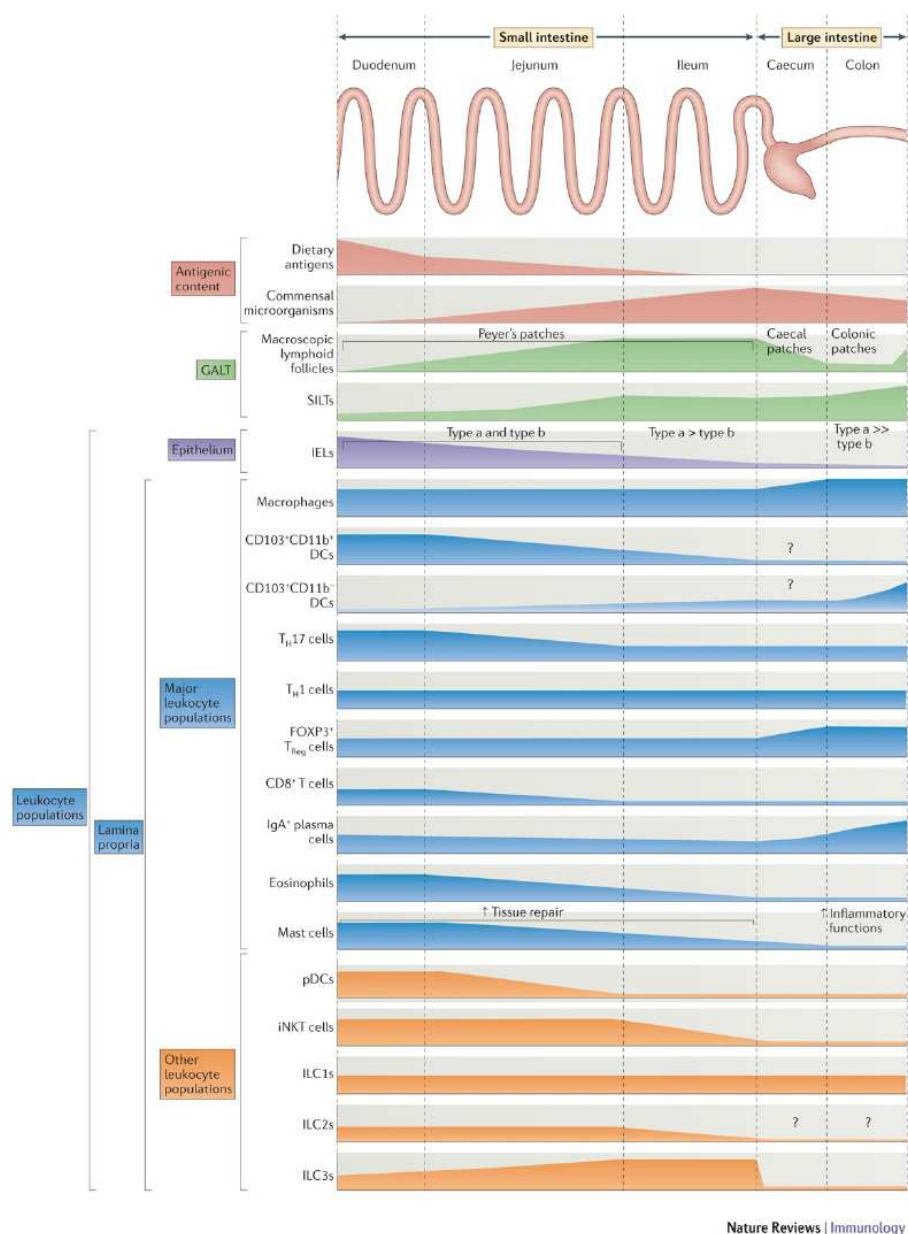


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1.5.2 HIV and persistence of the GALT-associated HIV reservoir

In addition to being the largest site of lymphoid tissue in the body, the gut has the greater proportion of activated memory CD4 T cells, the cell population which HIV preferentially infects. These cells are present in greater frequencies in the intestinal lamina propria than the peripheral blood or lymph nodes (252,262), and thus it is clear that GALT are key target sites for HIV infection. One study estimates that the GI tract contains up to 83-95% of all cells containing HIV in the body (76). Rapid and sustained infection of GALT T lymphocytes by HIV occurs rapidly upon infection, and severe depletion of uninfected CD4 T cells also occurs (262,263). HIV DNA levels have been found to be up to 5-fold higher in GALT than peripheral blood mononuclear cells (264). Studies have shown that GI and lymphoid tissues are sources for rebound viraemia and dissemination of virus to other tissue reservoirs (60,265–267). When people with HIV have started ART, HIV DNA levels persistent in rectal tissue despite declining in lymph nodes, with 98% of HIV RNA-positive cells were found in GALT in virally suppressed individuals on ART, suggesting that GALT may be an important source of HIV persistence even when individuals are virally suppressed on ART (268). Although the terminal ileum may be a more transcriptionally active reservoir site in the GI tract, when comparing different sites along the HIV DNA levels have not been found to be significantly different between sites in the GI tract in multiple studies (269,270).

HIV may be differentially present in CD4 T cell subsets in the GI tract compared to the blood. While central memory (T_{CM}) and naïve CD4 T cells are the predominant cells containing HIV in blood, T_{CM} and effector memory T cells (T_{EM}) are the main subsets harbouring HIV in GALT (266,271). However, other T cell subsets, including follicular T helper cells (T_{FH}) present in germinal centres of lymphoid follicles (272,273), long-lived Th17 cells (274), and tissue-resident memory CD4 T cells (T_{RM}) (275) may be important populations contributing to the tissue GALT-associated HIV reservoir. Although T_{RM} cells were thought to be primarily parked within tissues, a recent study reported that HIV reprograms resting peripheral T cells into a T_{RM} phenotype by the accessory protein Vpr, which may provide a viral mechanism to drive infected peripheral

blood CD4 T cells to migrate and persist into tissue (276). While this process has yet to be characterised in GALT, emerging data suggests that T_{RM} precursors in the blood may be induced by tissue signals to become tissue-bound T_{RM} cells (277), and the persistence of HIV within CD69 T_{RM} cells in adipose tissue have already been described (278).

Antiretrovirals and novel immunotherapeutics such as bNAbs may differentially penetrate GI and lymphoid tissue sites at lower levels compared to blood (229,279), leading to concerns around compartmentalisation of viral replication and resistance development in sanctuary sites. However, the data are mixed, with one study reporting evidence of diversity between areas of the GI tract (280), and other with none or limited evidence of compartmentalisation between different areas of GALT, and PBMCs(281,282) Further characterisation of HIV sequences between blood and tissue compartments may shed light on the development of resistance following administration of immunotherapeutics such as bNAbs.

1.5.3 Do bNAbs penetrate the GI mucosal tissue

Most clinical trial data in humans characterising the effect of bNAbs have been undertaken using blood samples. However, there is a paucity of pharmacokinetics data in other tissue compartments.

Due to the relatively large size and physiochemical characteristics of monoclonal antibodies (mAbs), including bNAbs, extravasation from blood into the tissue occurs primarily through convective transport and transcytosis (283). Convective transport of fluid between blood to tissue is driven by blood-tissue hydrostatic gradient, and the paracellular pores in the vascular epithelium providing a sieving effect. This is affected by the number, size, and tortuosity of pores as well as the shape, charge, and size of the mAbs (284). Transcytosis is the process of uptake and passage of molecules, such as mAbs, through the vascular epithelial cells, and is mediated via the FcRn (285). Subsequent interstitial distribution occurs through diffusion, convection, and binding affinity to target antigens within the tissue. The presence of target antigen would directly correlate with a greater volume of distribution (283). The extracellular matrix is another barrier to

tissue distribution of large mAb molecules. Clearance of antibodies from the tissue interstitial space is determined by antibody convection into the lymphatic drainage system. In a physiology-based pharmacokinetics model using data from radiolabelled mononuclear antibodies administered to humanised mice comparing different FcRn affinities, changes in the FcRn affinities did not appear to affect tissue clearance in gut tissue substantially (286). With these factors in mind, the tissue concentrations of bNAbs are likely to be substantially lower than serum bNAb measurements.

In HIV-negative individuals receiving a shorter acting non-LS variant bNAb VRC01, vaginal and rectal tissue concentrations were observed to be 10-fold lower than in paired serum samples. Although functioning, neutralising bNAb activity within the tissue was seen, breakthrough infections occurred when the tissue was challenged with high dose HIV ex vivo after day 8, reflecting a decay of functional antibody in tissue over time to sub-protective levels (229).

Filling the knowledge gap around bNAbs pharmacokinetics in tissue is crucial, particularly for participants living with HIV receiving bNAbs undergoing treatment interruption or viral rebound. If bNAbs do not penetrate to therapeutic levels in GALT, their efficacy at viral eradication might be limited, and if viral replication occurs under sub-therapeutic bNAb concentrations in tissue for substantial durations off ART driving selection of bNAb resistance, these have implications for the use of bNAbs as an effective immunotherapeutic strategy.

1.6 Summary

BNAbs represent a promising immunotherapeutic strategy associated with longer-term ART-free viral control for HIV treatment. Beyond a direct antiviral effect, early studies demonstrate the potential for a ‘vaccinal’ effect with bNAbs therapy, with persistent enhanced HIV-specific cellular responses that may be associated with post-interventional viral control and reduced intact HIV reservoir measurements in blood. However, it is unclear if bNAbs penetrate the gut tissue adequately to impact tissue-associated HIV reservoirs, of which GALT is the largest reservoir site in the body. The analysis of HIV reservoir, pharmacokinetics and immunological data from tissue samples of people living with HIV who receive bNAbs in clinical trials are crucial to understanding and improving the use of bNAbs as a cure or remission strategy for HIV treatment.

1.7 Thesis aims

Hypothesis

LS-bNABs are safe and effective for long-term viral control, and are able to penetrate gastrointestinal mucosal tissue to affect the gut-tissue associated HIV reservoir and mucosal immunity.

Research questions

1. Are LS-bNABs safe and effective for maintaining viral control for people living with HIV in cure-related ATI studies?
2. What serum LS-bNAB therapeutic thresholds are associated with loss of viral suppression and do LS-bNABs achieve comparable concentrations in rectal mucosal tissue?
3. Are there changes in gut-associated latent HIV reservoir measures and mucosal immunity associated with LS-bNABs?

Therefore, the aims of this thesis are to:

1. To evaluate the safety of LS-bNABs and ATIs, and their impact on time to viral resuppression after restarting ART in cure-related ATI studies including the RIO study.
2. To determine the clinical effectiveness of LS-bNABs on maintaining viral suppression in people diagnosed during primary or early HIV in the RIO study.
3. Determine a therapeutic threshold for LS-bNAB serum concentrations to maintain effective viral suppression through pharmacokinetic-pharmacodynamic (PKPD) modelling of serum bNAB and plasma HIV viral load measurements.
4. Assess if LS-bNAB concentrations are able to effectively penetrate into the rectal mucosa by comparing paired serum and tissue bNAB concentrations.
5. Measure changes in the gut-associated latent HIV reservoir and mucosal immunity after bNAB administration.

Chapter 2

Methods

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2.1 The RIO study design

2.1.1 Rationale and trial objectives

In this chapter I will describe the design of the RIO trial and the Gut sub-study, and methodology which underpins my thesis. The RIO trial investigates the use of two long-acting bNAbs 3BNC117LS and 10-1074LS in people who initiated ART during early or primary HIV infection (PHI), with the primary outcome being the time to HIV viral rebound after stopping ART for an extended period by week 20 after ART interruption. I have published the study protocol along with a discussion on the importance of community representation (246). Further details and methodology of the systematic review and meta-analysis undertaken are presented in chapter 5.

The RIO trial aims to answer two key research questions:

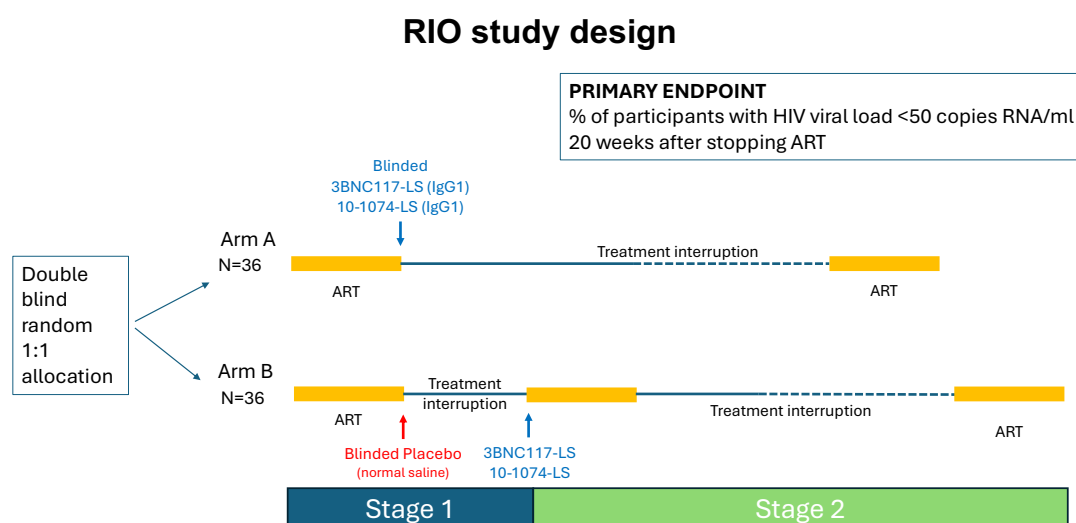
1. To determine if infusions of dual long-acting bNAbs are able to achieve ART-free viral suppression up to 20 weeks post infusion in people treated during early or primary HIV infection compared with placebo. This will inform the field on safety of this approach as well as to quantify the impact of the intervention on ART-free viral control.
2. To determine if receiving dual bNAbs at the time of ART restart when participants are likely to be viraemic following an ATI can confer post ART viral control after 6 months of ART, at a point when it is anticipated that plasma concentration of bNAbs will be below therapeutic levels.

The second aspect of the two-stage approach will explore the scientific question as to whether the lasting impact of dual bNAb treatment, or “vaccinal effect” of stimulating persistent HIV-specific CD8+ and NK cell activation, is sufficient to contain viral replication in human participants. An additional benefit of the two-stage design is that it allows all eligible interested study participants to receive bNAbs, improving acceptability and participation in the trial following feedback from community representatives when designing the study. However, to answer the research aims of this thesis, primarily data from the first stage comparing bNAbs to placebo are presented.

2.1.2 Study design (246)

The RIO study is a double-blinded, randomised, placebo-controlled phase II trial in two stages, summarised in Figure 2.1.2. The trial recruited 68 participants (of a target of 72 participants) for randomisation in a 1:1 allocation, to receive either a single dose of dual bNAbs or placebo infusion. Participants then undertook a closely monitored ATI two days after randomisation, until they reached the pre-defined criteria for restarting ART and unblinding. Participants unblinded to Arm A (bNAbs) were followed up until the end of the study 16 weeks after restarting ART. Participants unblinded to Arm B (placebo) were invited to the second stage of the trial, where they received an open-labelled infusion of the dual bNAbs. They remained on ART for at least 24 weeks after bNAbs infusion to allow wash-out of the bNAbs, followed by a second ATI. The monitoring schedule and criteria to restart ART during the second ATI was identical to the first ATI, and once ART is restarted, they were followed up until the end of the study 16 weeks after restarting ART. The study was approved by the UK London-Westminster Research Ethics Committee (REC) on the 12 Dec 2019 (REC reference 19/LO/1669) and began recruitment in March 2021. I was the trial physician responsible for recruitment at the lead clinical site and led the analyses of the primary and secondary outcomes.

Figure 2.1.2. Summary of the RIO study design (246)



2.1.3 Study cohort and criteria for inclusion

The target study population included eligible and consenting people living with HIV who commenced ART during confirmed PHI and who have remained with viral suppression below the level of detection (plasma HIV RNA <50 copies/mL) for at least one year.

The inclusion criteria were as follows (246):

- Aged ≥ 18 to ≤ 60 years old at screening
- Able to give informed written consent including consent to long-term follow-up.
- Willing and able to comply with visit schedule and provide blood sampling.
- Started ART within a maximum of six months of estimated date of primary or early HIV infection, based on one of the following six criteria:
 - a. Positive HIV-1 serology within a maximum of 24 weeks of a documented negative HIV-1 serology test result (including point of care tests (POCT)). The estimated time of infection is taken as the midpoint between the dates of the negative HIV-1 serology or POCT test and positive HIV test at diagnosis, unless there was clear evidence for a transmission event, in which case that date will be taken.
 - b. The date of a positive p24 antigen result and a negative HIV antibody test.
 - c. The date of a negative HIV antibody test with either detectable HIV RNA or proviral DNA.
 - d. RITA reported as “Incident” confirming the HIV-1 antibody avidity is consistent with recent infection (within the preceding 16 weeks). The estimated date of infection is assumed to be two months prior to the date of the incident test result.
 - e. The date of a weakly reactive or equivocal 4th generation HIV antibody antigen test
 - f. The date of an equivocal or reactive antibody test with less than 4 bands on western blot

- OR, started ART in early-stage infection, with nadir CD4 >500cells/ μ L and stable on ART with a suppressed undetectable HIV VL ‘target not detected’ using local assays for ≥ 1 year (a single viral load measurement >50 copies/mL but <500copies/mL during this time period is allowable).
- Stable on ART with suppressed undetectable HIV VL result using local assays for ≥ 1 years.
- No evidence of viral insensitivity to 10-1074 based on proviral sequencing algorithm.
- Hepatitis B virus (HBV) surface Antigen or HBV DNA, Hepatitis C virus (HCV) Ag or HCV RNA negative or HCV anti-core antibody negative
- No significant co-morbidities
- nadir CD5 >500 cells/ μ L or nadir CD4 > 250 cells/ μ L if meets PHI criteria
- Current CD4 count > 500 cells/ μ L or CD4:CD8 ratio >1
- On INSTI or boosted protease inhibitor (bPI) based regimen at time of randomisation, if previously on NNRTI at time of screening, participants would have their ART regimen switched at least 4 weeks prior to randomisation.
- Haemoglobin levels ≥ 120 g/L for males or ≥ 110 g/L for females
- Weight ≥ 50 kg
- Females capable of becoming pregnant must agree to use hormonal contraception, intrauterine device, intrauterine hormone-releasing system, or to complete abstinence from at least two weeks before the first bNAb/placebo infusion and for 20 months after the last bNAb/placebo infusion.
- Participants must have been vaccinated against coronavirus (COVID-19), at least 4 weeks prior to enrolment.

Participants with evidence of any of the following criteria were not eligible for the study:

- Previous ischaemic heart disease (including ST or non-ST myocardial infarction, 10-year cardiovascular risk estimated to be > 20%, stable angina, unstable angina, or stroke)
- Any current or past history of malignancy.

- Concurrent opportunistic infection or other comorbidity or comorbidity likely to occur during the trial e.g. malabsorption syndromes, autoimmune disease.
- Any contraindication to receipt of British HIV Association (BHIVA) recommended combination antiretrovirals (287).
- Human T Lymphotropic virus 1 (HTLV-1) co-infection
- SARS-Cov-2 infection confirmed by SARS-Cov-2 PCR positive result from nasopharyngeal swab up to 72 hours prior to randomisation/dosing visit.
- Individuals at high risk from severe COVID-19 disease who may be defined in accordance with National Health Service England guidance (288) as vulnerable and shielded
- Current or planned systemic immunosuppressive therapy
- Participation in any other clinical trial of an experimental agent or any non-interventional study where additional blood draws are required; participation in an observational study is permitted.
- History of anaphylaxis or severe adverse reaction to antibody infusions, or hypersensitivity to 3BNC117LS or 10-1074LS or to or any constituent products or excipients
- Treatment with intravenous immunoglobulin or other monoclonal antibody treatments planned during the duration of the trial.
- Clinically significant abnormal blood test results at screening including:
 - a. Moderate to severe hepatic impairment as defined by significant liver impairment with evidence of advanced fibrosis or cirrhosis with decompensation.
 - b. Alanine transaminase >5x upper limit of normal.
 - c. Estimated renal glomerular filtration rate < 60mL/min/kg.
 - d. Urine Protein Creatinine Ratio >30 mg/mmol.
 - e. International Normalised Ratio >1.5.

- Physical examination findings: Evidence of organ dysfunction or any clinically significant deviation from normal in physical examination and/or vital signs that the investigator believes is a preclusion from enrolment into the study.
- Active alcohol or substance use that, in the Investigator's opinion, will prevent adequate adherence with study requirements.
- Insufficient venous access that will allow scheduled blood draws as per protocol.
- Concern regarding likelihood of participant not taking precautions to prevent HIV transmission during treatment interruption period.
- Pregnancy or breastfeeding

2.1.4 Treatments and study procedures

BNAb Dosage and administration (246)

3BNC117LS and 10-1074LS were administered at a dose of 30 mg/kg and 10mg/kg respectively (246). Prior to infusion, each IMP is diluted to a final volume of 100mL in a single normal saline injection. Each IMP was then administered as a separate intravenous infusion over a period of approximately 30 minutes per bNAb. IMP infusion were administered through a 0.2 or 0.22 micron in-line filter.

Antiretroviral Treatment Interruption protocol (246)

Individuals were enrolled into the study and received either dual bNAb or placebo on the day of randomisation, and were asked to stop ART at least two days after the infusion. At the time of recruitment, all participants had been taking ART according to current best practice and national UK guidelines (287). At the time of randomisation, participants' ART regimen would have been on an INSTI or bPI; if on an NNRTI they must be switched to an INSTI or bPI at least 4 weeks prior to randomization. The ART regimen after viral rebound included an INSTI or bPI to minimize the risk of resistance. Other changes in ART outside the protocol-mandated ART interruption period, and any other concomitant medical therapy during the study were recorded.

Management of ATI:

Following interruption of ART all participants had their plasma HIV VL and CD4 count monitored using routine clinically validated assays. These were performed weekly for the first 8 weeks and then fortnightly until viral rebound has occurred, and/or ART was recommenced once any of the pre-defined ART re-start criteria had been reached. Participants were able to attend for additional visits for VL monitoring if they preferred.

2.1.5 Criteria for restarting antiretroviral therapy (246)

Participants were advised to restart ART during the ATI if they reached the following criteria:

1. Viral rebound confirmed by HIV VL measurement from venous blood:
 - a. Sustained HIV viral load ≥ 1000 copies/mL for 6 weeks $\pm$ 7 days.
 - b. HIV viral load $\geq 100\,000$ copies/mL for two readings, 7 days apart $\pm$ 7 days.
 - c. Instances outside of the above two categories but were close to these definitions and necessitate ART restart for clinical reasons. These instances will be individually reviewed by an independent panel (Endpoint Adjudication Committee) to confirm if they meet the definition for viral rebound.
2. CD4 count decrease to less than 350 cells/ μ L.
3. Clinical symptoms attributable to ATI.
4. Participant preference.
5. Major concerns over risk mitigation for HIV transmission.
6. New COVID-19 diagnosis during ATI.

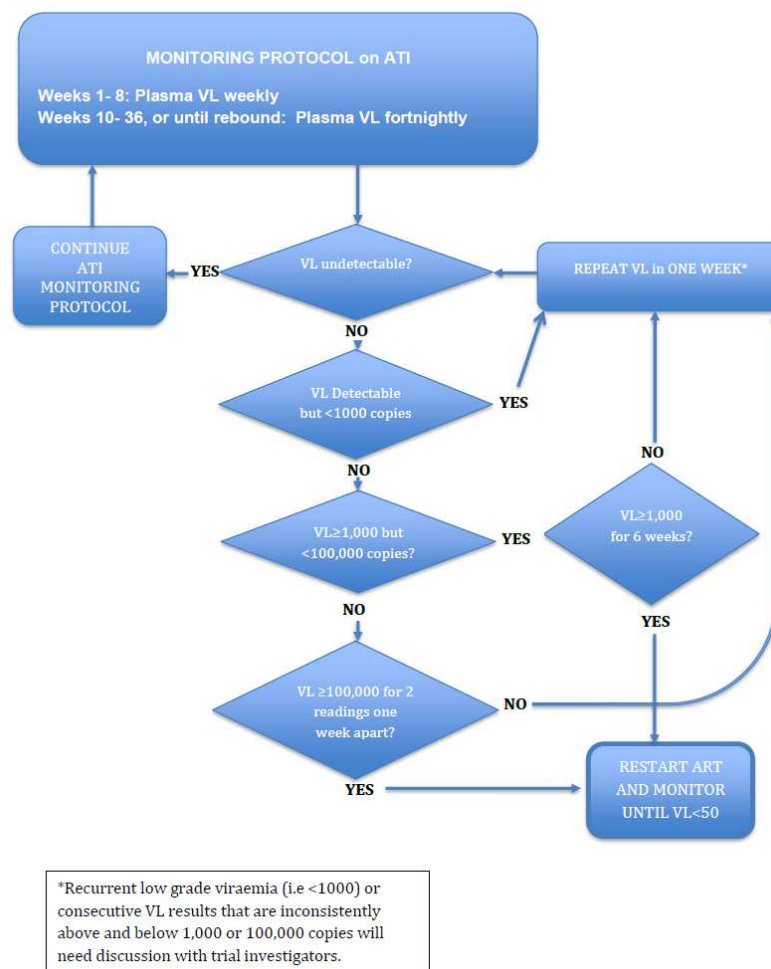
The clinical decision algorithm for management of detectable viral load and viral rebound is summarised in Figure 2.1.5. In addition,

- At any VL threshold, ART was restarted if the participant changed their mind during the treatment interruption.
- In cases when participants did not formally meet the criteria for viral rebound and ART restart, and yet have evidence of sustained viraemia (for example, viral loads >1000 copies/mL for less than six weeks). In these cases, an independent Endpoint Adjudication Committee reviewed these cases to determine if viral rebound was reached for the purposes of the primary outcome analyses.

- If there was a confirmed drop in CD4 count to < 350cells/μL or in the presence of symptoms that were attributed to the ATI, ART was recommenced.
- After restarting ART
 - Participants were followed up at two-weekly intervals until they achieved undetectable plasma viral load measurements, followed by monthly visits until the end of the study.

Participants who do not experience HIV rebound viraemia off ART within 20 weeks continued to remain off therapy for the duration of the study or until viral rebound occur. At the end of the study, all participants were recommended to re-start ART regardless of HIV viral load in accordance with national UK guidelines.

Figure 2.1.5. Monitoring protocol during ATI (246)



2.1.6 Mitigating safety concerns during COVID-19 outbreaks

COVID-19 pandemic measures

All study visits took place in COVID-19 free areas and participants will be provided appropriate Personal Protective Equipment, and study procedures were undertaken as per relevant national COVID-19 guidelines/practices (289). Risks and benefits of undertaking a clinical trial for people living with HIV during the SARS-CoV-2 pandemic were discussed with potential participants prior to enrollment (290).

Participants were only enrolled if they had received at least one dose of a SARS-CoV-2 vaccine, following the introduction of a protocol amendment to the original trial eligibility criteria. Even a single dose of the BNT162b2 mRNA vaccine (Pfizer BioNTech) and ChAdOx1 nCoV-19 vaccine (AZD1222) has been estimated to be high at 89% and 73% respectively (291), and participants were deemed eligible to join the study whilst awaiting subsequent vaccination doses to complete their vaccination course as recommended by the UK joint committee on vaccination and immunisation (292). In a cohort of people living with HIV, there were no differences in both humoral and cell-mediated immune responses produced by the ChAdOx1 nCoV-19 vaccine compared to a HIV-negative cohort (293).

If participants exhibited symptoms suggestive of COVID-19 during the duration of the study, participants were retested for SARS-CoV-2. If they were found to be PCR-positive for SARS-CoV-2, a decision would be made on a case-by-case basis by the clinical team, to evaluate if the participant should be advised to restart ART, and if they did, whether a repeat ATI would be undertaken in consultation with the participant, after clearance of SARS-CoV-2 and resolution of symptoms. All study participants were able to access recommended COVID-19 treatment as per UK National Health Service (NHS) guidance. A similar decision tree for repeating an ATI was undertaken for participants who restarted ART during their treatment interruption for any other acute medical reasons.

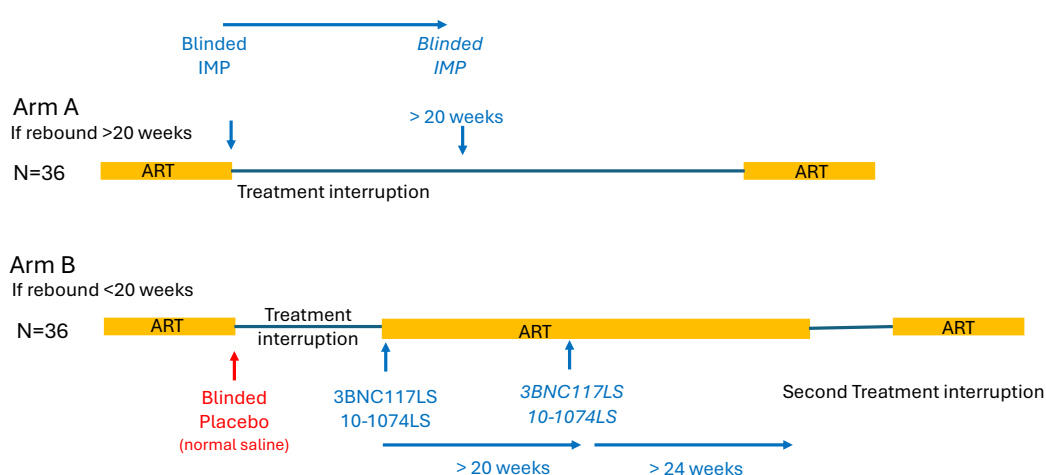
2.1.7 Second dose sub-study

Participants who reached week 20 or beyond and had not met the viral rebound and ART restart criteria, were offered a second dose of IMP. The IMP they received was dependent on the stage of the trial. For participants who had not reached viral rebound by week 20 in the stage 1 ATI, they received another blinded dose of bNAb or placebo as they had received during the initial randomisation, to retain the blinding from enrolment.

For participants in stage 2 who have received an open-labelled bNAb dose, they were offered a second open-labelled bNAb dose whilst on ART at least 20 weeks after the initial bNAb dose.

The second dose sub-study is summarised in figure 2.1.7.

Figure 2.1.7. Second dose sub-study design



2.1.8 Study outcomes (246)

The primary endpoint of the RIO study was the comparison of time to viral rebound at 20 weeks after initial ATI, during stage 1, summarised by hazard ratios.

The secondary endpoints for the RIO trial that are reported in this thesis are

1. Safety defined as the number and types of Adverse Events (AEs) and Serious Adverse Events (SAEs) by group

2. Length of time undetectable following ATI in the absence of detectable ART (comparing those who received bNAbs (Arm A) vs placebo (Arm B) during the 1st stage ATI).
3. Duration of remission analysed under multiple alternate definitions (e.g. VL<50copies/mL, <200copies/mL), comparing Arm A vs B and Arm B
4. bNAbs concentration in blood at dosing, weeks 2, 4, 8, 12, 24, 36, 48, time of viral rebound and end of study, summarised by mean.

This thesis will include the relevant safety and clinical outcomes from the study as described in chapters 4 and 5, serum bNAbs concentrations in chapter 6, and the outcomes of the RIO Gut sub-study in chapter 7. Other endpoints in the RIO protocol are beyond the scope of this thesis and will be released in accordance with the RIO study's dissemination plan (246).

2.1.9 RIO sample size calculations

The study sample size was calculated by the Imperial College Clinical Trials Unit statistical team, and originally powered to 90% power using a two-sided log-rank-test at the 5% significance level provided there are 31 participants per arm and no loss to follow-up or return to ART treatment prior to 36-week primary outcome. The original recruitment target of 72 participants was chosen in order to provide an allowance of approximately 10% return to ART treatment, and loss to follow-up, prior to the earlier of either 36-weeks or a primary event. A substantial amendment to the protocol was approved by the REC on the 22nd June 2022 to allow a sub-study offering a second dose of the investigational medicinal product (IMP) dose to participants at 20-weeks. Following the second dose sub-study amendment, the primary outcome timepoint was revised to 20-weeks, with no change in the overall power or sample-size required. With more participants expected to remain undetectable off ART by week 20 in the intervention arm, the overall power of the study would likely be increased with a shorter cut-off for the primary outcome.

2.2 The RIO Gut Sub-study

2.2.1 Sub-study aim and objectives

I designed and led a sub-study within the RIO trial to collect rectal biopsies, known as the RIO Gut sub-study. The aim of the RIO Gut sub-study was to investigate the tissue pharmacokinetics of the dual bNAbs 10-1074LS and 3BNC117LS and their impact on the HIV reservoir and mucosal immunity. The primary objective was to measure bNAb mucosal concentrations in rectal tissues sampled longitudinally from participants in the RIO study. A total of 27 participants were recruited to the RIO Gut sub-study. Secondary outcome measures were the quantification of the total or intact HIV proviral DNA levels in rectal tissue immune cells, and changes in immune cell populations.

2.2.2 Sub-study design and sampling timepoints

Participants consented to three key rectal biopsies at the time of enrollment and a number of optional rectal biopsies during the course of the sub-study. The main RIO study was double blinded, and this blinding was retained throughout enrolment into this sub-study until the time of unblinding in the main RIO trial schedule. Procedures were usually performed at the same time as the main trial schedule visits, to avoid additional visit burden for participants, however, participants could schedule their biopsies separate from the main trial visit within the stated timeframe if they preferred.

The key biopsies timepoints were as follow: the baseline rectal biopsies taken at time of enrollment visit (days -3 to -1) prior to infusion. Following treatment interruption (day 0), they were invited for a second rectal biopsy taken at day 28 (± 5 days). Invitation to the final rectal biopsy visit was taken at week 16 (± 5 days) post ART restart for Arm A participants concurrently with the main trial end of study visit. Arm B participants were invited to a rectal biopsy at week 12 (± 5 days) post ART restart, with paired serum sample for bNAb level at the same visit. Participants who missed any biopsy visit were able to continue participating for future biopsies.

Participants were able to consent to up to four further optional biopsies. These biopsies were taken at weeks 12 and 24 (± 14 days) during the ATI period, as long as participants remain off ART; and for Arm B participants, they were invited for a rectal biopsies 4 weeks (± 5 days) after their 2nd treatment interruption, and a final biopsy visit at week 16 (± 5 days) post ART restart.

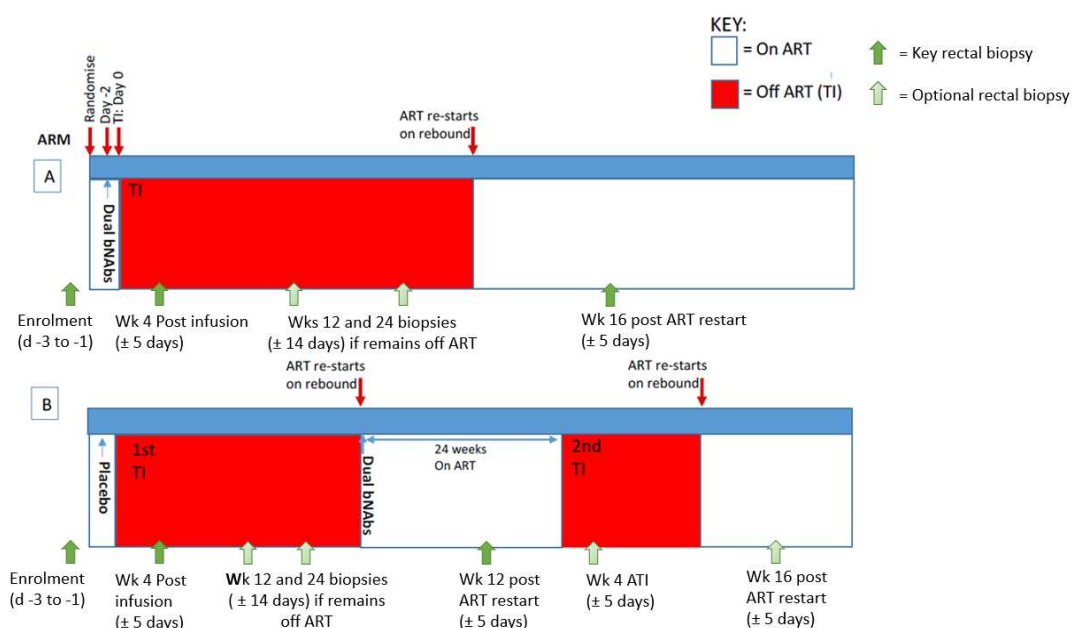
All sub-study procedures were performed in addition to the main trial schedule visits and procedures. Paired serum samples for bNAb concentration measurements were taken as covered in the main trial schedule, unless stated otherwise. At the end of the sub-study, all participants continued their study visits as per the main trial visit schedule. The study was designed to minimise unnecessary procedures for participants in the placebo arm during stage 1. Participants who receive placebo were expected to rebound prior to the optional week 12 biopsy visit.

For participants participating in the RIO second dose sub-study, there was no change in the three key biopsy visits; however, the optional visits were adjusted accordingly

- First two optional visits (stage 1 ATI weeks 12 and 24) remained the same irrespective if 2nd infusion has been administered.
- Third and fourth optional visits occurred 4 weeks after the 2nd ATI and 16 weeks after the 2nd ART restart respectively.

A summary of the participants visits is presented in Figure 2.2.2.

Figure 2.2.2: The RIO Gut sub-study design and sampling schedule.



2.2.3 Inclusion and exclusion criteria

Inclusion criteria

Enrolled or potential RIO study participants from any of the RIO study sites, who fulfill the main trial eligibility criteria, were eligible to participate. They were identified and offered the sub-study by their HIV clinicians at the study sites. Procedures were conducted at the lead site, Imperial College Healthcare NHS Trust.

Exclusion criteria

As for the main study exclusion criteria plus

- a. Documented bleeding disorders or taking anti-platelets, anti-coagulants, or any other medications associated with an increased bleeding risk.
- b. Individuals on immunosuppressive therapy
- c. Diagnosis of inflammatory bowel disease

2.2.4 Presumed HIV-negative controls

Colorectal biopsies from volunteers not known to be diagnosed with HIV were taken during routine clinically indicated colonoscopies or resections as part of gut tissue biobanks available from both Imperial College

London (Dr Carolina Herrera and Dr Paul Ziprin), and the Translational Gastroenterology and Liver Unit (TGU), University of Oxford (*TGU project number: 21-JF-ML-HIV*, Professor Paul Klenerman). These samples were used for the optimisation of assays described below.

2.2.4 Procedures

Informed consent: A medical or nursing professional who is part of the RIO research team counselled participants on the study rationale and obtained informed consent for the RIO GUT sub-study. Potential participants were given a minimum of 24 hours to read and consider the study Participant Information Sheet (PIS) before consent. During the consultation, screening for inclusion and exclusion criteria specific to the sub-study was undertaken (as outlined above).

Rectal biopsies: Rectal biopsies were carried out by myself in at the Clinical Trials Centre at Imperial College Healthcare NHS Trust. I discussed the procedure, possible risks and adverse effects prior to obtaining consent. Participants were advised to avoid anal products, objects including but not exclusive to douches, lubricants, and vibrators/dildos, butt plugs, or urethral sounds, or have receptive anal intercourse for 48 hours before and after the collection of rectal biopsies. Participants were signposted to request and complete routine sexually transmitted infection screens using self-sampling kits via home-testing pathways from their local sexual health services if they reported a history of receptive anal intercourse, to exclude the presence of rectally acquired sexually transmitted infections (STIs). Participants were asked to empty their bowels prior to the procedure. Participants will be asked about any adverse events from any preceding study visits, have their previous haematology and clotting results reviewed, and a directed physical examination will be performed if indicated during the procedure visit.

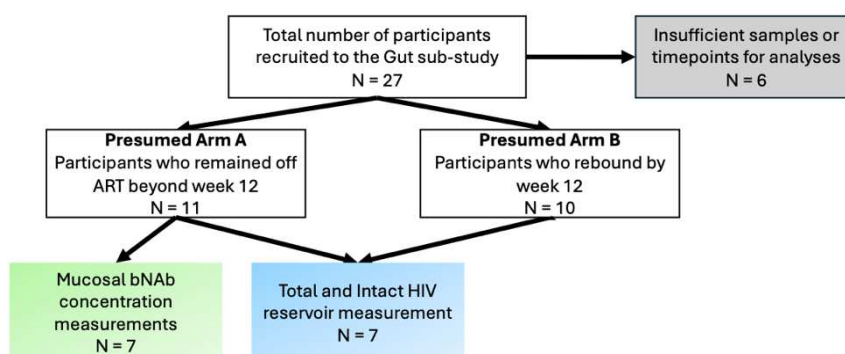
A chaperone was present during the procedure, usually the assisting nurse or other appropriate member of staff. During the procedure, the participant were asked to lie on their side with knees slightly bent, and a lubricated proctoscope was inserted to visualize the rectum. Up to twelve mucosal pinch biopsies were taken

using a Sarrett's forceps at random from 4-6 inches above the dentate line. As the biopsy sites are viscerally innervated, the procedure was painless, and no anaesthesia was required. Minor bleeding was managed with direct pressure and silver nitrate cautery as required. Duration of the procedure was 20 – 30 minutes, and participants were asked to recover in the clinic for a further 15 – 30 minutes prior to leaving the department.

2.2.5 Sample processing and storage

Samples were processed depending on the intended assay. Figure 2.2.5 shows a flowchart of assays for which participants samples were used for. 10-12 pinch biopsies from each timepoint per patient were pooled for either mucosal bNAb measurements or reservoir analysis.

Figure 2.2.5. Flowchart of which assays were samples from each participant used for.



For mucosal bNAb measurements, rectal samples were collected in pairs per cryovial without media, and immediately snap frozen at time of collection with a liquid nitrogen (LN₂) bath to -173°C. Samples from seven participants who remained undetectable beyond 12 weeks were subsequently stored at ultra-low temperatures (-150°C) and transported to the Tomaras lab, Duke University, USA, for further analysis. Seven remaining snapfrozen samples were used for DNA extraction and single genome envelope sequencing.

For reservoir measurements, 10 rectal biopsies from seven participants were collected and stored in cryopreservative medium (each cryovial contained 5 biopsies in 2mL of either 10% dimethylsulfoxide (DMSO)/fetal bovine serum (FBS), or Cryostor®CS10 freezing media, STEMCELL Technologies) and placed in a Mr. Frosty™ Freezing Container (Thermo Scientific) for controlled freezing in a -70 °C freezer overnight, before longer-term storage at -150°C until transportation to the relevant lab for analysis.

2.2.6 Managing adverse events

General safety and management of post-procedural concerns

Participants were advised that if they feel unwell after a study procedure, they should contact the HIV research team at their study site from 9am to 5pm, or attend their local emergency department out of working hours. Participants were informed that they should tell the doctor that they have been on this study and what procedure has been performed.

Concerns following rectal biopsy.

Participants were counselled that rectal biopsies were expected to heal within a week of procedure, and complications were rare (persistent bleeding 0.5%, proctitis or biopsy site infection 0.06%, perforation 0.06%). Participants were given verbal information regarding which symptoms should alert them to the possibility of a serious adverse event following their procedures, such as perforation or bleeding. In the event of these symptoms, they were advised to attend their local emergency department directly and inform the medical staff that they were involved in research, and the research unit should be informed.

Clinically significant incidental findings

Any unexpected medical conditions identified at the time of study procedures undertaken, would have been promptly referred for appropriate specialist advice and management. If participants reported any new rectal symptoms not related to previous study visits, they would have been asked to attend their local sexual health clinic to check if their symptoms were related to a sexually transmitted infection. If participants were diagnosed with a rectal sexually transmitted infection on their asymptomatic screens, they were advised to

complete treatment and had their procedure deferred until at least a week after the completion of treatment. Participants were advised that the procedure was not a screening test for malignancy or other bowel conditions.

2.3 Ethical considerations and approvals

2.3.1 Ethical approval by the Research Ethics Committee

Both the RIO study and the RIO Gut sub-study, and their subsequent study amendments, all received approvals from the London-Westminster REC (UK REC reference 19/LO/1669)., Health Research Authority (HRA) and Medicines and Healthcare products Regulatory Agency (MHRA). The RIO Gut sub-study was designed as a substantial amendment under the RIO study protocol. The studies had also received confirmation of capacity and capability from each participating NHS Trust. The studies were conducted in accordance with the recommendations for human research adopted by the 18th World Medical Assembly, Helsinki 1964 and later revisions.

2.3.2 Withdrawal from the sub-study

Participants were informed of their right to withdraw from the sub-study at any time for any reason prior to joining the study. Their study team also were able to withdraw patients from the sub-study in the event of inter-current illness, AEs, SAEs, cure, administrative reasons or other reasons.

Participants may withdraw their consent in the following scenarios:

- Withdrawal of consent from sub-study: Participants continued with the main RIO trial schedule. If they have undertaken any sub-study procedures, they were asked if they continued to give consent for any samples or data to be used for ongoing research.
- Withdrawal from individual study procedure: Participants who withdraw or were unable to undertake any individual biopsy visit, were still eligible to undertake future biopsies.

Participants were informed that their withdrawal from the sub-study would not impact participation in the overall RIO trial or their routine clinical care.

2.4 Laboratory analyses

2.4.1 Serum and tissue bNAb measurements

Serum 10-1074-LS or 3BNC117-LS IgG levels were measured by anti-idiotypic enzyme-linked immunosorbent assay (ELISA) by Dr Kelly Seaton, Tomaras Lab, Duke University. The assay has been validated for use in human samples. Briefly, high-bind 384 well plates were coated overnight with anti-idiotypic antibody specific to 10-1074 or 3BNC117. Plates were then washed, blocked, and washed again and diluted samples, standards, and controls were added. Plates were then washed and incubated with an anti-human IgG-HRP detection antibody, followed by washing, addition of tetramethylbenzidine substrate and then stop solution. Plates were then read at 450 nm to obtain an optical density (OD) reading. Infused mAb concentration in each sample was determined based on a standard curve.

Several criteria were used to determine if data from an assay were acceptable and could be statistically analyzed. Any positive sample with coefficient of variation between replicates greater than 20% was repeated. If the blank well on the plate exceeded 0.1 OD, the plate was repeated. The half maximal effective concentration (EC₅₀) measured on the standard curve also had to be within 3 standard deviations of the historical mean EC₅₀ as determined by optimization assays.

10-1074-LS and 3BNC117-LS standard curve concentrations were determined based on A₂₈₀ absorbance using the manufacturer's IgG extinction coefficients of 1.68 and 1.69, respectively. Samples with optical density less than the limit of detection (LOD) are negative and samples with optical density greater than the LOD are considered positive. For 3BNC117-LS, the LOD in HIV seropositive serum is 1265 ng/mL. For 10-1074-LS, the LOD is 197 ng/mL.

Measurement of bNAb concentrations in tissue was performed by Kelly Seaton, Duke university, using the standardized single molecule counting (SMC) technology (SMCxPro) assay using a quantitative fluorescent sandwich immunoassay technique to measure analyte in a matrix. Briefly, the anti-idiotypic capture antibody specific to either 3BNC117 or 10-1074 is labelled and then coated onto microparticle beads. Labeled and

coated microparticle beads are mixed with samples, standards, and controls. The bNAb in samples and standards was detected by a labeled anti-human secondary antibody detection conjugated to a fluorophore, which forms a sandwich complex. After several rounds of washing to remove excess unbound reagents, the complexes were broken apart by the addition of an acidic elution buffer and the released labeled detection molecules (which are now proportional in amounts to the analyte) are quantified by a combination of sensitive detectors that together perform SMC and fluorescence detection. The in-well LOD for 10-1074-LS and for 3BNC117-LS-GT in rectal tissue was 231 pg/mL.

Tissue bNAb concentrations were normalised to total protein concentrations measured using the QuantIT kit (Thermofisher™), as well as total tissue IgG antibodies measured using a custom binding antibody multiplex assay using rabbit anti-human IgG light chain capture, and anti-human IgG detection, both performed at Duke university by Dr Kelly Seaton.

2.4.2 Isolation and fluorescence-activated cell sorting (FACS) of mucosal CD45 cells from rectal biopsy samples

Cryovials containing frozen rectal tissue (10 pooled biopsies per sample) were stored in either a 10% DMSO/2% FBS medium or Cryostor®CS10 media at -150°C. These cryovials were thawed in a warm water bath set at 37 °C for about 1minute 40s. The thawed vial contents were poured over a 100 µm filter on a 50mL falcon, and washed with a complete RPMI-1640 (cRPMI) media containing 1% penicillin/streptomycin and 10% FBS. Further wash steps were undertaken by transferring the biopsies to 5mLs of RPMI/FBS, then transferring the biopsies to warm phosphate buffered saline (PBS) to be incubated for 1 minute at RT with gentle shaking.

The biopsies were digested using a two-step process. Firstly, the biopsies were incubated in 5mL of 1% ethylenediaminetetraacetic acid (EDTA) in Hank's Balanced Salt Solution (HBSS) in a universal container with a magnetic stirrer set at 350 rounds per minute (RPM) on a magnetic plate at 37 °C for 15 minutes. The biopsies were then washed with 2% FBS in PBS solution on a 40 µm filter, before transferring into universal

container containing 4 mL of RPMI-1640 media containing 25mM Hepes solution, 500µL of 10mg/mL collagenase IV (Sigma C5138) and 500µL of 10mg/mL collagenase VIII (Sigma C2139). The biopsies were then incubated with a magnetic stirrer on a magnetic plate at 37 °C set at 500 RPM for 30 minutes.

After digestion with collagenase, the contents of the universal container were transferred passed through a 40 µm filter into a 50mL falcon tube on ice, and the container washed with cold 2% FBS in PBS. The falcon tube was then centrifuged at 1700rpm for 7 minutes at 4 °C, followed by the removal of the supernatant. The remaining cell pellet resuspended in 400µL of 2% PBS in FBS and transferred to a 1.5mL Eppendorf tube on ice. The Eppendorf tube was further centrifuged at 2700RPM for 5 minutes at 4 °C, before removing the supernatant, and resuspending the cell pellet in 200µL of 1 in 1000 LiveDead dye in PBS solution and incubated under aluminium foil for 20 minutes.

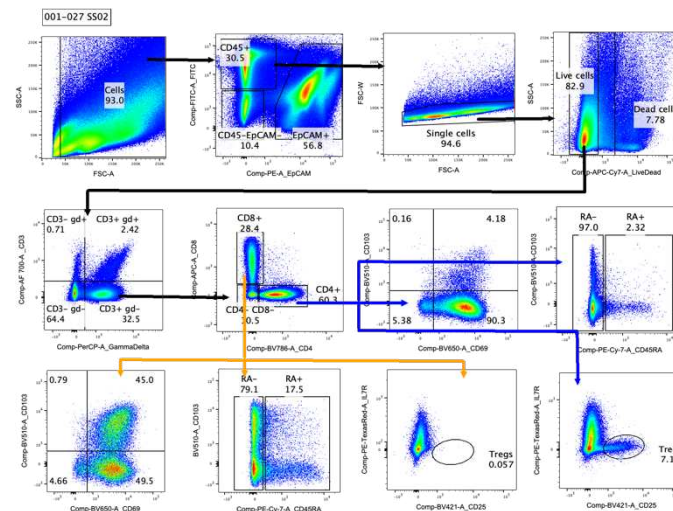
The sample was then washed with 800µL 2%FBS/PBS, centrifuged at 2700RPM for 5 minutes at 4°C, before removing the supernatant. The cell pellet was incubated with the 200µL of solution containing the remaining staining panel (Table 2.4.2) under aluminium foil for 25 minutes at 4°C. The cells were subsequently washed with 800µL of 2%FBS/PBS, centrifuged at 2700RPM for 5 minutes to remove the supernatant, and resuspended in 100µL of 4% PFA for 15minutes at RT for fixation. The samples were then washed with 800µL of 2%FBS/PBS, centrifuged at 3500RPM for 5 minutes to remove the supernatant, and transferred to a FACS tube for cell sorting.

Table 2.4.2: Staining panel for FACS of gut tissue biopsies

Marker	Colour	Titers
LiveDead	FVS780	1:1000
EpCAM	PE	1:320
CD45	FITC	1:100
CD3	A700	1:100
CD4	BV786	1:400
CD8	APC	1:40
CD45RA	PeCy7	1:200
CD69	BV650	1:80
CD103	BV510	1:320
TCR gamma delta	BB700	1:100
CD25	BV421	1:100
IL7R	PECF594	1:100

All samples were acquired on the BD FACSAria™ Fusion Flow cytometer with a 100µM nozzle in yield mode at the Vandekerckhove lab, Ghent University in collaboration with Dr Marion Pardons. Calibration was done prior to sorting of samples using single-stained beads. Samples were sorted for live CD45+ cells based on the following gating strategy: Total cells by forward and side scatter -> CD45+ cells -> Single cells by width against area of forward scatter -> live cells (Figure 2.4.2). Data were analysed using FlowJo Software v10.10.1 (BD Life Sciences).

Figure 2.4.2: FACS gating strategy for cell sorting.



Sorted CD45+ cells were centrifuged at 14000RPM for up to 10 minutes to completely remove the supernatant, and the resultant dry cell pellet was taken for direct cell lysis without for PCR requiring DNA extraction using the DirectPCR Lysis Reagent (Viagen), by incubating at 45 minutes at 85°C.

2.4.3 Total and Intact HIV DNA quantification in rectal tissue samples

Total and intact proviral DNA reservoir measurement of the direct cell lysate by a multiplex 5-probe digital PCR assay (86) by Dr Marion Pardons and Ms Ytse Noppe, Ghent University. The primers and probes are described by Delporte et al. (86) and target the LTR, *gag*, *pol*, *env*, and packaging site (ψ) regions of the HIV genome, and linked to the ROX, Cy5, ATTO550, HEX, and FAM fluorophores respectively. All primer and probe sequences were manufactured by Integrated DNA Technologies. The assay was performed on the QIAcuity Four platform (Qiagen, Germany). The reaction mix includes 10 μ L 4x concentrated, ready to use QIAcuity Probe Master Mix (Qiagen), 2 μ L of each primer/probe set, 10 μ L template gDNA and nuclease free water to 40 μ L total volume per reaction. The resultant PCR reaction mix are then transferred to a 26K 24-well nanoplate (Qiagen) and sealed, and the mix is partitioned on the QIAcuity Four digital PCR system. The PCR program used involves 2 minutes at 95°C to activate the QIAcuity probe mix, followed by 40 cycles of 94°C for 30 seconds and 56°C for 60 seconds. Dilution curves were measured in 20 replicates per dilution point to obtain three standard curves, and a total of 288 negative controls were measured to establish the

limit of blank (LoB). Participant samples were run in triplicates, with two positive controls and two negative controls.

The measurements for total and intact HIV DNA were normalised to RPP30, a human housekeeping gene, as a marker of the total number of human cells in each sample, as described by Bruner et al (81). Primers and probes were used as described by Bruner et al (81). The dPCR program was the same as described for the 5-probe assay PCR program described above. Each sample was diluted 100 times before adding to the reaction mix.

I analysed the reservoir measurements using a R Shiny app software described by Delporte et al. (86) and thresholds adjusted manually if the default thresholds did not fit individual samples. Intact HIV DNA was quantified based on either two regions (*ψ*, *env*) from the classical IPDA (81) or all five regions from the multiplex assay. Total HIV DNA was quantified based on the presence of LTR regions detected. Intact HIV DNA levels were adjusted for DNA shearing and normalised per million cells using the RPP30 measurements.

2.4.4 DNA extraction from rectal tissue

For samples used for single genome envelope amplification and sequencing, DNA was extracted using the DNeasy® Blood and Tissue kit (Qiagen), in accordance with the manufacturer's protocol for purification of total DNA from animal tissues (Spin-Column protocol). In summary, pooled tissue biopsies from each timepoint were washed with PBS, before 180µL of Buffer ATL and 20µL of proteinase K were added to the microcentrifuge tube containing the samples. The sample was mixed by vortexing, and incubated at 56°C on a shaking platform at 800 rpm overnight for complete lysis. 4µL of RNase A (100µg/mL) was added, vortexed, and incubated for 2 minutes at RT. 200µL of buffer AL is added and mixed by vortexing, followed by 200µL of 100% ethanol and mixed by vortexing to form a homogenous solution. The mixture was pipetted into the DNeasy Mini spin column placed in a 2mL collection tube and centrifuged at 8000rpm ($\geq 6000g$) for 1 minute. The flowthrough was subsequently discarded. The spin column was placed in a new 2mL collection tube and 500µL of buffer AW1 was added and centrifuged at 8000rpm ($\geq 6000g$) for 1 minute. The flowthrough was

subsequently discarded. The spin column was placed in a new 2mL collection tube and 500µL of buffer AW2 was added and centrifuged at 14000rpm ($\geq 20000g$) for 3 minutes. To prevent carry over of ethanol, the column was placed in an empty collection tube and centrifuged for another minute at 14000rpm ($\geq 20000g$). The column was then placed in a microcentrifuge tube and 200µL of the elution buffer AE (pre-warmed to 70°C) was added onto the DNeasy membrane and incubated at room temperature for 5 minutes. The column was then centrifuged at 8000rpm ($\geq 6000g$) for 1 minute. The last step was repeated in a new microcentrifuge tube to increase DNA yield.

2.4.5 Single genome amplification and intact envelope sequencing

Nested Envelope PCR amplification. Extracted DNA were amplified using a nested envelope PCR protocol, with one of three primer sets: Default protocol (Outer reaction primers: Nested_Env_5Outer, Nested_Env_3Outer; Inner reaction protocol: Nested_Env_5Inner, Nested_Env_3Inner), Long protocol (LP) (Outer reaction primers: Nested_Env_5_B3R3, Nested_Env_3_R3B6R; Inner reaction protocol: Nested_Env_5Inner, Nested_Env_3Inner), and the LP807 protocol (Outer reaction primers: Nested_Env_5_B3R3, Nested_Env_3_R3B6R; Inner reaction protocol: Nested_Env_5Inner, Nested_Env_3Inner_807). The list of primers and thermocycler protocols are listed in Table 2.4.5.

The DNA samples were first serially diluted to a target of fewer than 33% positive wells (<32wells in a 96 well plate) to maximise the distribution of a single copy DNA template in each positive well. A master mix was made up of the following: For the default outer reaction and all inner reactions, a 14.25µL mix per well was made up of 11.5125µL Molecular-grade DNase/RNase-free water, 1.5µL 10X PCR buffer, 0.6µL 50mM DNA-free Magnesium Chloride, 0.0375µL DNA-free Invitrogen™ Platinum™ Taq (Fisher Scientific), 0.3µL deoxynucleotide triphosphate (dNTP) mix (Fisher Scientific, 10mM each), 0.15µL each of the forward and reverse primer. 1µL of the DNA sample was added as the template to the mix per well, for a total reaction volume of 15.25µL per well. For the LP and LP807 outer reaction reactions, a 19µL mix per well was made up of 15.3µL Molecular-grade DNase/RNase-free water, 2µL 10X PCR buffer, 0.8µL 50mM DNA-free

Magnesium Chloride, 0.1 μ L DNA-free Invitrogen™ Platinum™ Taq (Fisher Scientific), 0.4 μ L deoxynucleotide triphosphate (dNTP) mix (Fisher Scientific, 10mM each), 0.2 μ L each of the forward and reverse primer. 1 μ L of the DNA sample was added as the template to the mix per well, for a total reaction volume of 20 μ L per well. After amplification of the inner reaction, the samples were diluted five-fold, and underwent DNA electrophoresis on a pre-cast 96well 1% Invitrogen™ agarose E-gel (Fisher Scientific) for 12minutes on an E-base™ (Fisher Scientific) under program EG as per manufacturer's instructions, with an E-gel sizing DNA ladder marker (Fisher Scientific). If >33% of wells were positive for appropriately sized Env PCR products (~3000bp), the experiment was repeated with serial dilutions of the DNA sample to achieve the target dilution for single genome copies. Positive well products were used for DNA library preparation described in the next step.

DNA Library preparation. DNA library preparation is performed using the Nextera Library PrEP kit (Illumina®). The amplified Env products are diluted three-fold with Molecular-grade DNase/RNase-free water. For the tagmentation of samples from a 96-well plate, a tagmentation master mix comprising of 132 μ L of Tagmentation buffer and 26.4 μ L of tagmentation enzyme was mixed and 1.5 μ L of the mix was distributed to each well of the reaction plate. 1 μ L of the PCR product was added into each well and incubated at 55°C for 5 minutes. The barcode index adapters (8x i5 and 12x i7) were prepared as index master mixes by adding 18 μ L of 2X KAPA HiFi HS Ready Mix polymerase (Roche) and 6 μ L of i7 index adapters, and 27 μ L of 2X KAPA polymerase and 9 μ L of i5 index adapters respectively. 2.5 μ L of each i5 and i7 index adapter master mix were added to each well using a multichannel pipette resulting in 96 individual permutation of the two adapters per well for individual barcoding. The reaction plate was then placed in the thermocycler to run the tagmentation protocol listed in Table 2. Following tagmentation, the resultant product was cleaned up using Ampure XP beads (Beckman Coulter™). 8 wells of tagmentation product were pooled in a 1.5mL Eppendorf tube and 136 μ L of Ampure XP beads added and incubated at RT for 5 minutes. The Eppendorf tubes were then placed on a magnetic stand and incubated for 1 minute, followed by removal of the supernatant

without disturbing the beads on the side of the tube. The beads were washed with 70% ethanol 4 times and left to dry for 2 minutes after removing the last ethanol wash step. After removing the Eppendorf tubes from the magnetic stand, the beads were resuspended in 100µL of Tris-EDTA (TE) buffer and incubated for a further 2 minutes. The Eppendorf tubes were placed on the magnetic stand again, and the supernatant was collected from each tube and pooled into a single DNA Lobind Eppendorf tube for the DNA library.

Table 2.4.5. List of HIV Envelope nested single genome amplification primers and thermocycler protocols

List of primers			
Target region	Primer name	Sequence (5' – 3')	Position (HXB ref)
Env	Nested_Env_5Outer	TAGAGCCCTGGAAGCATCCAGGAAG	5853 – 5877
	Nested_Env_3Outer	TTGCTACTTGTGATTGCTCCATGT	8913 – 8936
	Nested_Env_5_B3R3	TGGAAAGGTGAAGGGGCAGTAGTAATAC	
	Nested_Env_3_R3B6R	TGAAGCACTCAAGGCAAGCTTTATTGAGGC	
	Nested_Env_5Inner	CACCTTAGGCATCTCCTATGGCAGGAAGAAG	5953 – 5983
	Nested_Env_3Inner	GTCTCGAGATACTCGTCCCACCC	8882 – 8904
	Nested_Env_3Inner_807	GTCTCGAGACGCTGGTCTACTC	8882 – 8904
Thermocycler protocols			
	Temperature	Time	No. of cycles
Env Default Outer Protocol	94 °C	2 minutes	1x
	94 °C	15 seconds	35x
	58.5°C	30 seconds	
	68 °C	4 minutes	
	68 °C	15 minutes	1x
	4 °C	For storage	
Env Long Outer Protocol	94 °C	2 minutes	1x
	94 °C	15 seconds	45x
	55°C	30 seconds	
	68 °C	4 minutes	
	68 °C	15 minutes	1x
	4 °C	For storage	
Env Inner Protocol	94 °C	2 minutes	1x
	94 °C	15 seconds	44x
	61°C	30 seconds	
	68 °C	4 minutes	
	68 °C	15 minutes	1x
	4 °C	For storage	
Tagmentation protocol	72 °C	3 minutes	1x
	98 °C	2minutes 45 seconds	1x
	98°C	15 seconds	8x
	62 °C	30 seconds	
	72 °C	1 minute 30 seconds	
	4 °C	For storage	

Sequencing

The DNA library was sequenced using the MiSeq System (Illumina®), and MiSeq Reagent Kits v3 (Illumina®) at the Frater lab, University of Oxford. The output was delivered in demultiplexed fastq files.

2.5 Statistical and bioinformatics data analyses

2.5.1 Meta-analysis of time to viral suppression after treatment interruption

I conducted a systematic review and meta-analysis of recent studies to determine the proportion of people living with HIV participating in ATI cure-related clinical studies, who achieve viral suppression following ART re-initiation. A full description of the search strategy and statistical methodology is presented in Chapter 4.

2.5.2 Sample size considerations for the Gut sub-study

Sample size calculations for recruitment into the gut sub-study were based on previous pharmacokinetics studies of a similar bNAb (VRC01) used in participants without HIV (229), where rectal tissue VRC01 normalised to total protein levels were 10-fold lower than seen in serum. Four participants receiving bNAbs will be required to provide 80% power to detect differences between tissues and serum levels, however due to the blinded nature of sub-study recruitment from the main trial double the numbers (total target of n=8) would be required to power the bNAb measurements for the primary sub-study outcome. The assumption was that a similar number was required for the other exploratory outcomes (n=8 for total and intact HIV reservoir measurements, and n=8 for Envelope sequencing) and finally to address potential withdrawal of consent for study procedures or inadequate timepoints or tissue samples, the recruitment target was rounded up to 30 participants in total.

To minimise procedure burden, three key biopsy visits have been identified to provide meaningful data on tissue bNAb concentrations at baseline, peak (week 4) and following ART restart (week 12 or 16 post ART restart). Although there is a maximum number of 7 biopsies for participants, participants in the placebo arm are unlikely to undertake the optional week 12 and 24 biopsies during the first treatment interruption, from previous reports they were likely to experience viral rebound and ART restart prior to week 12 after stopping ART .

Although the numbers for the exploratory outcomes for reservoir measurements, and flow cytometry of gut tissue were small and may not be sufficiently powered for statistical differences between groups, the results

contribute to a deeper mechanistic understanding of the impact of bNAbs to fill a knowledge gap and allow the generation of further hypotheses for developing future cure-related trial strategies.

2.5.3 Pharmacokinetics (PK) analyses of serum and tissue bNAb measurements

Population and individual PK analyses of serum and tissue bNAb measurements were estimated using both one and two-compartment models with the MonolixTM software. Monolix estimates PK parameters using a non-linear mixed effects model with a stochastic approximation of the expectation maximisation (SAEM) algorithm. The covariate model was built according to a Stochastic Approximation for Model Building Algorithm (SAMBA) algorithm (294), with the criteria for final model acceptance based on the lowest Bayesian Information Criteria (BIC) score. The following covariates were tested: Age, Log(Weight), Virus clade (B vs non-B or unknown clade), study arm, and presence of predicted baseline bNAb resistance mutations using a validated algorithm (295). The model that best fit the data for either bNAbs was a two-compartment model with a central compartment (V), a peripheral compartment with a rate of transfer to and from represented by the constants k12 and k21, and a linear elimination with a rate constant k, with parameters transformed into the input parameters A, B, α , β using the equations below. 90% prediction intervals were calculated using the R package RsmLx, with 1000 bootstrap replicates.

Equations

$$V = 1/(A+B)$$

$$k_{21} = \alpha - A \cdot V \cdot (\alpha - \beta)$$

$$k = (\alpha \cdot \beta) / k_{21}$$

$$k_{12} = \alpha + \beta - k_{21} - k$$

2.5.4 Building a direct inhibitory pharmacokinetic-pharmacodynamic model of the effect of bNAb concentration on viral dynamics to predict bNAb rebound concentrations and IC80 values

A direct inhibitory pharmacokinetic model was used where the action of inhibition is defined by the following parameters: The baseline effect (E_0), maximal fraction of inhibition (I_{max}), the half maximal inhibitory concentration (IC_{50}), and sigmoidicity of the curve (γ). The eighty percent of the maximal inhibitory concentration (IC_{80}) value was calculated using the equation $\text{Log}_{10}(IC_{80}) = \text{Log}_{10}(IC_{50}) + (1/\gamma) * (\text{Log}(80/(100-20)))$.

The population parameters from the best fit pharmacokinetics model were fixed, and plasma viral load data from participants in Arm A who experienced viral rebound were included. Initially individual bNAbs models were built using the COSSAC method for the best fit model. Subsequently both bNAbs were modelled in a combined model using a Bayesian approach to determine the overall effect based on the lowest resultant effect (viral load). Data from Arm B participants were subsequently included, with the additional condition that if participants were on ART at a particular timepoint, the viral load was assumed to be below the limit of detection at 1 copies/mL. The best fit model was chosen based on the lowest BIC score.

2.5.5 Phylogenetic analyses and bNAb resistance prediction

Baseline HIV proviral DNA from PBMCs and rebound HIV RNA sequences from the RIO trial were provided by Dr Panagiota Zacharopoulou, Frater lab, University of Oxford. In brief, phylogenetic analyses comparing baseline PBMC proviral DNA sequences and HIV RNA sequences were done using R packages phangorn and ape. Maximum likelihood nucleotide trees were built using phangorn with 1000 bootstrap replicates, comparing four commonly used nucleotide substitution models (JC, K80, HKY, GTR) (296–299) to select the optimal model by lowest BIC. Trees were rooted on the HXB2 reference sequence, and annotated in MEGA (v11.0.10).

bNAb resistance prediction were also computed by Dr Penagiota Zacharopoulou, Frater lab, University of Oxford, using a custom computational pipeline previously described by Gaebler et al. (176). In brief, the

model was trained using paired neutralisation and sequencing data from the CATNAP database, and initially discards non-HIV sequences before aligning the remaining sequences against a reference HIV sequence to build consensus sequences for each sample, which each sample's reads are re-aligned to. Individual reads that have less than 70% nucleotide concordance in more than one position within *env* are discarded, to build a final consensus sequence which is translated to an amino acid sequence for each sample. The sensitivities to both 10-1074 and 3BNC117 are assigned using a machine learning algorithm described by West et al. (295) The specific amino-acid residues associated with resistance in this algorithm are as follow: for 10-1074 325D/N, 330H/Y, 332N, 333notP, 334S/T; for 3BNC117 279D/N, 456R, 457D, 458G, 459G, and the output is a binary prediction of resistant or not resistant for each sequence.

2.5.5 Descriptive analyses of changes in gut-associated HIV reservoir measures and mucosal immunity

Descriptive frequencies and proportions of total and intact proviral reservoir measures, and mucosal immune cell populations on flow cytometry analyses were compared and plotted in R. Comparison using chi-squared tests and t-tests or Mann-Whitney U tests were performed as appropriate.

Chapter 3

Development of a high throughput assay for HIV DNA quantification and intact sequence verification

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3.1 Introduction

A key marker for evaluating cure interventions is the size of the latent HIV reservoir, however there are many challenges for accurately quantifying the reservoir. Firstly, the transcriptionally silent nature of the reservoir means there are no cellular surface markers or protein targets available to detect quiescent cells containing HIV apart from the integrated proviral genome itself, although assays that detect intracellular RNA may be able to discriminate between low-level transcriptionally active reservoir cells (105–107). The cells may be stimulated to become transcriptionally active to produce viral proteins, however, this process is likely to change the phenotype of the host cell. Assays involving cell stimulation may still underestimate the true replication-competent reservoir as not all cells containing intact provirus may be activated (63,79). Second, due to the error-prone nature of the pre-integration reverse transcription step in the viral life cycle, the vast majority of integrated provirus is defective with large deletions or hypermutations and are therefore unlikely to be replication-competent (74,75). Thirdly, the overall frequency of cells containing HIV is rare, even more so in people who were diagnosed and treated during PHI (124,300). Finally, the bulk of the HIV reservoir is present in tissue rather than blood, particularly in gut, lymph nodes, genital tract, respiratory tract, and the central nervous system, all of which are difficult to adequately sample with existing techniques (79,268,269).

There are numerous assays currently in use or being developed to estimate the HIV reservoir, as summarised in Table 1.2.3, Chapter 1. These assays may be divided into the following broad categories: 1) Quantitative viral outgrowth assays and its derivatives; 2) PCR or sequencing based methods to measure the intact HIV reservoir; 3) molecular assays measuring total HIV DNA or cell-associated HIV RNA; 4) Assays measuring transcriptionally or translationally active virus; 5) Other strategies such as the ultrasensitive assay to measure residual viraemia. Each assay has their advantages and limitations as described in Table 1.2.3, and none of the existing assays have been validated to predict outcomes of these strategies apart from closely monitored ATIs in clinical trials.

For high throughput analyses in large clinical trials such as RIO, particularly with low HIV DNA copy samples such as gut tissue biopsies obtained from the RIO Gut sub-study, it has been suggested that molecular assays such as the IPDA (81) or its derivatives may be the most suitable assay for estimating intact the HIV DNA reservoir size (78) due to its low cost, high-throughput nature and low cell numbers requirement. Drawbacks of the assay include clade dependence and the possibility of primer/probe mismatches, and the inability to recover proviral sequences for further genomic characterisation of the reservoir. Alternatively, the Q4PCR assay (82) may provide qualitative analyses of proviral sequences in addition to the quantification of the intact HIV reservoir. In a head-to-head comparison between the IPDA and Q4PCR, although they were positively correlated, the IPDA identified a median 19-fold higher intact provirus reservoir estimates than the Q4PCR (84), suggesting the possibility of the IPDA overcalling defective proviruses as intact due to bias of amplifications towards smaller products or probe misidentification, and inefficient amplification within the Q4PCR. The differences in cell normalisation methods may also contribute to the discrepant intact proviral estimates.

Droplet microfluidics may provide a solution to address the time and labour-intensive serial limiting dilution step for single-genome analyses. Water-in-oil (WO) emulsions droplets are used in the IPDA (81) to encapsulate DNA for analyses on the BioRAD QX200 Reader for analysis, however these droplets are unable to be sorted for downstream analyses on the commercial platform. Fluorescence-activated droplet sorting (FADS) platforms are available but require custom and highly specialised equipment limiting their use (301,302). Brower et al. (303) first demonstrated the isolation and recovery of nucleic acid sequences of interest using standard fluorescence activated cell sorting (FACS) platform by double encapsulating WO droplets in an outer aqueous sheath, which allows an electrical charge to be applied to the droplet for sorting, with post-sort recovery efficiencies >70%. This would allow high-throughput analyses of single-genomes of interest including the rare intact HIV proviral DNA sequences from a mixed sample of defective proviral sequences and human genomic DNA. In this chapter, I describe the development and optimisation of a high-

throughput assay to sort HIV DNA for quantification and intact sequences verification, the barriers of achieving this assay, and alternatives assays considered for quantification of the latent HIV reservoir from tissue sample

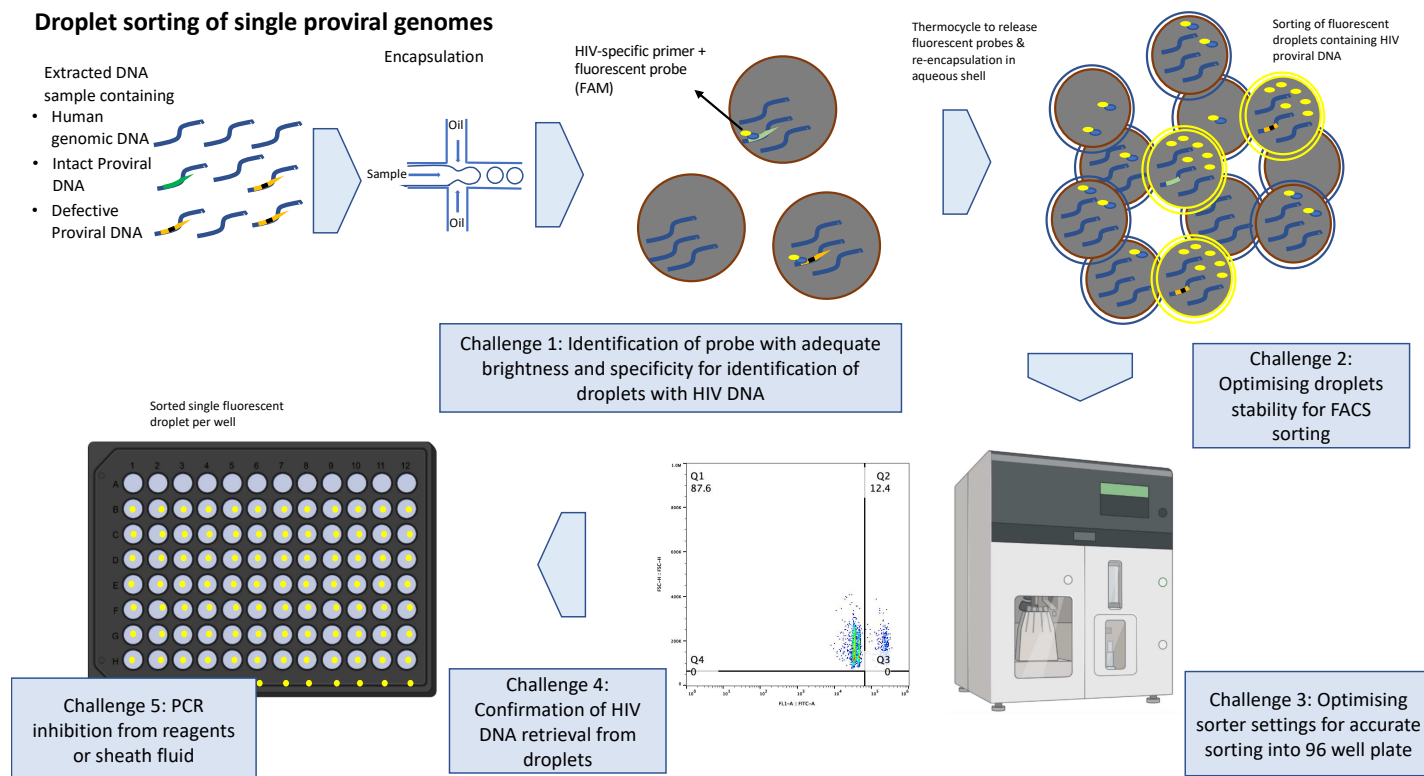
3.2 Methods

3.2.1 Proposed assay workflow and challenges

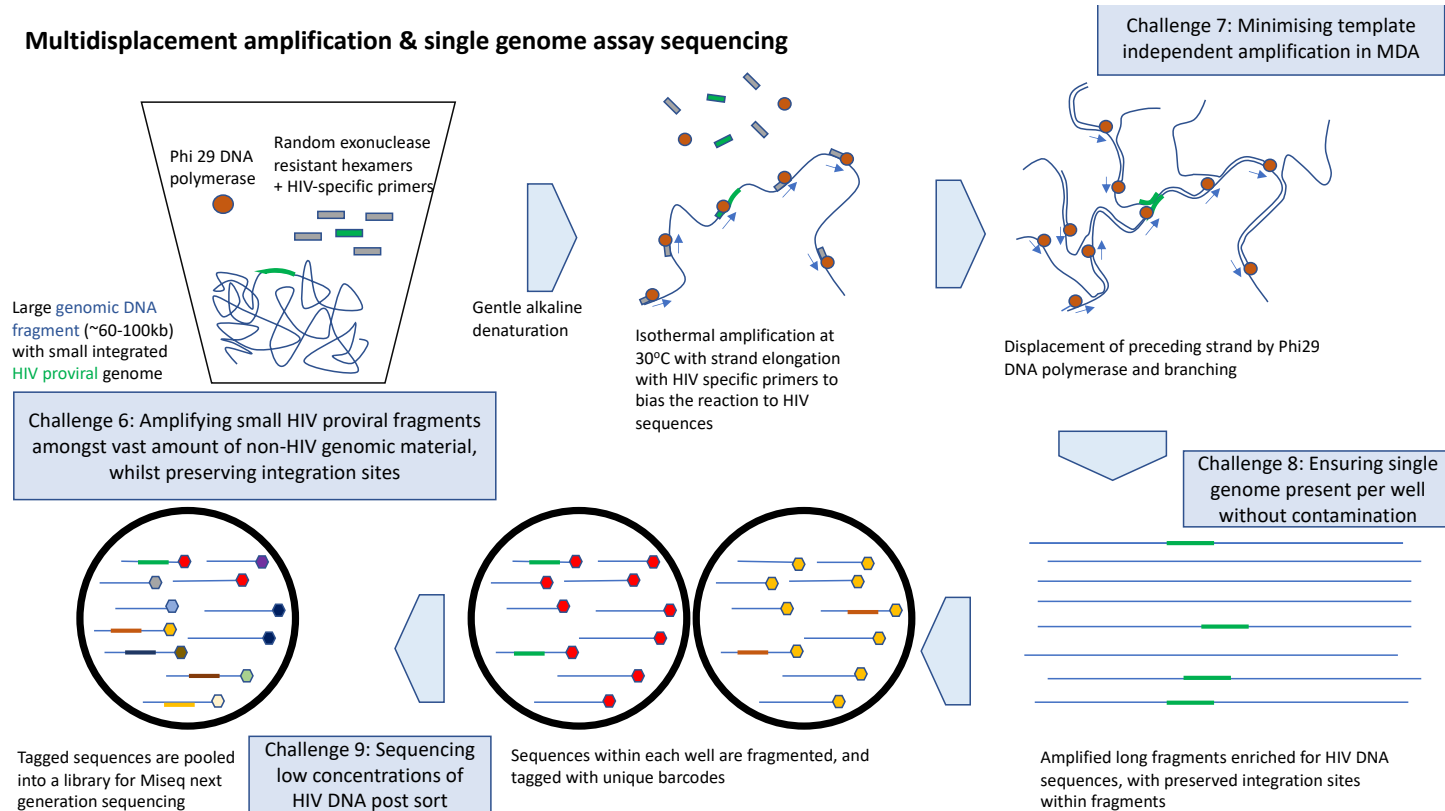
The proposed assay summarised in Figure 1 would encapsulate extracted DNA with primers and fluorescent probes targeting conserved HIV regions in double emulsion water-oil-water (WOW) droplets. This would allow selection of droplets containing single copies of HIV genomes for downstream single genome amplification of the HIV proviruses present in the sample. The use of non-specific primers and multi-displacement amplification would allow amplification of all genomic material in the well, including the HIV and host genomic templates, which could be bioinformatically aligned to interrogate full-length HIV sequences and corresponding interrogation sites. The steps taken to address the challenges described at each step of the assay are subsequently described.

Figure 3.2.1 Proposed assay workflow and challenges

Droplet sorting of single proviral genomes



Multidisplacement amplification & single genome assay sequencing



3.2.2 Standards and positive controls

DNA Standards: DNA extracted from 8E5/LAV and ACH2/LAI cell lines was provided by Dr Mathew Jones, postdoctoral scientist at the Frater lab. Each 8E5 cell line containing one HIV provirus per cell which is replication incompetent (304); and ACH2 cell lines, containing replication competent virus, with reported increased number of integration sites with time and cell passage (305). 8E5 DNA was extracted using QiaAmp DNA Blood Mini kit (Qiagen, Germany) and diluted to 3-fold dilution series as described by Jones et al(304). ACH2 DNA was similarly extracted and diluted to 1-log standards. DNA concentrations were measured using QUBIT flurometry (Table 3.2.2). Primer and probe sequences are available in Appendix 1. ACH2 standards demonstrated a 10 – 15 fold increase in copy numbers per cell compared to 8E5.

***Gag* PCR products**

8E5 extracted DNA were amplified using outer nested *gag* primers to produce solutions of 1418bp long fragments. Figure 3.2.2 shows the position of the fragment (blue) and the packaging site amplicon (green) contained within, used to signal droplets containing HIV DNA.

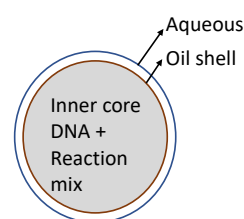
Both *env* and *gag* PCR products were used for optimisation of the assay.

3.2.3 Generation of double emulsion droplets using a microfluidics platform

Figure 3.2.3.1 summarises the composition of the double emulsion droplets. These droplets are approximately 40-50µm in diameter and average inner core volume of 14 picolitres. The droplets generation workflow had been previously developed by Dr M Jones and Dr L Parolini (Postdoctoral scientists, Frater lab).

Figure 3.2.3.1 Composition of double emulsion droplets

Droplets	
Size	~40-50µm diameter
Inner core	Reaction mix + surfactant (0.5% Igepal Ca630)
Oil shell	1% EA surfactant in Hydrofluoroether (HFE) oil
Outer Aqueous shell	0.1% Sodium Dodecyl Sulfate (SDS) in deionized water

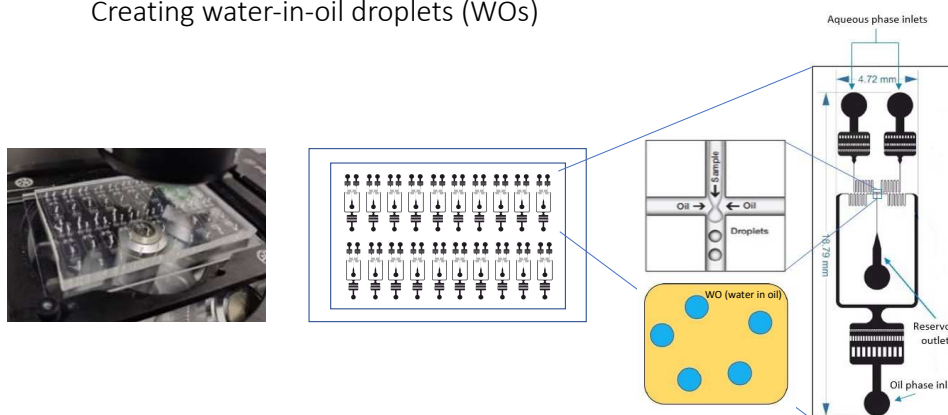


During the single encapsulation stage, extracted DNA libraries were encapsulated within WO droplets using a single emulsion device (Figure 3.2.3.2). 1% EA surfactant (Raindance technologies, Lexington, MA) in Hydrofluoroether (HFE) oil (3M) is used to form an oil shell around each WO droplet, which are collected in PCR tubes under a layer of mineral oil, which provides a thermoprotective layer during thermocycling.

Figure 3.2.3.2. Single emulsion encapsulation device schematic

Single emulsion encapsulation device

Creating water-in-oil droplets (WOs)

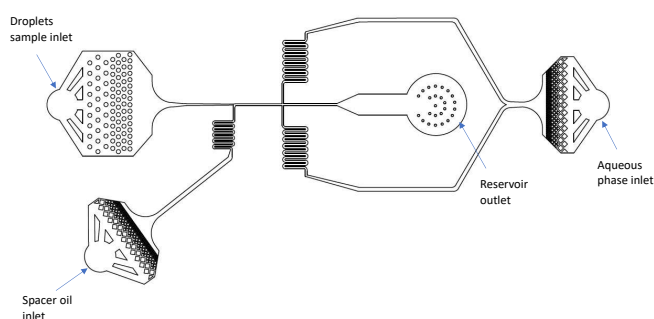


Device design by Lucia Parolini

The WO droplets are subject to thermocycling to release fluorescent probes at the PCR conditions listed in the previous section. Post thermocycling, the WOs are re-encapsulated in a double emulsion encapsulation device (Figure 3.2.3.3.), using a pressure pump to maintain the pressure (~130 – 180mbar) input of the droplet sample. 0.1% sodium dodecyl sulfate (SDS) (Sigma-Aldrich) in deionised water is used for the external aqueous sheath.

Figure 3.2.3.3. Double emulsion encapsulation device schematic

Double emulsion encapsulation device Creating water-in-oil-in-water droplets (WOWs)

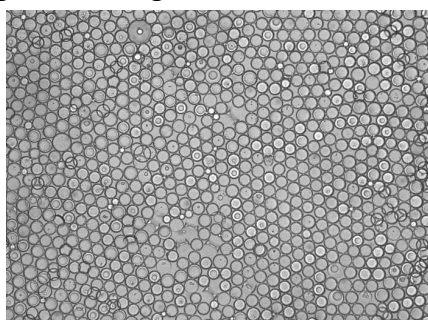


Device design by Lucia Parolini

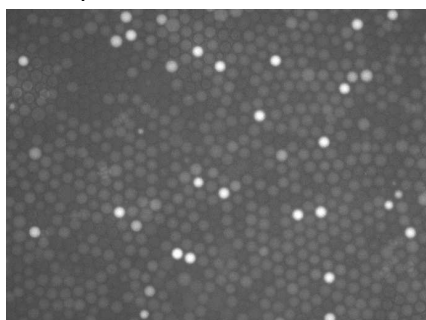
This workflow has the capacity to generate a high frequency of droplets (1620droplets/s). For a sample containing 10 000 Copies/ μ L of DNA, the mean rate of droplets containing a single copy of HIV fragment under poisson-distribution is 1 per 71 droplets (Figure 3.2.3.4).

Figure 3.2.3.4 Microscopy images of microfluidic droplets

a. Brightfield images of double emulsion droplets



b. Droplets with FITC fluorescence indicating HIV DNA.



3.2.4 Primers, probes and polymerase chain reactions

A list of primers, probes, their sequences and corresponding positions against the HIV reference sequence HXB2 is available in the Appendix Table 1.

PCR conditions:

PCR amplification of FAM-labelled probes: 1 minute at 93°C, followed by 45 cycles of 15 seconds at 93°C and 1 minute at 60°C, followed by enzyme deactivation at 98 °C for 10 minutes, then cool at 4°C for storage.

Nested PCR reaction for *env*: Conditions as listed in Chapter 2, Table 2.

Nested PCR reaction for *gag*:

1st round - 2 minutes at 94°C, followed by 35 cycles of 15 seconds at 94°C, 30 seconds at 58.5°C, and 3 minutes at 68 °C, on completion followed by incubate at 68 °C for 15 minutes before cooling at 4°C for storage.

2nd round - 2 minutes at 94°C, followed by 45 cycles of 15 seconds at 94°C, 30 seconds at 61°C, and 3 minutes at 68 °C, on completion followed by incubating at 68 °C for 15 minutes before cooling at 4°C for storage.

3.2.5 Fluorescence activated droplet sorting

Droplets were sorted using the Sony MA900 cell sorter. After optimisation, droplets were sorted using a 130µm nozzle chip, at single-cell 3-drop mode. Default bead-calibrated sort-delay values were used.

Droplets were sorted into FACS tubes or 96-well U bottom plates.

3.2.6 Multidisplacement amplification (MDA)

MDA reactions were performed using the Repli G single cell kit (QIAGEN) and a modified protocol from the original manufacturer's instructions (personal communication from M. Lichterfield). The protocol involves 10µl reaction mixes consisting of 5.8µl reaction buffer, 0.4µl of Phi29 DNA polymerase, 1.8µl of water and 2µl of DNA template), and a reduced reaction time for 4 hours at isothermal temperatures at 30°C.

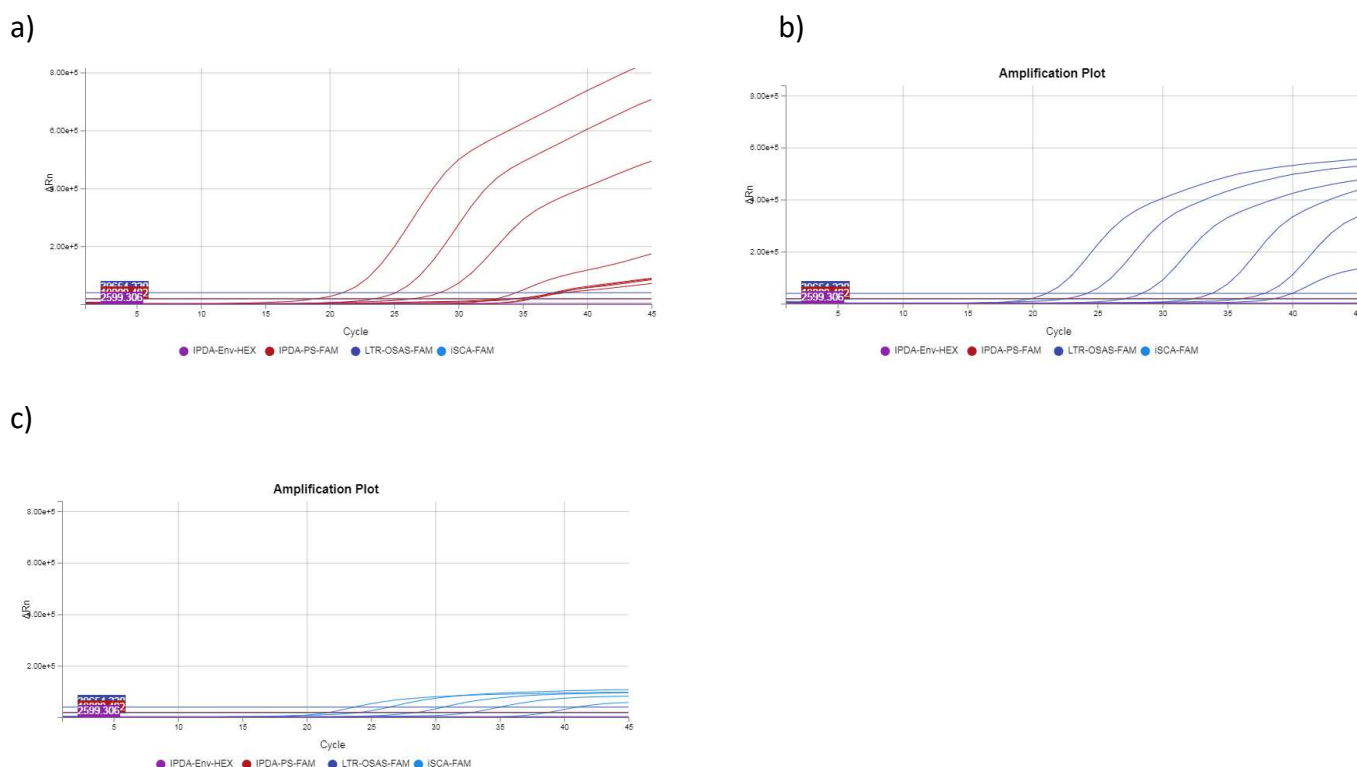
3.3 Results

3.3.1 Identifying probes

Three probe candidates to conserved regions in the HIV genome, previously characterised and published, were assessed. These FAM-labelled probes included those targeting the packaging site (ψ) region described in the IPDA (81), 5' LTR region in a total HIV DNA assay (304), and the integrase-encoded *pol* region in the integrase single-copy assay (ISCA) (306).

As the assay required the largest difference in fluorescence to isolate the target population, end-point fluorescence was used to determine the optimal probe for the assay. The FAM-labelled ψ probe was selected as it demonstrated the highest end-point fluorescence of the 3 probes when measured in bulk qPCR reactions (Figures 3.3.1 a-c).

Figures 3.3.1 qPCR plots assessing different primer and probe candidates



(Left to right, top to bottom) a.) qPCR plot of 8e5 standards vs negative controls, using the IPDA primer/probes to the PS (ψ) site. b). qPCR plot of 8e5 standards vs negative controls, using the total DNA assay primer/probes to the 5' LTR site. c) qPCR plot of 8e5 standards vs negative controls, using ISCA primer/probes to the *pol* region

3.3.2 Optimising sorter settings

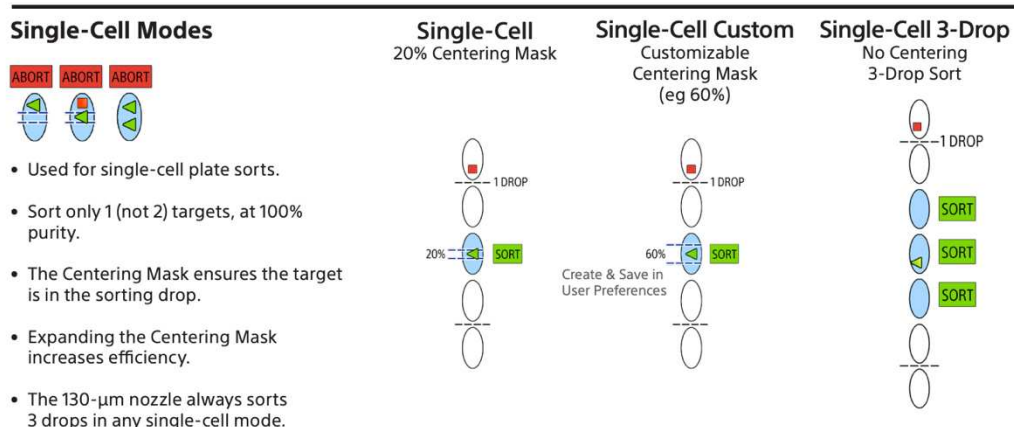
Selecting chip nozzle size

Initial attempts using a 100mm nozzle chip was unsuccessful, but the use of a larger 130mm nozzle chip allowed analysis of droplets on the analyser. The size of the droplets (~40-50mm) are larger than conventional bacteria or lymphocyte cells (~10mm) used for sorting, and may be too large for a 100mm nozzle chip. My findings are in line with previous reports that larger nozzle sizes are preferable to minimise droplets loss during FACS sorting from the high shear rates and forces generated during sorting (303,307).

Sort modes and target calibration

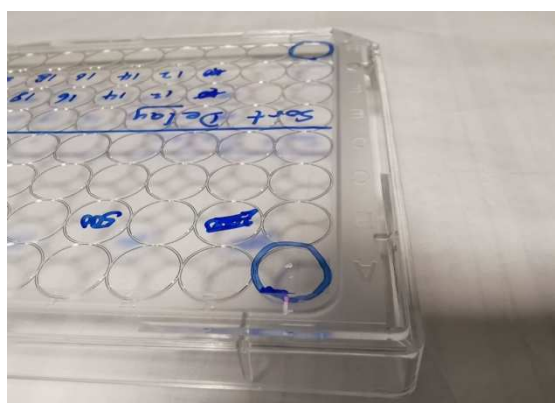
Calibration of the sorter was performed on 96-well U-bottom plates with cover lids (SARSTEDT Ag & Co. KG, Germany). Using the single-cell mode (for regular cells) and single-cell 3-drop mode (for larger cells) (Figure 8.), droplets were aimed at 4-corners target wells. Various sort modes are available with the Sony FACS machine to allow calibrating of the sort to achieve different balances of purity vs yield of the sorted samples (Figure 3.3.2). The single cell modes resulted in off-target positions, while the single-cell 3-drop modes resulted in on-target positions within the wells, and was the recommended sort mode for the 130mm nozzle chip.

Figure 3.3.2.1. Sony MA900 sorting modes. (308)



To check and adjust the trajectory of the side streams targeting alignment, target calibration was required prior to each sort. 30-50 drops of sheath fluid, followed by an aliquot of the sample (double-emulsion droplets) were targeted at the centre of the four corners of a covered 96 well plate. If the trajectory were off-centre of the lid of the target well, the nozzle was adjusted to re-centre the targeting system, before the sort proceeded, to minimise off-target sorting into a 96 well plate. The results of successful target calibration of the droplets in the centre of the target well lid are shown in Figure 3.3.2.2.

Figure 3.3.2.2. Droplet calibration using single cell 3-drop mode

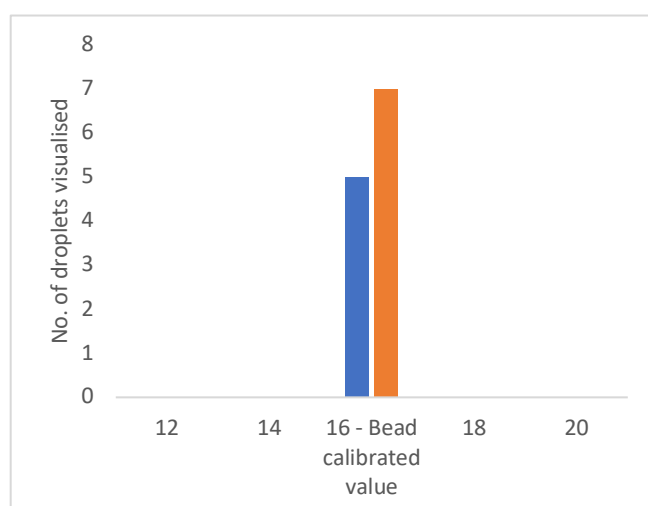


Sort delay calibration

Brower et al. (303) reported that accurate sorting of WOW droplets was dependent on optimal calibration of the droplet sort delay - the time between the droplet passing through the laser excitation within the analyser and when a charge is applied to the droplet after it leaves the nozzle for sorting. For the Sony SH800, bead-calibrated sorting was optimal for post-sort recovery of all droplet populations, unlike the BD Aria II where droplet delay had to be calibrated at different values from the Accudrop values. I aimed to determine if optimal delay times for the Sony MA900 platform differed from the bead-calibrated values.

50-droplets were sorted per well containing 100ml 0.1% SDS solution, two replicates for each sort delay time (delay time values 12, 14, 16, 18, 20 droplet cycles). The median bead-calibrated sort delay value was 16. Post sort the number of droplets visualised under microscopy was counted and displayed in Figure 3.3.2.3. The optimal sort delay time was at the bead-calibrated value. The low recovery of droplets visualised was possibly due to droplet lysis due to the transfer time from the sorter room to the microscopy room.

Figure 3.3.2.3. Droplet count post sort by delay times (bars represent duplicates)



3.3.3 Confirmation of HIV DNA retrieval after sorting

Extracted DNA from ACH2 samples at 10,000 copies/mL concentration were double encapsulated and sorted using the γ primer and probe set, previously selected. FITC-positive droplets (gate Q2, figure 3.3.3.1) were sorted to wells containing 1, 10, 50 droplets. 500 FITC-positive wells were also sorted into 100ml of 0.1% SDS for visualisation. Droplet lysis was achieved using the 'dry plate' technique described by Brower et al. (303): droplets are sorted onto a dry plate and left to dry for 30-60s, allowing changing interfacial tensions to break droplets releasing the genomic material within. Post-sort, droplets within the 500-droplet well were visualised for confirmation of FITC enrichment (Figure 12).

Figure 3.3.3.1: Flow plot of droplets containing genomic material from ACH2 HIV cell line standard, gated by FITC fluorescence

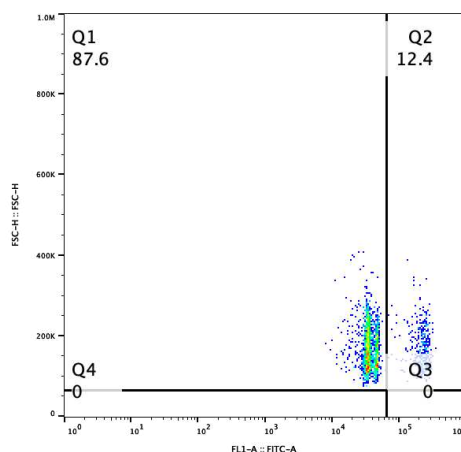
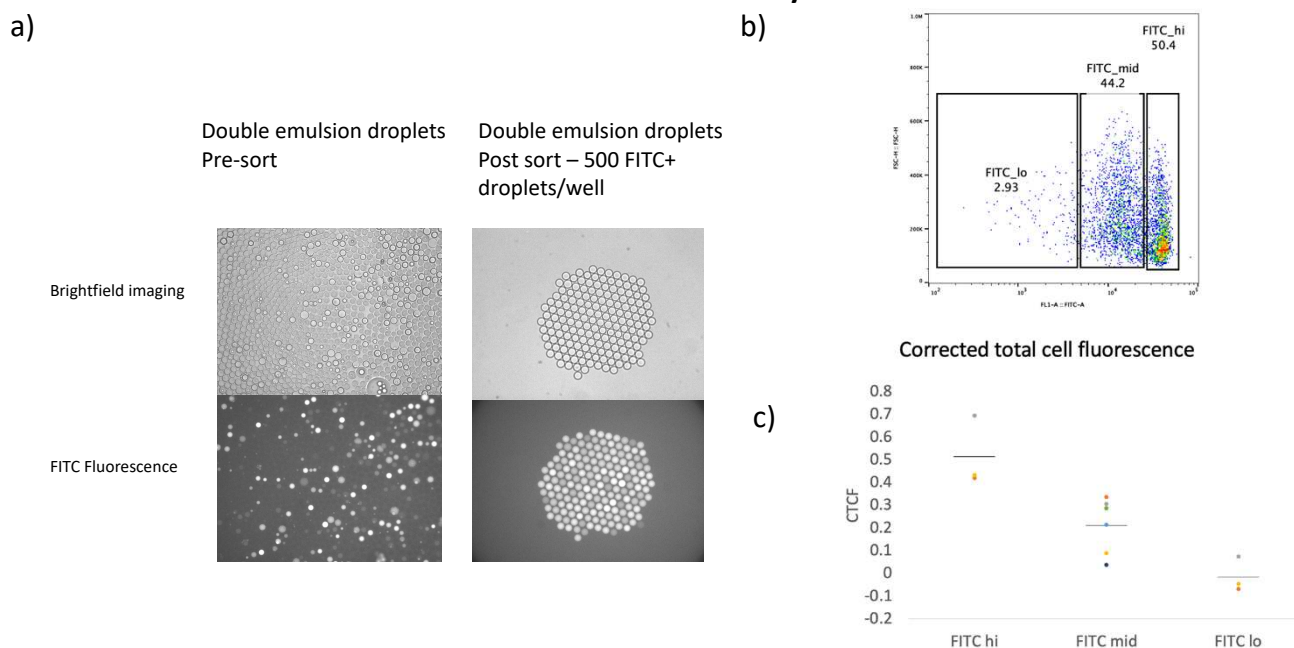
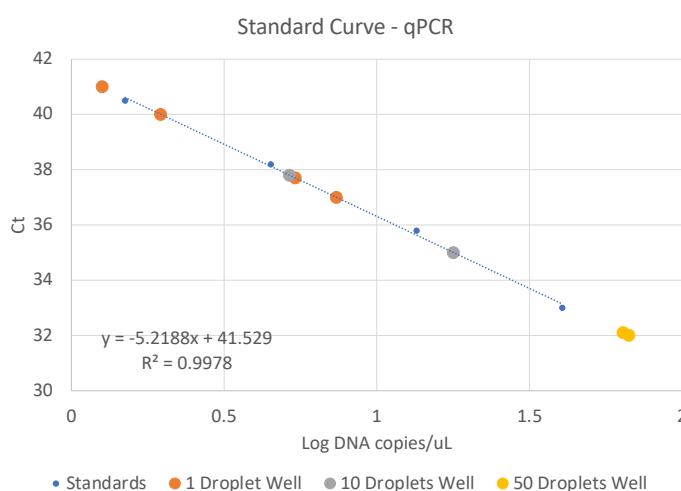


Figure 3.3.3.2 a) Microscopy brightfield and FITC fluorescence imaging of droplets pre- and post- sort. b) Droplets flow plot sorted by high, medium and low FITC fluorescence, c) corresponding corrected total cell fluorescence by well.



DNA from wells containing 1, 10 and 50 droplets were eluted in 10ml of water and added to a reaction mix containing primers and probes targeting the *env* region of HIV (81), and qPCR was performed with standards of known HIV concentrations to confirm the retrieval of HIV DNA post sorting (Figure 3.3.3.3.)

Figure 3.3.3.3. Standard curve comparison of qPCR results of wells containing 1 vs 10 vs 50 droplet wells using HEX-labelled probes for *env* regions



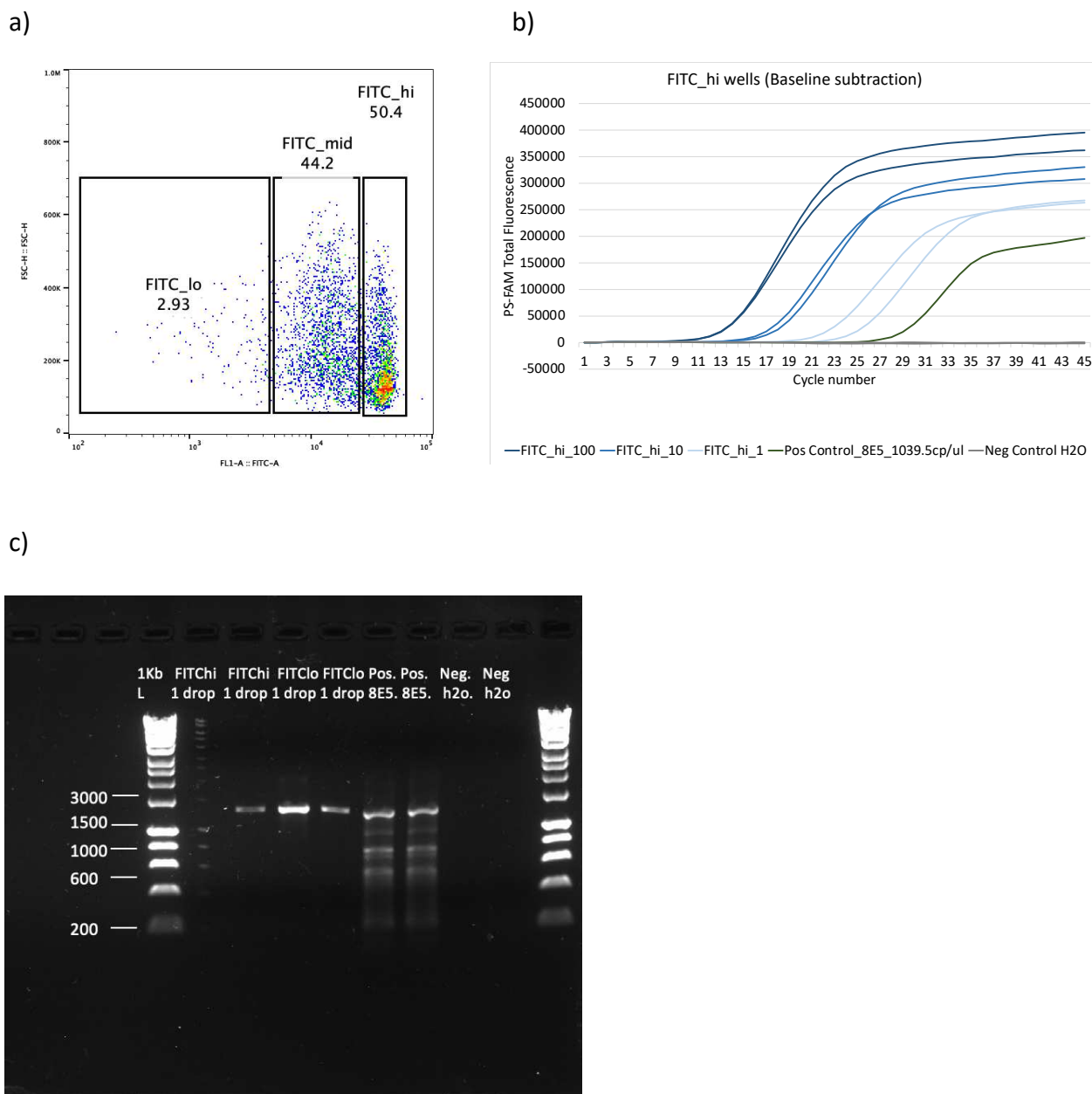
Ct = PCR cycle threshold when a target becomes detectable. (the higher the value, the lower the concentration of target).

Amplification of larger HIV fragments post-sort

The previous experiment was repeated, and droplets were sorted into single droplet wells from both FITC-negative (gate Q1) and FITC-positive (gate Q2) populations. DNA from dry plates was eluted in 10ml of water and added to reaction mixes for two nested reactions, one specific for a 1300bp *gag* region, and the other to a 3000bp *env* region. Reactions were unsuccessful with no visible bands on gel electrophoresis (Data not shown). Repeat experiment with droplets sorted into a well of 100 droplets was also negative for visible bands on nested PCR reactions (Data not shown). Two possible reasons were 1) the presence of PCR inhibitors in the droplet encapsulation or sorting reagents; or 2) the concentration of DNA available was too low for longer-distance amplification using nested reactions.

The latter was tested using a sample containing only amplified *gag* PCR products. The starting DNA concentration was 8.22ng/μl, which in a single 14pl droplet would contain approximately 31000 fragments. The droplets were into 100, 10 and single droplet per well and a confirmatory qPCR was ran using the same ψ primer and probes. As the qPCR may be amplifying existing ψ within the droplets, a long-distance PCR was carried out using the inner *gag* primers, which confirmed the retrieval of *gag* from single droplet wells (Figure 3.3.3.4 a-c). Of note, HIV *gag* fragments were also seen in the FITC-lo wells. When pooled DNA from post-sorted droplets containing *gag* or *env* products were sequenced, the run failed with mostly small fragments ~100bp rather than full-length *gag* or *env* products seen.

Figure 3.3.3.4 a) Flow plot of droplets containing *gag* fragments; b) qPCR post sort of 100, 10 and 1 droplet wells containing *gag* fragments; c) Gel image following PCR of inner *gag* fragments from the wells containing single droplets.



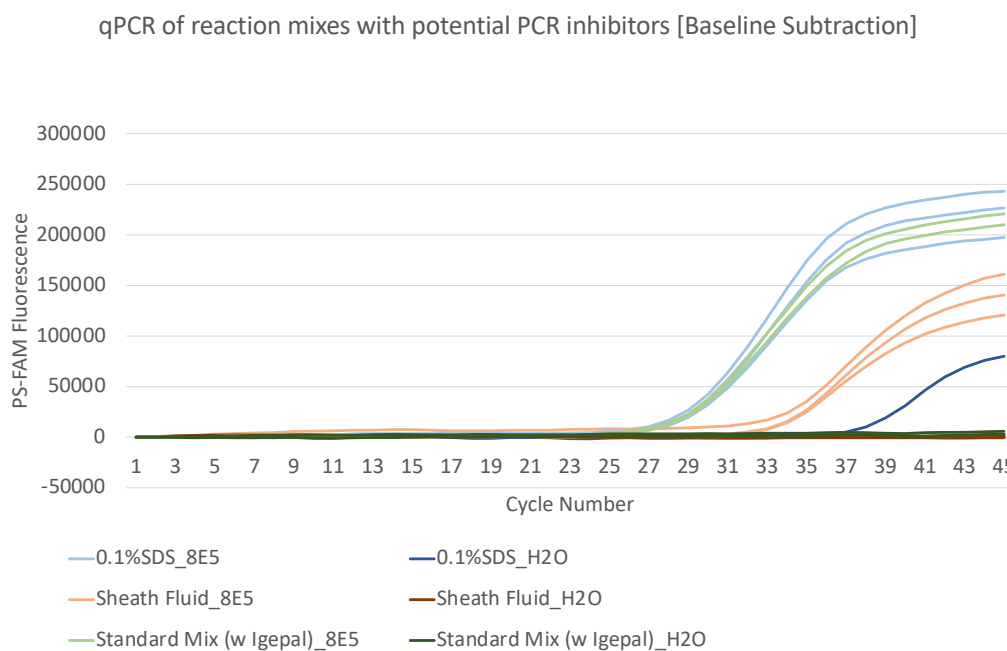
3.3.4 Investigating sources of inhibition of polymerase chain reaction

Potential PCR inhibitors were tested in bulk qPCR reactions using γ primers/FAM-labelled probe, on both 8E5 positive controls (1093.5copies/ml) and water for negative controls. qPCR reaction mixes were prepared

including 1X sheath fluid (Final concentration 0.304X), 0.1% SDS (Final concentration 0.0304%), and Igepal (Final concentration 0.5%).

The qPCR results (Figure 3.3.4) suggest sheath fluid is associated with inhibition of qPCR reactions, while SDS and Igepal surfactant are not. Sheath fluid contains HEPES buffer, sodium chloride, and phenoxyethanol, of which the phenoxyethanol is the most likely to contribute to inhibition of PCR due to the presence of both phenol and ethanol groups in the molecular structure (309). However, this inhibition has been shown to be overcome with higher concentrations of starting DNA material within the droplets, allowing downstream amplification.

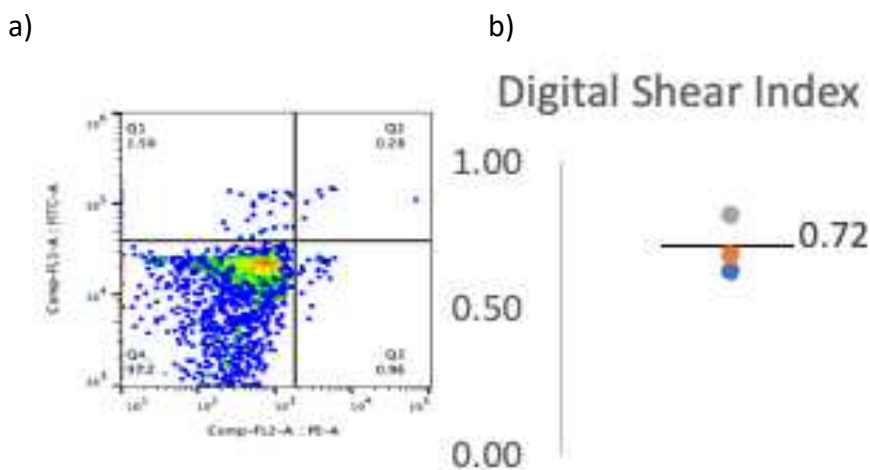
Figure 3.3.4: qPCR reaction mixes with potential PCR inhibitors



3.3.5 Quantification of DNA shearing through the microfluidics device

To investigate the hypothesis that failure to amplify sorted DNA fragments was due to excessive fragmentation of DNA during the encapsulation process, I measured the digital shear index (DSI) of the droplets after encapsulation. DNA from 8E5-positive controls were encapsulated with 2 fluorescent probes to the human housekeeping gene *RPP30* 7kb apart (comparable to the distance between the PS and *env* IPDA targets), and the ratio of the double positive droplets to sum of single and double positive droplets were quantified after sorting. The higher the DSI value, the greater the degree of shearing of DNA, with a DSI value of 1.00 meaning 100% of DNA in a sample were sheared. The mean DSI value was 0.72 (range 0.53 – 0.86) (Figure 3.3.5), suggesting a high degree of DNA fragmentation contributing to loss of signal for DNA amplification. The shearing occurs after the first encapsulation step prior to the PCR reaction (mean DSI 0.73), and the use of a high molecular weight DNA extraction protocol did not improve the degree of shearing (DSI 0.76 vs 0.73).

Figure 3.3.5. Digital shear index of sorted double emulsion droplets containing RPP30 probes



a) Flow plot measuring DSI, each axis represents a probes targeting end regions on *RPP30*, a 11kb housekeeping gene. Droplets containing only a single probe (Q1 or Q3) represent fragmented DNA, and double positive droplets (Q2) represent intact *RPP30* DNA fragments. B) Triplicate results of DSI experiments demonstrating excessive DNA fragmentation following DNA encapsulation, with a mean DSI value of 0.72.

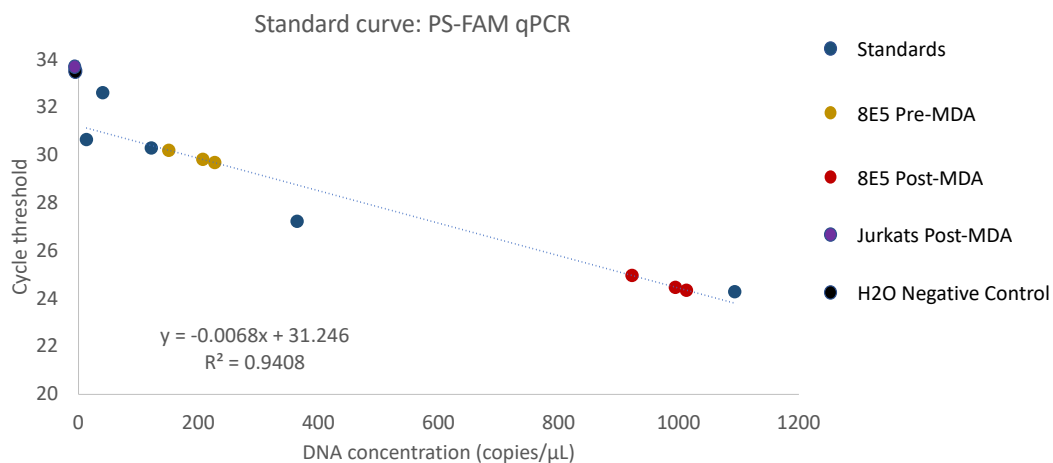
3.3.6 Multidisplacement amplification as a strategy for enrichment of HIV DNA

One proposed solution to address the low concentrations of HIV DNA following sorting was non-specific multidisplacement amplification (MDA) of sorted genetic material using the Phi29 DNA polymerase. Phi29 DNA polymerase allows amplification of single proviral genome and the flanking integration sites and host genomic DNA through a non-biased amplification step with random hexamers at isothermal temperatures (30°C). The resulting branching DNA polymerisation generates 1000 – 10000 identical copies of the starting genetic material at high fidelity (95), suitable for downstream screening for intact provirus, integration site analyses to name a few applications. Einkauf et al. used the MDA approach in a matched integration site and proviral sequencing (MIP-Seq) assay (95), to interrogate the integration sites of intact proviruses compared to defective proviruses in individuals on ART.

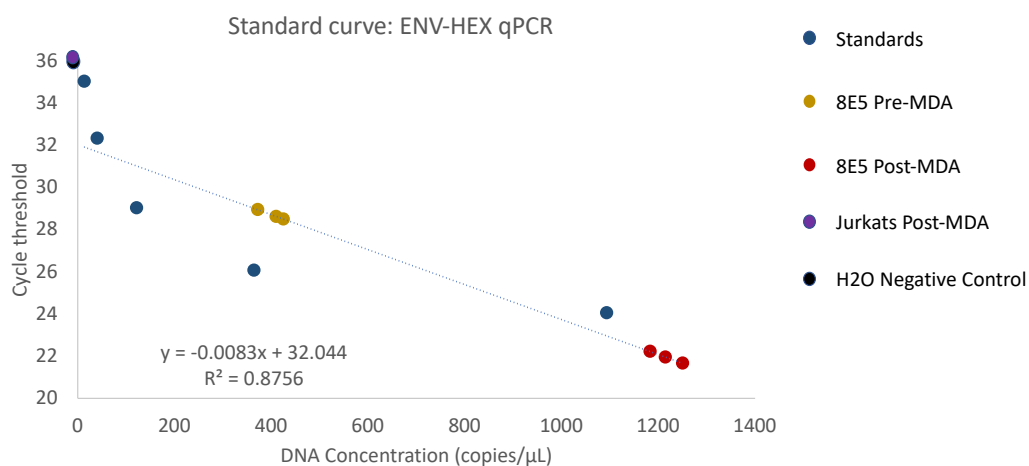
To prove the concept, an experiment was conducted in bulk using three samples: DNA extracted from 8E5 containing HIV DNA, DNA extracted from HIV-negative Jurkat cell lines, and a negative H₂O control. These samples underwent multidisplacement amplification, and the resultant pre-MDA and post-MDA product underwent confirmatory qPCR with both probes, demonstrating increased DNA concentrations following amplification for both Env and ψ targets (Figure 3.3.6.1).

Figure 3.3.6.1 a) qPCR standard curves pre- and post-MDA using a) PS-FAM and b) *env*-HEX probes

a)



b)



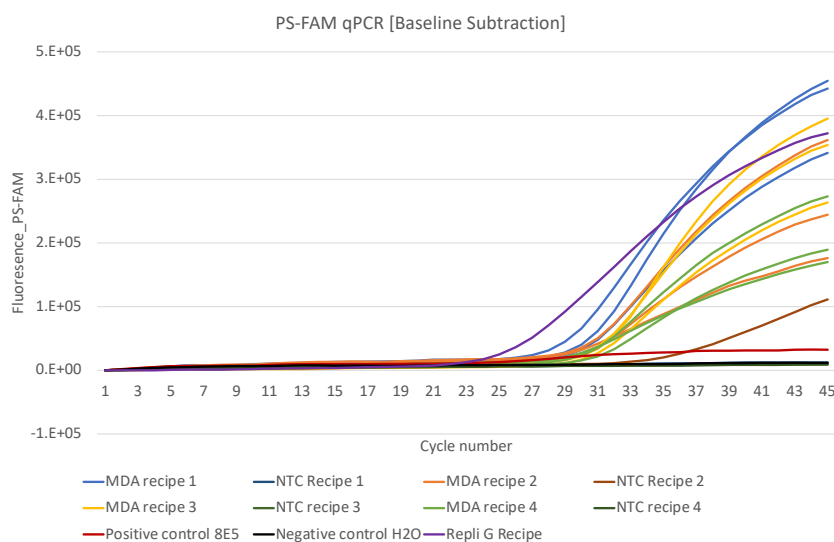
Cycle threshold = PCR cycle which the target becomes detectable (the higher the value, the lower the concentration of target).

Optimising MDA reaction mix in bulk

The MDA reaction is non-specific due to the use of random hexamers, and when used to amplify DNA containing both HIV and host genomic DNA, the relatively minute HIV-proviral sequences are lost in the vast non-HIV genomic material present (HIV vs human genome size: 1:640 000). Previous protocols such as MIP-Seq which uses MDA for non-specific amplification, still require subsequent HIV-specific primers for a nested near-full length amplification to identify HIV sequences for downstream analysis (95). Another issue is template-independent amplification, where amplification of nucleic acids occurs in the absence of template DNA, particularly in ultralow concentrations, due to dimerization and self-priming of random hexamers at high concentrations (310).

To address both issues, we looked at designing custom primers for use with Phi-29 DNA polymerase, to allow us to spike HIV-specific primers into the reaction mix to increase specificity of the multidisplacement amplification towards HIV. Primers were modified at the 3' end to include 2 phosphorothioate (PTO) bonds to be resistant to the 3' -5' exonuclease activity of Phi29 DNA polymerase. Primers were designed in collaboration with Panagiota Zacharopoulou and Daniel Coneyworth, Frater Lab. Primers were either shortened PTO modified versions of existing primers, or sequences identified through using SWGA (311), a primer design toolkit, as well as a manual search along the HXB2 reference sequence for specificity along the entire length of the HIV sequence with minimal overlap with non-HIV human genome sequences. Primers were kept at 11-12 nucleotides length to maintain their melting temperatures under 40°C, the optimal temperature reported for Phi29 DNA Polymerase activity(312). Primers were added in sequence (recipe 1: 2 primers, recipe 2: 4 primers, recipe 3: 6 primers, and recipe 4: 8 primers) to the MDA reaction mix, comprising of 2.5mM of each Primers, 5mM dNTPs, 2.5mg/mL recombinant albumin, 0.6U/ml Phi29 DNA polymerase, and 1X reaction buffer. The amplified MDA products were analysed with qPCR using FAM-labelled y probes, and results were comparable to results from Repli G reactions (Figure 3.3.6.2).

Figure 3.3.6.2. qPCR results following MDA reactions with various custom primer recipes



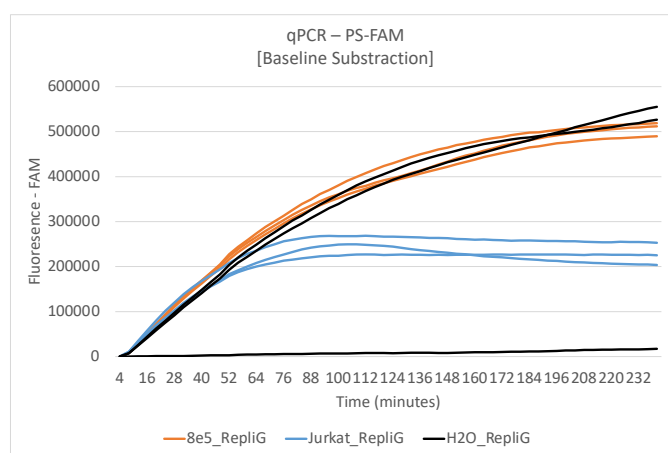
MDA = Multidisplacement Amplification; NTC = Negative Control

3.3.7 Template independent amplification remains a challenge for multidisplacement amplification reactions

Reductions in MDA reaction volumes to nanoliter droplet reactors have been reported to reduce contaminant DNA templates, amplification bias, and non-specific amplification (313). For this workflow, the use of MDA reactions in droplets provides other advantages, including simultaneous release of fluorescent probes under isothermal conditions, forgoing the need for an intermediate thermocycling step, and amplified fragments would provide greater amounts of template DNA within each single droplet well, overcoming the earlier challenges of the inability to retrieve HIV DNA due to the ultralow DNA concentrations.

I performed the MDA in a bulk reaction using an PTO-modified γ primers (500nM) and FAM-labelled probes (200nM), and the Repli G MDA reaction mix, on the following samples: 8E5 standard control, DNA from HIV-negative jurkat cell-lines, and H₂O water control at 30°C for 4 hours. Figure 3.3.7 shows Phi-29 DNA polymerase is capable of releasing fluorophores discriminating DNA samples containing HIV vs non-HIV template DNA. However, in the absence of template DNA (H₂O negative control), non-specific binding between the hexamers and probes results in fluorescence suggesting template independent amplification.

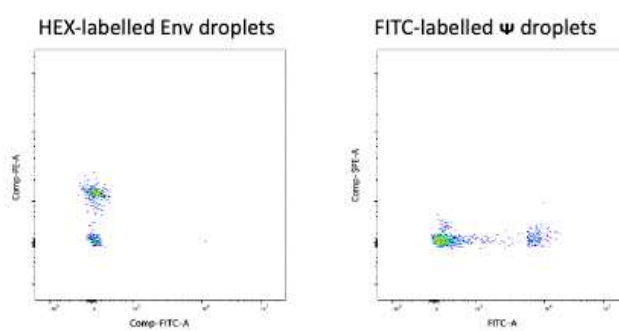
Figure 3.3.7. qPCR plots of bulk MDA reactions with FAM-labelled γ probes



3.3.8 Modification of assay to measure intact proviral DNA using Env and ψ probes

I modified the assay to include two probes to ψ and *env* used in the IPDA assay(81). The aim was to quantify the amount of double-positive droplets containing intact proviral DNA, even if we were unable to amplify or sequence DNA from sorted droplets downstream. Using droplets containing either HEX-labelled *env* and FITC-labelled ψ probes and a positive control (8E5 HIV cell line DNA), I was able to identify distinct fluorescent positive double emulsion droplets on FACS (Figure 3.3.8.1).

Figure 3.3.8.1 FACS plot of single-probe droplets containing positive controls

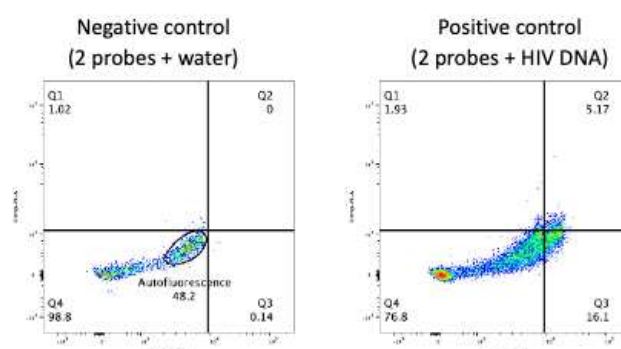


However, when positive droplets with both probes and HIV were ran, there was not a clearly defined double-positive population, as there appeared to be a population of autofluorescent droplets even when no HIV DNA was present (Figure 3.3.8.2). Neither switching the probe to Cy5, or varying the concentration of the probes were able to improve the performance of the assay (Data not shown).

Figure 3.3.8.2 FACS plot of droplets containing two probes with or without HIV DNA

3.4 Discussion

I have successfully shown encapsulated DNA may be enriched for, based on the probes using standard FACS



that droplets- successfully sorted and presence of fluorescence equipment. However,

there remains significant barriers to successful amplification and recovery of DNA targets due to 1) DNA fragmentation during the droplet encapsulation protocol, 2) presence of PCR inhibition including the cell

sorter sheath fluid which may be overcome by the presence of high concentration of DNA for downstream amplification following sorting. Non-specific MDA reactions may provide a solution to amplify the sorted DNA material, however, template independent amplification and the preceding DNA fragmentation during encapsulation remains barriers to successfully achieving full-length amplification of the starting genomic material.

Of these reasons, the excessive degree of DNA fragmentation is the greater challenge to a successful assay. Bruner et al. reported a mean DSI of 0.32 ± 0.07 within the IPDA, and in my findings the degree of DNA shearing was more than double this value. The use of a device with shorter and wider microchannels, to reduce the shear forces was designed and tested by Lucia Parolini and Daniel Coneyworth, Frater lab, however, there was no reduction in the degree of DSI compared to the original device. DNA fragmentation is likely to occur during the bubble formation due to hydrodynamic shearing (314), a fundamental step in this workflow.

Due to the time limits of my project and urgent need to measure the reservoir sizes of the tissue biopsies sampled, I was not able to pursue further solutions to overcome the challenges from the high degree of DNA fragmentation and negligible yields of the sorted samples. However, possible solutions include the encapsulation of single cell genomic material in a hydrogel that captures and protects the high-molecular mass DNA and polyadenylated RNA for droplet sorting, as described in the recently published FIND-Seq method (108). This assay allows for more in-depth whole transcriptomic analysis in addition to reservoir quantification.

MDA technology is a promising strategy to allow unbiased amplification of all genomic material after sorting, and has now been incorporated in a number of reservoir assays (95,98,101). Although my assay was unsuccessful, Sun et al. described the SIP-Seq method (98) where individual provirus encapsulation into droplets with MDA reagents, allow direct amplification of proviral sequences including integration sites. The

use of droplets decreases reagent use by 100-fold and decreases doublets. However, this technique is technically complex and involves specialised microfluidic merger devices and dielectrophoretic sorting equipment. Analyses of clonal sequences may also be limited due to coverage variation from MDA bias and read cross-contamination. Non-specific amplification, or template independent amplification remains an issue, likely resulting from primer dimer formation, and the products of MDA reactions may result in chimeric DNA rearrangements (315).

I was unable to optimise a modified IPDA by using two probes to ψ and *env*. This may have been due to the autofluorescent properties of the double-emulsion droplets containing the two probes, or compensation errors when analysing droplets containing both probes. However, even if successful, the assay remains more time consuming and provides few advantages over the existing commercially available IPDA.

Following an interim analysis of the assay progress, and the stop-go criteria of this project (Table 3.4), a decision was made to seek an alternative validated, high-throughput assay for reservoir quantification in gut tissue. The 5-region multiplex Rainbow digital PCR assay (86), available at the HIV Cure Research Centre, University of Ghent, provided a more sensitive and specific assay for the quantification of both total and intact reservoir sizes and had been validated in gut tissue. Thus, I undertook the tissue reservoir measurements in collaboration with Dr Marion Pardons and Professor Linos Vanderkerckhove, HIV Cure Research Centre, University of Ghent.

Table 3.4. Summary of assay development progress

Challenges	Progress	Status
1. Identification of probe with adequate brightness and specificity for identification of droplets with HIV DNA	Able to distinguish distinct fluorescent and non-fluorescent droplets seen under microscopy and on flow analyses	Successful
2. Optimising droplets stability for FACS sorting	Visualised stable double emulsion droplets post encapsulation with clear positive/negative fluorescence differentiation on flow analyses	Successful
3. Optimising sorter settings for accurate sorting into 96 well plate	Demonstrates droplets in centre of wells using sorter plate calibration Able to visualise fluorescent-only droplets post sorting.	Successful
4. Confirmation of HIV DNA retrieval from droplets 5. PCR inhibition from reagents or sheath fluid	Partially successful nested <i>gag</i> and <i>env</i> fragments of HIV DNA from single sorted droplets containing 8e5 cell line DNA, or successful nested <i>gag</i> amplification of sample containing <i>gag</i> fragments only	Partially successful
6: Amplifying small HIV proviral fragments amongst vast amount of non-HIV genomic material, whilst preserving integration sites	Demonstration of successful amplification of HIV proviral sequence in bulk, but not in single droplets	Partially successful
7. Minimising template independent amplification in MDA 8. Ensuring single genome present per well without contamination 9. Sequencing low concentrations of HIV DNA post sort	Unable to retrieve HIV DNA sequences from sorted DNA	Unsuccessful

Chapter 4

Safety and time to viral resuppression outcomes for bNAbs and ATI studies

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4.1 Introduction

One of the key concerns for participants and researchers around cure-related studies is the safety of undertaking ATIs. Concerns primarily include the risk of onward HIV transmission, impact on health and immune status, and need for partner protection (316–318). In a survey of people diagnosed during PHI in the UK, which I had previously published (319), survey participants considering ATI studies were most concerned about onward HIV transmission, however they were accepting of higher viral load thresholds to restart as long as frequent clinical monitoring was undertaken. Studies involving shorter, closely monitored ATI studies and cure-related interventions, including bNAbs, have been shown to be safe (222). There are now recommendations available for conducting studies involving ATIs (219). A systematic review and meta-analysis reported that studies with more frequent viral load monitoring, higher baseline CD4 counts, and undetectable viral loads (VL) were reported to be safe with negligible risk of adverse events (0%, 95%CI 0-1%) or development of ART drug resistance (0%, 95%CI 0-1%) (221).

However, although safety data are routinely available during the period of ATI in clinical trials, there has been limited data or recommendations on viral resuppression rates following ART reinitiation after ATI. Studies have reported that the reservoir containing total and replication-competent HIV remains stable following short periods of ATI (223,224,320), but the small sample sizes and shorter duration of ATI may limit generalisability of their findings to larger studies with longer periods of treatment interruption. It also remains unclear what effect novel immunotherapeutics such as broadly neutralising antibodies (bNAbs) have on the HIV reservoir following ATI (224), although one study suggest bNAbs were associated with a modest reduction in the intact proviral reservoir (176). For participants living with HIV and their healthcare providers, the concerns of onward HIV transmission remain the greatest concern when considering participating in clinical trials involving ATIs (321), and data to demonstrate the safety and ability to achieve viral suppression after restarting ART in ATI studies is paramount to addressing these concerns.

As discussed in chapter 1, exogenous bNAbs have been shown to be safe and to date there have been no reported cases of severe drug-related adverse reactions associated with either the short acting or long-acting LS-variants of 3BNC117 and 10-1074 given intravenously (M Caskey, personal communication), however, there have only been small studies of the LS-variants of these bNAbs. There was one case of a severe infusion-related reaction related to the 2nd 3BNC117 infusion in the TITAN study, which resolved with intravenous fluid, antihistamine and corticosteroid therapy without any long-term sequelae (175). A systematic review of ATI studies which included three trials (n = 37 participants) involving bNAbs did not identify safety concerns during the ATI (221). Serious adverse events associated with bNAbs were only reported in one phase one study of subcutaneous 10E8VLS injections, where seven of eight participants experienced erythema and induration at the injection site (248) related to the mode of administration.

To further evaluate the safety of ATIs and cure-related interventions, I undertook and published a systematic review and meta-analysis (322) to determine the the impact of receiving any trial interventions, including bNAbs, on viral resuppression rates compared to undertaking ATI without interventions (ATI only). I also separately reported adverse events and time to viral resuppression outcomes from an extended ATI following LS-bNAbs administration from the placebo-controlled double-blinded randomised RIO study.

4.2 Methods

4.2.1 Systematic review search strategy

A structured systematic literature search of the Medline, Embase, and Web of Science databases was performed, an initial search on the 2nd March 2023, and an updated literature search on 22nd April 2024. Two reviewers (myself (MJL), and Mile Eason (ME), Imperial College London) read and ascertained the relevance of the studies independently. Discordant results were adjudicated by a third reviewer, Sarah Fidler (SF), Imperial College London. The full protocol was registered on Prospero registry (Registration Number

CRD42023403809) and follow the recommendations from the PRISMA statement. Full search terms and results from the final literature search are available in Appendix Tables 2 - 4.

Inclusion Criteria: Clinical studies with an ATI were included. An ATI was defined as a protocol determined temporary pause in ART with a viral rebound and regular viral load measurements. Only studies with viral suppression data after ART restart were included, defined as studies with plasma viral load (VL) measurements for participants until at least one VL <50 copies/mL. Frequency of viral load monitoring, originally defined as at least fortnightly in the original inclusion criteria on the Prospero registry, was dropped to increase the number of studies available and allow analysis of the association of frequency of viral load monitoring on the time to viral load suppression and all studies with at least one VL measurement post ART was included.

Exclusion Criteria: Any studies prior to 2015, pre-prints, abstracts, letters, reviews, commentary articles, opinion articles, animal studies and In vitro studies were excluded. This timeframe was selected to reflect modern ART regimens usage amongst study participants and minimise study heterogeneity. Studies with children and adolescent participants (<18 years old) were excluded, and stem cell transplant recipients were also excluded due to the unique characteristics of participants and distinct mechanism of action featuring potentially fatal toxicities, their inclusion potentially impacting on the generalizability of the study findings.

4.2.2 Outcomes definitions

Primary outcome

The time taken to viral suppression <50 copies/mL following ART restart after ATI, stratified by cohorts receiving intervention and undertaking ATI, or undertaking ATI only.

Secondary outcomes

Proportion of participants that experienced viral suppression <50 copies/mL by week 12 after ART restart. Other factors associated with time to viral suppression were analysed using both a univariable and multivariable a cox proportional hazards regression model. Covariables include plasma HIV viral load at ART restart, nadir CD4, CD4 at study enrolment, duration since HIV diagnosis, ART regimens, duration of ATI, and frequency of HIV VL monitoring after restarting ART.

4.2.3 Data extraction and collection

Extracted data included VL measurements during ATI and after ART restart, participant demographics, HIV-associated disease characteristics (nadir and baseline CD4 counts, duration since HIV diagnosis, virus clade, presence of ART resistance-associated mutations), ART regimens restarted, ART restart criteria, interventions received during study, and any adverse events including development of drug resistance during and after ATI.

ME extracted the data and MJL was responsible for verification of the extracted data. If data were not available from published manuscripts or associated supplementary material, these data were requested from the corresponding study author.

4.2.4 Assessment of study quality and bias

As viral resuppression rates are not included in the primary or secondary outcomes of these studies included, they are unlikely to drive publication bias based on whether participants achieve viral resuppression after restarting ART. Thus, a formal publication bias assessment was not performed beyond a funnel plot assessment.

A modified Newcastle Ottawa scale (323) and Cochrane Risk of Bias 2 (ROB2) tool (324) were used to assess quality and bias of non-randomised studies and randomised controlled trials (RCT) respectively. The Grading

of Recommendation, Assessment, Development and Evaluation (325) classification was used to evaluate overall quality of evidence. Two reviewers (ME & MJL) independently assessed bias within each study. Differences were adjudicated by a third reviewer (SF).

4.2.5 Adverse events definition

Adverse events (AEs) were defined as any untoward medical occurrence in a participant who was administered a medicinal product. Serious adverse events (SAEs) were defined as any events that meets any of the following criteria (246)

- Results in death
- Is life threatening
- requires hospitalisation or prolongation of existing inpatient's hospitalisation,
- results in persistent or significant disability or incapacity,
- is a congenital abnormality or birth defect.
- Important adverse events or reactions that do not meet the above criteria but may jeopardise a participant or require intervention to prevent one of the other outcomes above.

All AEs and SAEs were recorded throughout the study in an online trial data collection system (OpenClinica), and coded using the MedDRA hierarchy. The management of all AEs and SAEs were taken in accordance to Good Clinical Practice guidelines(326), including the rapid reporting of all SAEs within 24 hours to the study sponsor. The assignment of causality as well as decisions on management of AEs were undertaken by trial physicians at each study site.

4.2.6 Safety data and time to viral resuppression data retrieval from the RIO study

The double-blind, placebo-controlled randomised RIO study design and methods are described in full in chapter 2. The number of AEs and SAEs from each study arm, along with the time from restarting ART to viral resuppression <50copies/mL was obtained from OpenClinica and presented descriptively. Further statistical analyses for the time to viral resuppression was undertaken as per the meta analysis, as described below. Time to viral suppression was similarly defined as the time from restarting ART till the first reading <50 copies/mL. Sustained viral suppression was defined as viral load <50 copies/mL for two or more readings at least four weeks apart after achieving the first reading <50 copies/mL.

4.2.7 Statistical analysis

All analyses were performed using (R version 4.2.2 (2022-10-31))(327). A Kaplan-Meier curve was plotted stratified by IA or ATI-alone subgroups to display time-to-viral-suppression results of all study participants. A multivariable time-to-event analysis was undertaken using cox proportional-hazards model to examine the effects of different demographics, HIV-related and study-related characteristics on the hazard risk (HR) of achieving viral suppression over time. A forward stepwise model approach for variable selection was performed, variables were included in the multivariable model if there was a univariable association below the threshold of $p = 0.3$. The variables included in the univariate and the final multivariate models are presented in Table 4.

A random effects meta-analysis was performed using the R package 'meta' (327,328) Higgins I^2 test was used to calculate study heterogeneity. Participants from studies with multiple arms were pooled within cohorts undertaking IA or ATI-only protocols and treated as separate cohorts for the proportional meta-analysis. Stratified meta-analysis of proportion effect estimates (ES) compared participants in IA subgroup with participants in the ATI-alone subgroups with results presented in forest plots and pooled ES with 95% confidence intervals, using χ^2 -squared test to assess for subgroup differences.

A sensitivity analysis was performed to assess the weight of the effect of bNAbs alone compared to all interventions received. Participants who received any other interventional therapies apart from bNAbs were excluded, and the Cox proportional-hazards model was repeated.

4.3 Results

4.3.1 Studies included in the systematic review

1073 papers were screened under the search terms, the initial search resulted in 22 studies involving ATIs in their study design which met all inclusion and exclusion criteria (80,132,176,183–185,188,190,329–342), and the updated literature search revealed a further study (175), resulting in 23 studies in total. Of these studies, 13 studies published or communicated sufficient data for inclusion in this systematic review (Figure 4.3.1.). Authors from the remaining 10 studies were contacted but were unable to provide the necessary VL data following ART restart within the timeframe requested, and these studies were excluded from the meta-analysis. Characteristics and study design of the 23 studies are summarised in Table 4.3.1.

Figure 4.3.1. Flow chart of study selection process (322)

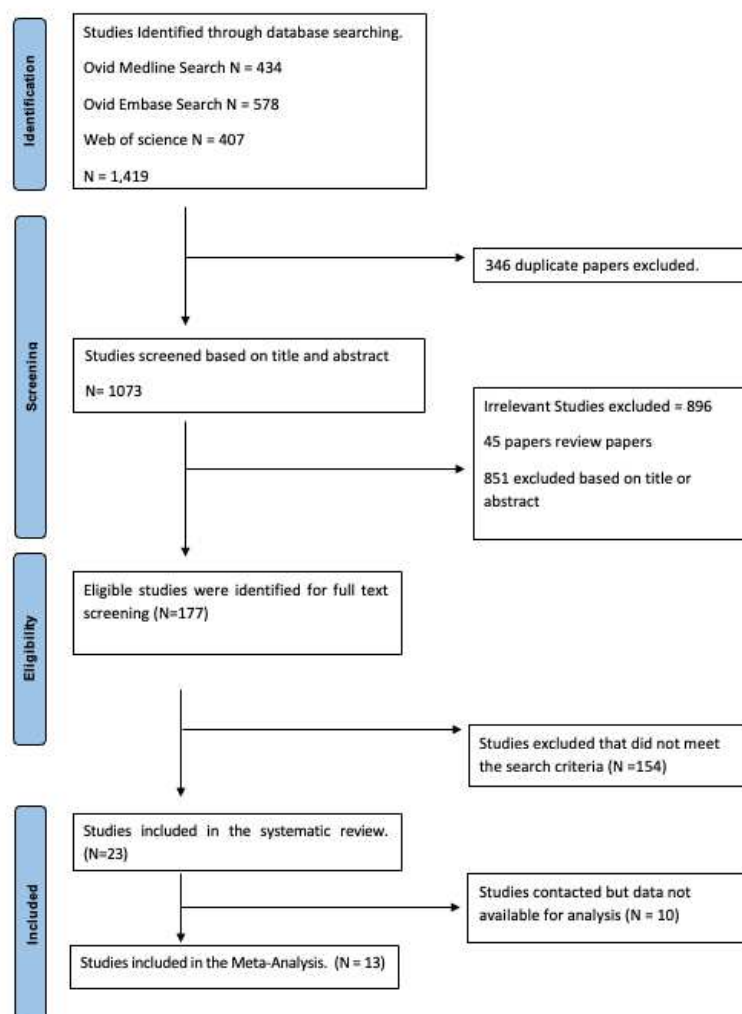


Table 4.3.1. Summary of ATI studies included in the systematic review. (322)

First Author	Publication Year and Journal	Number of Participants that underwent ATI	Intervention with ATI or ATI only	Details of Study	VL Criteria to restart ART	Monitoring Frequency of VL after ART restart	Gender	Median Age (Years)	Median ART duration prior to study entry (Years)	Median CD4+ count at baseline (Cells/ μ L)	Post ART restart Data Status
Castagna	J Antimicrob Chemother 2019	9	ATI Only	Observational ATI study	Two consecutive HIV VL >50 copies/mL (14 days apart).	Not stated in manuscript.	9 M	50.7	16.9	737	Available included in the meta-analysis
Mendoza	Nature 2018	15	Intervention with ATI	Open Label Single Arm Participants included in this study received three infusions of 30mg/kg 10-1074 + 3BNC117 antibodies. They then had an ATI.	Two consecutive measurements of HIV VL $\geq$ 200 copies/mL	Fortnightly until <50 copies/mL. Every 2 weeks thereafter.	1F 14 M	40	5	654	Available included in the meta-analysis
Scheid	Nature 2016	13	Intervention with ATI	Non-randomised dose finding study Arm 1: Two doses of 3BNC117 + ATI Arm 2: Four doses of 3BNC117 + ATI	Two consecutive measurements of HIV VL $\geq$ 200 copies/mL (weekly measurements)	Weekly	1 F 12 M	31	6	720	Available included in the meta-analysis
Gaebler	Nature 2022	26	Intervention with ATI	Open Labelled RCT. Arm 1: Received bNAbs 3BNC117 and 10-1074 during ATI. Arm 2: Took ART whilst receiving 3BNC117 and 10-1074 and then underwent ATI.	Weeks 0-26: Two consecutive measurements of HIV VL $\geq$ 200 copies/mL (weekly measurements) Week 26-38: Four weekly HIV VL >1000 Copies/mL;	Monthly	3 F 23 M	49.5	10	675	Available included in meta-analysis

					Week 38-48: Two consecutive pVL >1000 measured every other week						
Gruell	Lancet Microbe 2022	20	Intervention with ATI	Group 1 underwent two treatment cycles of antibody each consisting of 3BNC117 infusions (30mg/kg) + three romidepsin infusions (5mg/m2). Group 2 Two treatment cycles each consisting of three romidepsin infusions (5mg/m2).	Two consecutive measurements of HIV VL ≥ 200 copies/mL	Fortnightly until <20 copies/mL	3 F 17 M	Arm 1: 40 Arm 2: 51	Arm 1: 5 years Arm 2: 10 years	Arm 1: 716 Arm 2: 600	Available included in the meta-analysis
Cohen	J Exp Med 2018	15	Intervention with ATI	Open Label. Received 3BNC117 infusions and then had an ATI.	Two consecutive measurements of HIV VL ≥ 200 copies/mL	Weekly	1F 14M	43	11	688	Available included in the meta-analysis
Gunst	Nat Med 2022	20	Intervention with ATI	Open labelled RCT. Arm 1: ATI Only Arm 2: ART+3BNC117 then ATI Arm 3: ART + romidepsin then ATI Arm 4: ART + 3BNC117 + romidepsin then ATI	Two consecutive measurements of HIV VL ≥ 5000 copies/mL (weekly measurements)	Every fourth week until VL were undetectable on two consecutive measurements.	2 F 18 M	37	0	529	Available included in the meta-analysis
Tebas	J Clin Invest 2021	14	Intervention with ATI	Open labelled study. Arm 1: Gene modified CCR5 CD4+ T-Cells. Arm 2: gene modified CCR5 CD4+ T-cells + CTX and then ATI.	Consecutive measurements of HIV VL ≥ 100 000 copies/mL over three weeks	Monthly	1 F 13 M	44	N/A	693	Available included in the meta-analysis

Kroon	J Virus Erad 2020	15	Intervention with ATI	Open labelled RCT. Arm 1: Vorinostat/ Hydroxychloroquine / Maraviroc + ART followed by ATI Arm 2: ART followed by ATI only.	Two consecutive measurements of HIV VL ≥ 200 copies/mL	Fortnightly until 34 Weeks after	2 F 13 M	Group 1: 28 Group 2: 26	3.4	Group 1: 634 Group 2: 1079	Available included in the meta-analysis
De Scheerder	Cell Host Microbe 2022	11	ATI only	Observational ATI study	Two consecutive measurements of HIV VL ≥ 200 copies/mL (3 days apart) or a single HIV VL ≥ 1000 copies/mL	Post ART restart at 4 weeks and at 12 weeks. If detectable at 4 weeks, VL repeated monthly until undetectable.	1 F 10 M	40	3.5	688	Available included in the meta-analysis
Pannus	J Int AIDS Soc 2020	16	ATI Only	Observational ATI study	Two consecutive measurements of HIV VL ≥ 1000 copies/mL (3 days apart) or a single HIV VL ≥ 10000 copies/mL	Post ART restart at 4 weeks and at 12 weeks.	15M 1 F	N/A	4	N/A	Available included in the meta-analysis
Bailón	Nat Med 2022	45	Intervention with ATI	Double blinded RCT. Arm 1: Received a combination of DNA.HTI, MVA.HTI and ChAdOx1.HTI I vaccines and then an ATI. Arm 2 received ATI + placebo.	Eight consecutive measurements of HIV VL ≥ 10000 copies/mL or a single HIV VL ≥ 100000 copies/mL	Post ART restart HIV VL was monitored at weeks 4 and 12.	44 M 1F	36	N/A	745	Available included in the meta-analysis
Gunst	Nat Med 2023	46	Intervention with ATI	Double blinded RCT.	Sustained HIV VL ≥ 1000 copies/mL for ≥ 4 weeks or	Monthly	39 M 7 F	Age by arm: P/P 54	Time on ART by arm (years):	CD4 count by arm	Available for 33 of 46 participants,

				Arm 1: Placebo/placebo (P/P) Arm 2: lefitolimiod/placebo (L/P) Arm 3: placebo/bNAb (P/B) Arm 4: lefitolimod/bNAb (L/B)	confirmed VL $\geq 100\,000$ copies/mL			L/P 44 P/B 51 L/B 48	P/P 8 L/P 9 P/B 8 L/B 8	P/P 743 L/P 694 P/B 1027 L/B 832	included in the meta-analysis
Leal	Front Immunol 2021	29	Intervention with ATI	RCT. Testing Intranodal vaccine. Arm 1: DCV3 Arm 2: DCV3 with PEG-INF Arm 3: placebo Arm 4: placebo + PEG-INF	A single HIV VL $\geq 100\,000$ copies/mL	Every four weeks until 12 weeks after ART restart.	29 M	46	N/A	752	Not available. Not included in meta-analysis.
Bar	NEJM 2016	24	Intervention with ATI	Combination of two Open label studies (ACTG 5340 + NIH trial 15-I-0140) of VCR01.	A sustained (≥ 2 weeks) HIV VL >1000 copies/mL; HIV VL ≥ 200 copies/mL followed by a confirmation level of 1000 copies/mL or three consecutive measurements of >200 copies/mL	Monthly	22 M 2 F	A530 cohort 38 NIH trial 51	A530 Cohort 4.7 NIH Trial 10.0	A530 Cohort 896 NIH trial 724	Not available. Not included in meta-analysis
Sneller	J Infect Dis 2020	22	ATI only	Observational ATI study	HIV VL >1000 copies/mL for ≥ 4 weeks	HIV VL measured weekly for the first 4 weeks then monthly thereafter for	20 M 2 F	51	7.7	767	Not Available. Not included in the meta-analysis

						up to 52 weeks					
Crowell	Lancet HIV 2019	18	Intervention with ATI	Double Blinded RCT. Participants either received VRC01 or a placebo and ATI.	Confirmed HIV VL >1,000 copies/mL	Not stated in manuscript.	18 M	29	3	717	Not available. Not included in the meta-analysis.
Colby	Nat Med 2020	26	Intervention with ATI	RCT. Receive vaccine or placebo.	Two consecutive measurements of HIV VL ≥ 200 copies/mL (weekly measurements)	Not stated in manuscript.	26 M	25	1.9	625	Not available. Not included in the meta-analysis.
SenGupta	Science Translat Med 2021	25	Intervention with ATI	RCT. Participants received either vestolimod or placebo and then had ATI.	Not stated in manuscript.	Not stated in manuscript.	21 M 4 F	52 Vestilamod 41 Placebo	2.7 Vestilamod 3.2 Placebo	752 Vestilamod 952 Placebo	Not available. Not included in the meta-analysis.
Liu	J Clin Invest 2021	6	Intervention with ATI	Open labelled CAR T cells study.	Two consecutive measurements of HIV VL ≥ 200 copies/mL	Every 3 Weeks.	6 M	30	3.9	530	Not available. Not included in the meta-analysis .
Rutsaert	J Virus Erad 2019	30	Intervention with ATI	RCT. Arm 1: 50mg ABX464 and ATI Arm 2: 150mg ABX464 and ATI. Arm Three Placebo and ATI	Not Available in the Manuscript	Every 14 days until viral suppression	29 M 1 F	Arm 1 45.2 Arm 2 38.4 Arm 3 48.3	N/A	Arm 1: 1004 Arm 2: 926 Arm 3: 698	Not available. Not included in the Meta-analysis.
Vibholm	AIDS 2019	9	Intervention with ATI	Arm 1: MGN1703 with ART and then ATI Arm 2: ART with MGN1703 and ATI with MGN1703	Two consecutive measurements of HIV VL ≥ 5000 copies/mL	Not stated in manuscript. Only states they were followed until	11M 1 F	51.5	6.3	635	Not available. Not included in meta-analysis.

						suppression .					
Colby	Nat Med 2018	8	ATI only	Observational ATI study	Confirmed HIV VL >1,000 copies/mL, HIV VL rise of ≥0.5 log ₁₀ copies/m L per day provided that the last VL was >1,000 copies/mL, a single HIV VL >10,000 copies/mL	Not stated in the manuscript.	7M 1F	29	2.8	577	Not available. Not included in the meta- analysis.

4.3.2 Characteristics of the studies included in the meta-analysis

Three observational studies and ten interventional studies, of which six were randomised controlled trials and four were non-randomised trials, were included in the meta-analyses. The interventions studied include bNAb in 7 studies (80,132,175,176,183–185), and individual studies involving HIV vaccines (340), latency reversal agents only (331), and CCR5 gene edited T-cell infusions (330). Three studies were observational studies of participants undertaking ATIs only without any intervention (329,332,333).

Exact criteria to restart ART differed between studies however it was determined by plasma HIV VL in all studies (Table 4.3.1), with additional criteria based on CD4 thresholds, safety concerns or participant/investigator preferences. Notably two studies had a much higher VL threshold (>100,000 copies/mL over one and three weeks respectively) for ART restart than the other included studies (175,330). After resumption of ART six studies monitored the VL either weekly or fortnightly, two studies monitored participants VL monthly, and two measured VL at weeks 4 and 12 post ART restart. Two studies included did not state in their manuscripts how often VL would be monitored after ART was resumed (80,329).

4.3.3 Study participant demographics

A total of 213 participants underwent ATI from the 13 included studies in this meta-analysis. The median age at enrolment was 41 years (range 19 - 68) and of those studies with gender available, 182 (91.5%) were male, 17 (8.5%) female, and 1 (0.5%) transgender female. Median time from HIV diagnosis to enrolment was 5.5 years (range 0 – 37). Median CD4 count at study enrolment was 706 cells/ μ l (range 320 - 2156). Characteristics of study participants undergoing ATI by studies is presented in Table 4.3.1.

27/213 (12.7%) participants did not experience an undetectable VL by week 12 after restarting ART. Table 4.3.3 summarises the characteristics of participants who did not experience viral suppression by week 12. 20 of them had received an interventional study drug (8 bNAbs, 3 bNAbs with TLR-9 agonist, 4 TLR-9 agonist, 5 CCR5-edited CD4 T cells with or without cyclophosphamide, and 1 who received romidepsin only) and 7

underwent ATI-only protocols. 26/27 were male, the median age was 51 years old (range 31 – 62). Due to the frequency of VL monitoring after restarting ART, it is possible 9 participants may have achieved undetectable VL results within 12 weeks of restarting ART if monitored more frequently. Of the remaining 19 participants with detectable VL results >50 copies/mL beyond 12 weeks, they often had higher plasma VL at time of ART restart (median 74900 copies/mL, range 12251 – 5610000 copies/mL), and 7/19 restarted on ART regimens not containing integrase inhibitors (Table 4). One individual (807) did not demonstrate persistent viral resuppression and experienced ongoing low-level viraemia (VL range 44 – 710 copies/mL) beyond 12 weeks, with only one VL result <50copies/mL at day 471 after restarting ART.

There were no adverse events reported in the included studies related to restarting ART after the ATI.

Table 4.3.3 Individual characteristics of participants who did not suppress by week 12 (322)

Study	Participant ID	ATI with Intervention or ATI only	Intervention received	Age	Gender	Clade	Nadir CD4 (cells/ μ l)	CD4 count at study baseline (cells/ μ l)	Duration of ATI (weeks)	ART regimen restarted	Mean interval between visits post ART restart (Weeks)	Viral load at ART restart (copies/mL)	Time taken from ART restart to 1 st undetectable VL (Weeks)
Castagna et al. J Antimicrob Chemother 2019	3371	ATI only	ATI only	48	M	F	360	814	3	ABC/3TC/ATV/r	4.2	180634	12.6
	4844	ATI only	ATI only	51	M	F	402	1512	3	ATV/r	12	12251	24.0
	5220	ATI only	ATI only	62	M	F	212	586	5	TDF/FTC/EFV	4.7	209265	28.0
	6127	ATI only	ATI only	50	M	B	386	552	5	TDF/FTC/RPV	6.1	50396	18.2
Gaebler et al. Nature 2022	5101	Intervention + ATI	3BNC117 + 10-1074	52	M	B	Not available	671	8	TDF/FTC/DTG	3.3	639760	20.0
	5125	Intervention + ATI	3BNC117 + 10-1074	34	M	B	450	1006	33	TDF/FTC/DTG	3	19300	15.0
Gruell et al. Lancet Microb 2022	03-16-B	Intervention + ATI	Romidepsin	62	M	B	Not available	Not available	6	TAF/FTC/EVG/c	4.7	57070	14.0
Mendoza et al. Nature 2018	9248	Intervention + ATI	3BNC117 + 10-1074	52	M	B	310	620	13	TAF/FTC/DRV/r	1.9	18200	13.0
	9249	Intervention + ATI	3BNC117 + 10-1074	49	M	B	426	1070	4	ABC/3TC/DRV/r	2.2	60000	20.0
Tebas et al. J Clin Invest 2021	101	Intervention + ATI	CCR5-edited CD4+ T cells	32	M	Not available	Not available	563	16	TDF/FTC/EFV	4.275	9637	17.1
	102	Intervention + ATI	CCR5-edited CD4+ T cells	49	M	Not available	Not available	870	16	TDF/FTC/ATV/r	4	28435	16.0
	202	Intervention + ATI	CCR5-edited CD4+ T cells + CTX	60	M	Not available	Not available	512	17	TDF/FTC/RPV	4.5	56198	18.0
	205	Intervention + ATI	CCR5-edited CD4+ T cells + CTX	45	M	Not available	Not available	456	16	TDF/FTC/ATV/r	5.3	35910	15.8
De Scheerder et al. Cell Host Microbe 2019	STAR 002	ATI only	ATI only	38	M	B	438	2000	8	TAF/FTC/EVG/c	8.6	59900	25.7
Gunst et al. Nat Med 2023	109	Intervention + ATI	3BNC117 + 10-1074	57	M	B	Not available	1410	10	ABC/3TC/DTG	Not available (monthly in protocol)	43600	14.9
	114	Intervention + ATI	Lefitolimod + 3BNC117 + 10-1074	31	M	B	Not available	610	26	TDF/FTC/DRV/c		40100	18.4
	115	Intervention + ATI	Leftolimod	53	M	Not available	Not available	840	4	TAF/FTC/DTG		1150000	31

	119	Intervention + ATI	3BNC117 + 10-1074	50	M	B	Not available	780	12	TAF/FTC/BIC		74900	18.1
	134	Intervention + ATI	Leftolimod	58	M	B	Not available	600	6	TAF/FTC/BIC		32200	17.1
	601	Intervention + ATI	3BNC117 + 10-1074	52	M	B	Not available	Not available	30	TDF/FTC/DTG		173000	17.0
	801	Intervention + ATI	Leftolimod + 3BNC117 + 10-1074	52	M	B	Not available	1076	11	ABC/3TC/DTG		2100000	18.4
	822	Intervention + ATI	Leftolimod + 3BNC117 + 10-1074	54	M	B	Not available	703	8	ABC/3TC/DTG		400000	15.3
	301-13	Intervention + ATI	Leftolimod	31	F	C	Not available	750	6	ABC/3TC/DTG		328000	15
	301-4	Intervention + ATI	Leftolimod	44	M	Not available	Not available	630	3	TDF/FTC/DTG		5610000	23.0
	807 *	Intervention + ATI	3BNC117 + 10-1074	40	M	C	Not available	1561	14	ABC/3TC/DTG		370000	67.3
	205	ATI only	ATI only	54	M	B	Not available	Not available	12	TAF/FTC/DRV		18000	13.3
	502	ATI only	ATI only	61	M	B	Not available	930	5	ABC/3TC/DRV/r		333000	20.1

* This participant continued to experience low level viraemia during the follow up period 2 years after restarting ART

4.3.4 Multivariable time-to-event analysis

There was no difference in time to viral suppression between participants in the IA group compared to those who underwent ATI alone ($p=0.22$) (Figure 4.3.4). When additional variables were adjusted for in a Cox proportional-hazards model (Table 5), participants in the IA group were less likely to achieve viral suppression compared to those undergoing ATI alone (adjusted hazard ratio (aHR) 0.61, 95%CI 0.40 – 0.94, $p=0.026$). A greater plasma HIV VL at ART restart (log copies/mL) (aHR 0.47, 95%CI 0.38 – 0.59, $p<0.001$), a longer interval between VL measurements after restarting ART (aHR 0.60, 95%CI 0.52 – 0.68, $p<0.001$) were associated with less likelihood of achieving viral resuppression over time, and the use of integrase inhibitors containing regimens was associated with greater likelihood of achieving viral suppression over time (aHR 1.88, 95%CI 1.25 – 2.82, $p = 0.002$). There was also a small effect associated with the duration since HIV diagnosis (aHR 0.93, 95%CI 0.90 – 0.97, $p < 0.001$). All participants in the included studies eventually achieved at least one VL reading <50 copies/mL after restarting ART.

In the sensitivity analysis, there was a non-significant trend towards more rapid time to viral suppression for participants who receive bNABs without any other interventions compared to those undergoing ATI alone ($p=0.055$) (Figure 4.3.4.2). However, this trend was reversed once adjusted for covariables (aHR 0.0.28, 95%CI 0.16 – 0.51, $p = <0.001$). Other covariables associated with time to viral suppression were consistent with the primary outcome analysis. (Table 4.3.4.2).

Figure 4.3.4.1. Kaplan-Meier plot of proportion of participants with undetectable plasma viral loads (<50copies/mL) following ART restart after ATI (weeks). (322)

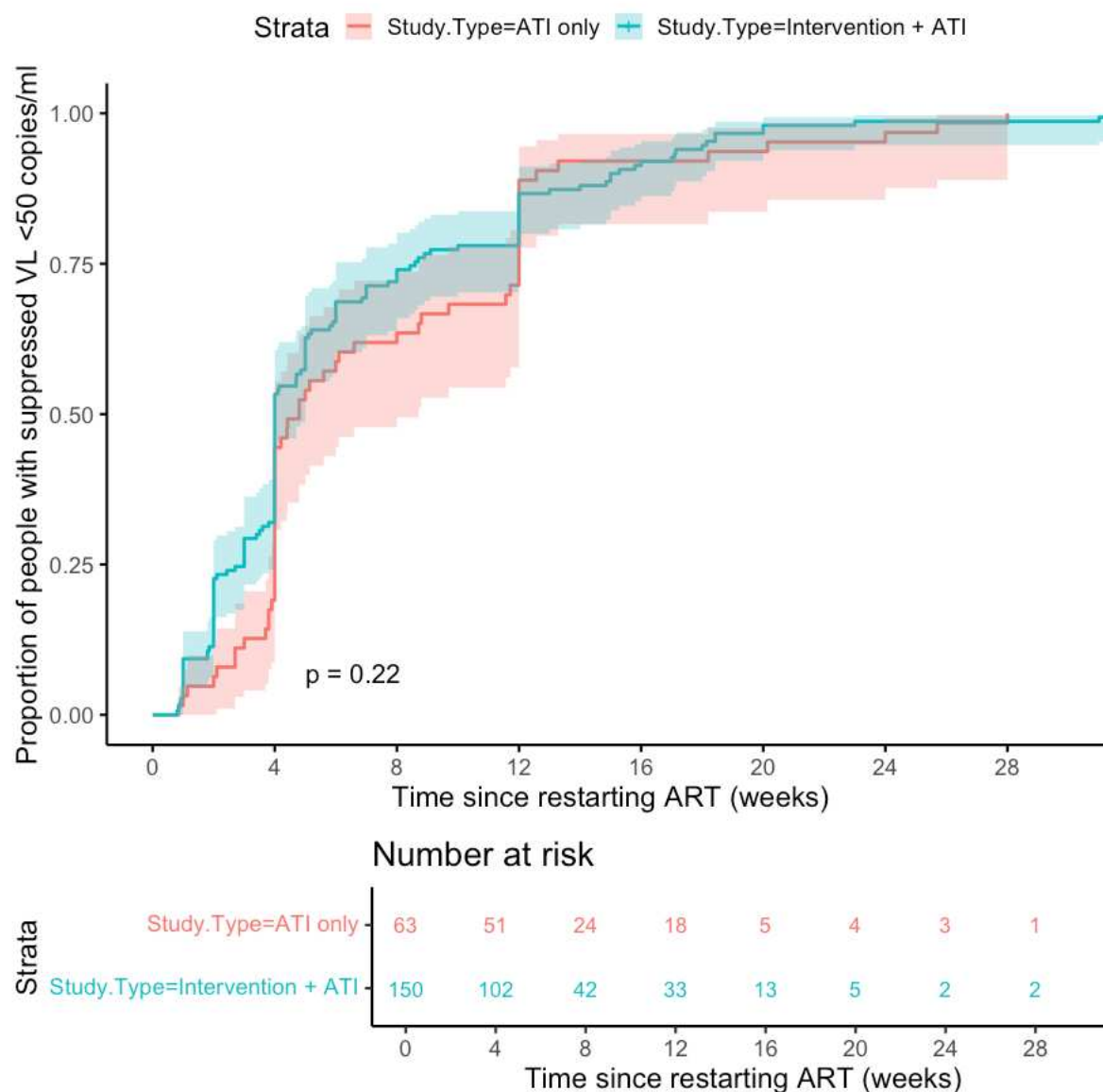


Figure legend. Kaplan Meier survival plot of proportion of participants with suppressed plasma viral load <50 copies/mL against time in weeks. The red line represents participants undergoing ATI only protocols, and blue line represent participants who received an interventional study drug with ATI as part of their study protocol. The red and blue shaded areas represent the 95% confidence intervals of their respective lines. Large changes in proportions at weeks 4 and 12 reflect study protocols where monitoring was undertaken only at these times after restarting ART. The table below shows the numbers at risk at 4-weekly intervals stratified by receipt of interventional study drug with ATI or ATI-only protocols. Datapoints are censored past week 32. Abbreviations: ART – antiretroviral therapy; ATI – analytical treatment interruption; VL - viral load.

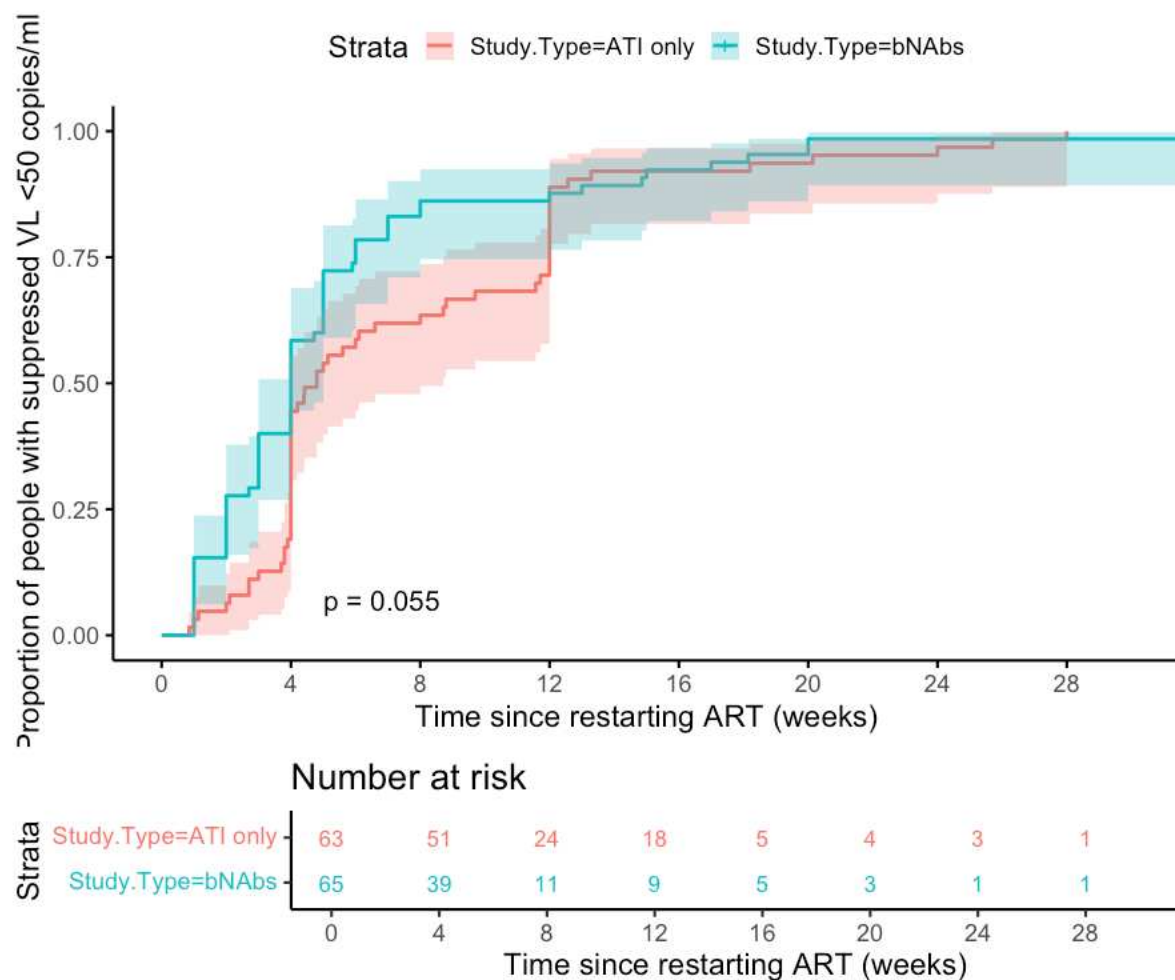
Figure 4.3.4.2. Sensitivity analysis: Kaplan Meier plot for people in bNAb + ATI vs ATI only (322)

Figure legend. Kaplan Meier survival plot of proportion of people with suppressed plasma viral load <50 copies/mL against time in weeks. The red line represents participants undergoing ATI only protocols, and blue line represent participants who received bNAb with ATI as part of their study protocol. The red and blue shaded areas represent the 95% confidence intervals of their respective lines. The table below shows the numbers at risk at 4-weekly intervals stratified by receipt of interventional study drug with ATI or ATI-only protocols. Datapoints are censored past week 32. Abbreviations : ART – antiretroviral therapy; ATI – analytical treatment interruption; bNAb – broadly neutralising antibodies; VL - viral load

Table 4.3.4.1. Multivariable cox proportional-hazards regression model (322)

		N (%) / Mean (SD)	HR	Univariable 95% CI	p-value	HR	Multivariable 95% CI	p-value
Patient characteristics								
Age (years) (per 10-year increase)		41.7 (10.7)	0.80	0.70 – 0.91	0.001	0.97	0.81 – 1.15	0.719
Gender ⁺	Female (Ref)	17 (8.5%)						
	Male	182 (91.5%)	0.70	0.43 – 1.16	0.171	0.72	0.40 – 1.31	0.282
HIV-related characteristics								
Plasma HIV viral load at ART restart (Log copies/mL)		4.4 (0.9)	0.56	0.48 – 0.66	<0.001	0.56	0.46 – 0.68	<0.001
Nadir CD4 (per 100 cells/ μ l increase)		544.6 (287.5)	1.00	0.94 - 1.06	0.914			
CD4 at study enrolment (per 100 cells/ μ l increase)		777.3 (299.8)	0.95	0.91 - 1.00	0.039	0.96	0.91 – 1.01	0.155
Duration since HIV diagnosis (years)		7.4 (7.4)	0.97	0.95 - 0.99	0.008	0.93	0.89 – 0.96	<0.001
ART regimen containing Integrase Inhibitors	No (Ref)	52 (24.5%)						
	Yes	161 (75.5%)	1.19	0.86 – 1.63	0.286	1.74	1.16 – 2.59	0.007
Study characteristics								
Duration of ATI in weeks		9.9 (8.1)	1.00	0.99 – 1.02	0.830			
Frequency of HIV VL monitoring between ART restart and viral suppression (weeks between visits)		3.4 (2.1)	0.73	0.67 – 0.79	<0.001	0.59	0.52 – 0.68	<0.001
Study type	ATI only (Ref)	63 (29.6)						
	IA	150 (70.4)	1.18	0.88 – 1.58	0.279	0.61	0.40 – 0.94	0.026

+ A transgender participant was not included in the multivariable analysis due to n <5.

Abbreviations: Ref – Reference category; ART – antiretroviral therapy; ATI – analytical treatment interruption; IA – intervention with ATI protocol; HR – hazard ratio.

Table 4.3.4.2. Multivariable analysis for people in bNAbs + ATI only vs ATI only (322)

		N (%) / Mean (SD)	HR	Univariable 95% CI	p-value	HR	Multivariable 95% CI	p-value
Patient characteristics								
Age (years) (per 10-year increase)		42.4 (10.9)	0.79	0.67 – 0.94	0.007	0.89	0.72 – 1.11	0.295
Gender ⁺	Female (Ref)	10 (8.8)						
	Male	104 (91.2)	0.73	0.38 – 1.41	0.346			
HIV-related characteristics								
Plasma HIV viral load at ART restart (Log copies/mL)		4.3 (0.9)	0.55	0.45 – 0.68	<0.001	0.52	0.42 – 0.66	<0.001
Nadir CD4 (per 100 cells/ μ l increase)		490.3 (277.7)	1.00	0.93 – 1.07	0.745			
CD4 at study enrolment (per 100 cells/ μ l increase)		803.8 (310.0)	0.92	0.87 – 0.98	0.007	0.96	0.89 – 1.02	0.197
Duration since HIV diagnosis (years)		8.5 (7.6)	0.98	0.96 – 1.01	0.120	0.97	0.93 – 1.00	0.057
ART regimen containing Integrase Inhibitors	No (Ref)	31 (24.4%)						
	Yes	97 (75.6%)	1.31	0.87 – 1.99	0.197	2.12	1.25 – 3.59	0.005
Study characteristics								
Duration of ATI in weeks		9.7 (8.2)	1.00	0.98 – 1.02	0.799			
Mean interval of HIV VL monitoring between ART restart and viral suppression (weeks)		3.3 (2.0)	0.71	0.64 – 0.80	<0.001	0.49	0.40 – 0.61	<0.001
Study type*	ATI only (Ref)	63 (49.6)						
	bNAbs and ATI	65 (50.4)	1.37	1.08 – 2.21	0.075	0.27	0.15 – 0.49	<0.001

+ A transgender participant was not included in the multivariable analysis due to n < 5.

Abbreviations: Ref – Reference category; ART – antiretroviral therapy; ATI – analytical treatment interruption; IA – intervention with ATI protocol; HR – hazard ratio.

4.3.5 Meta-analysis

The pooled proportion of participants who experience viral suppression <50 copies/mL within 12 weeks of restarting ART after an ATI was 86% (95%CI 77 - 92%) (Figure 4.3.5). There was a moderate degree of heterogeneity present across all studies ($I^2=45\%$, $p=0.02$). There was no difference between participants who underwent IA compared to ATI-alone (87% vs 84%, $p=0.68$).

Figure 4.3.5. Forest plots of primary outcome events stratified by receipt of intervention with ATI or undergoing ATI-only protocols. (322)

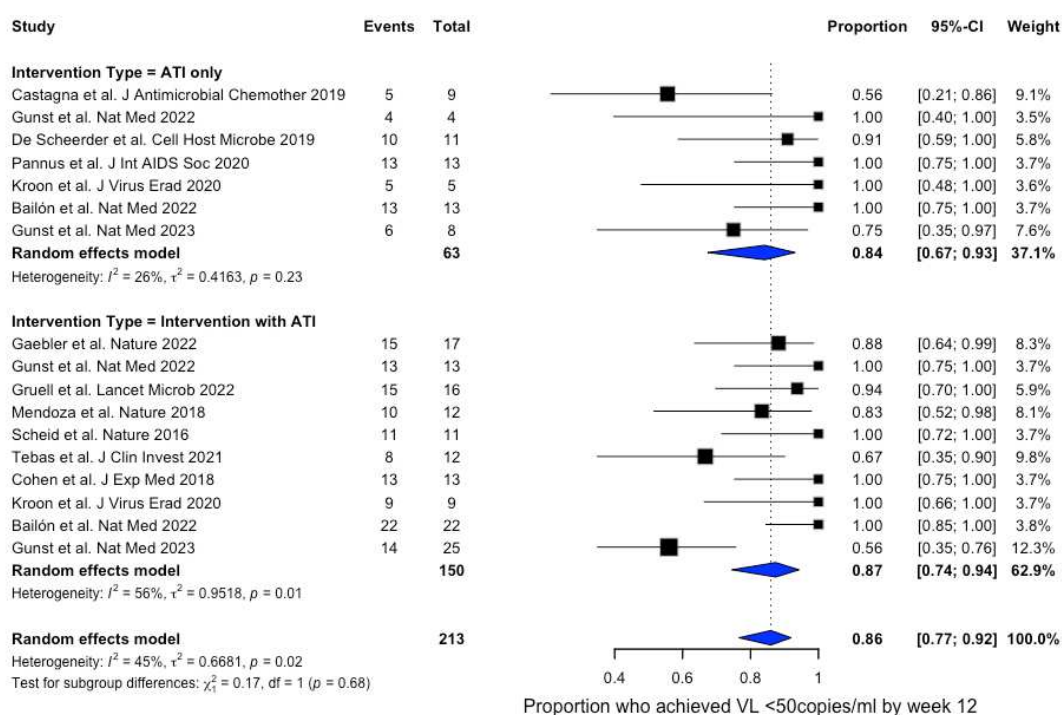


Figure 4.3.5 Legend. Studies are identified by the name of the first author, journal and year of publication. Primary outcome events were defined as participants who experienced viral suppression with plasma HIV viral load <50copies/mL) by week 12 after restarting ART. Study weights (%) are from the random-effects analysis and represented by individual box sizes. The dashed line represents the overall proportion of primary outcome events across all participants. Abbreviations: ATI - Analytical Treatment Interruption; CI – Confidence Interval.

4.3.6 Assessment of study quality and bias

The modified Newcastle Ottawa scale (323) was used to assess for bias in the single-arm cohort and observational studies (Table 4.3.6). All studies had a predominantly male population, with strict co-morbidities exclusion criteria. Three studies with longer follow up intervals (monthly or greater) were considered likely to introduce a bias for obtaining the primary outcome (Table 4.3.6). Bias was assessed for RCTs using the Cochrane ROB2 tool (324) and four studies assessed raised some concerns for bias raised in domains 3 (missing outcomes data) and domain 4 (measurement of the outcome) due to the limited VL monitoring following ART restart likely contributing to bias in the time to achieving viral suppression, due to the knowledge of intervention arms that may influence the frequency of monitoring following ART restart , respectively (Table 4.3.6).

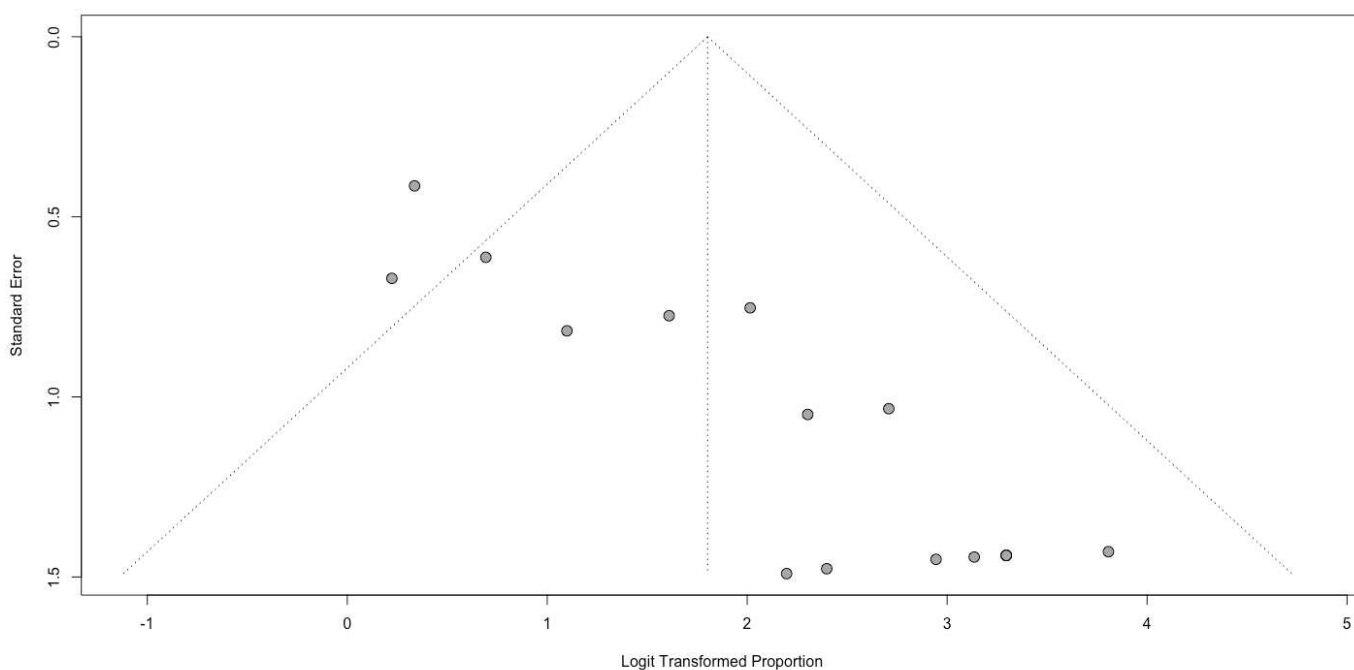
Table 4.3.6. Quality & Risk of bias assessment (322)

Non-randomised studies							
Modified Newcastle-Ottawa Score	Representativeness of the cohort	Ascertainment of exposure	Outcome was present not at the start	Assessment of outcome	Adequate follow up duration	Loss to follow up accounted for	Total
Mendoza et al. Nature 2018	0	1	1	1	1	1	5
Scheid et al. Nature 2016	0	1	1	1	1	1	5
Cohen et al. J Exp Med 2018	0	1	1	1	1	1	5
Castagna et al. J Antimicrob Chemother 2019	0	1	1	1	0	1	4
Tebas et al. J Clin Invest 2021	0	1	1	1	0	1	4
De Scheerder et al. Cell Host Microbe 2022	0	1	1	1	1	1	5
Pannus et al. J Int AIDS Soc 2020	0	1	1	1	0	1	4
Randomised studies							
Risk of Bias 2 score domains	1. Randomisation process	2. Deviations from intended interventions	3. Missing outcome data	4. Measurement of the outcome	5. Selection of the reported result		Overall
Bailón et al. Nat Med 2022	Low risk	Low risk	Some concerns	Some concerns	Low risk		Some concerns
Gaebler et al. Nature 2022	Low risk	Low risk	Some concerns	Some concerns	Low risk		Some concerns
Gruell et al. Lancet Microbe 2022	Low risk	Low risk	Low risk	Low risk	Low risk		Low risk
Gunst et al. Nat med 2022	Low risk	Low risk	Some concerns	Some concerns	Low risk		Some concerns
Gunst et al. Nat med 2023	Low risk	Low risk	Some concerns	Some concerns	Low risk		Some concerns
Kroon et al. J Virus Erad 2020	Low risk	Low risk	Low risk	Low risk	Low risk		Low risk

Table legend: Modified Newcastle-Ottawa Score: Each score represents the number of stars given per category, to a maximum NOS score of six for a well-designed study with minimal or no bias, and zero for a flawed study with substantial bias across all domains.

Although the funnel plot showed asymmetry in the publications (Figure 4.3.6), this is unlikely to be due to publication bias as discussed earlier. Alternative explanations may include participants demonstrating late viral suppression associated with high viral loads prior to restarting ART, lower rates of integrase inhibitor use, and infrequent viral load monitoring which led to their outlier positions with low standard error rates.

Figure 4.3.6. Funnel plot of included studies (322)

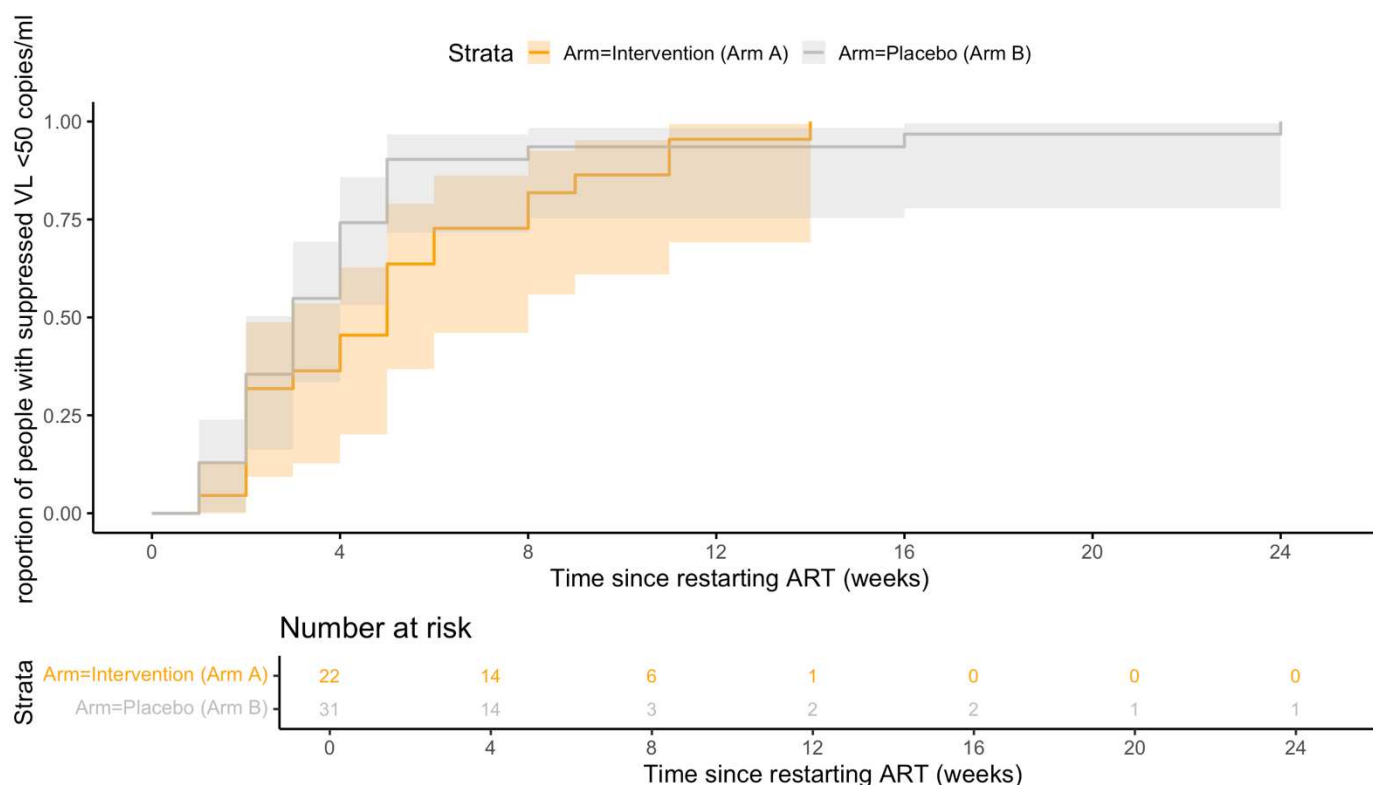


4.3.7 Time to viral suppression after LS-bNABs and an extended ATI in the RIO study

The RIO study described in chapter 2 involves LS-bNABs along with an open-ended ATI with VL-based ART restart criteria (246), and I looked at the time to viral suppression outcomes of the RIO participants after restarting ART after their first treatment interruption.

Of the 68 participants in RIO, 53 experienced viral rebound and restarted ART. The median duration of the ATI in the RIO study was 36.5 weeks (range 10 – 112 weeks) for participants in the interventional arm (Arm A), compared to 8 weeks (range 2 – 46) in the placebo arm during their first ATI (Arm B). There was no overall difference in the time to viral suppression after restarting ART for Arm A compared to Arm B participants (HR 0.69, 95% CI 0.39 – 1.21, $p=0.191$) (Figure 4.3.7).

Figure 4.3.7.1. Survival curve of time to viral suppression by arm in the RIO study



When adjusted for confounding variables, greater peak HIV viral load during ATI was associated with a lower hazard likelihood of achieving viral resuppression over time (aHR 0.63, 95%CI 0.46 – 0.85, $p = 0.003$), and use of integrase inhibitors was associated with an increased hazard likelihood of achieving viral resuppression over time (aHR 2.89, 95%CI 1.13 – 7.36, $p=0.026$). Those in Arm A (participants in receipt of bNAbs in the intervention Arm) were not different compared to Arm B in their hazard likelihood of achieving viral resuppression (aHR 0.67, 95%CI 0.27 – 1.66, $p=0.390$) (Table 4.3.7.1).

Three of 53 participants (5.7%) took longer than 12 weeks to suppression <50 copies/mL, and a further 3 did not meet the criteria for sustained viral suppression and experienced viral blips or low-level viraemia. The peak viral load was high in these most of these six participants (median 371500, range 6354 – 10000000 copies/mL), and the two participants with peak viral loads less than 100 000 copies/mL were not on ART regimens containing integrase inhibitors. The characteristics of these six participants are reported in Table 4.3.7.2. Of note, one participant in Arm B (001-013) developed a new K65KE and D67DEG mutation conferring NRTI resistance when they experienced low level viraemia after restarting ART, not present at baseline or after first ATI, despite reporting excellent ART adherence.

Table 4.3.7.1. Multivariable analysis for time to viral resuppression for participants in the RIO study

			Univariable			Multivariable		
Patient characteristics		N (%) / Mean (SD)	HR	95% CI	p-value	HR	95% CI	p-value
Age (years) (per 5-year increase)		40.0 (9.7)	0.90	0.78 – 1.06	0.205	1.00	0.83 – 1.19	0.967
HIV-related characteristics								
Peak HIV viral load during ATI (Log copies/mL)		4.7 (1.0)	0.77	0.59 – 1.01	0.060	0.63	0.46 – 0.85	0.003
ART regimen containing Protease Inhibitors	No (Ref)	48 (90.6%)	0.33	0.13 – 0.87	0.024			
	Yes	5 (9.4%)						
ART regimen containing Integrase Inhibitors	No (Ref)	8 (15.1%)	2.06	0.95 – 4.47	0.068	2.89	1.13 – 7.36	0.026
	Yes	45 (84.9%)						
Study characteristics								
Duration of ATI in weeks		22.6 (25.6)	1.00	0.99 – 1.01	0.889			
Frequency of HIV VL monitoring between ART restart and viral suppression (weeks between visits)		1.6 (0.7)	0.53	0.32 – 0.86	0.011	0.67	0.33 – 1.39	0.286
Study Arm	Arm B	31 (58.5)	0.69	0.39 – 1.21	0.191	0.67	0.27 – 1.66	0.390
	Arm A	22 (41.5)						

Table 4.3.7.2. Participants in the RIO study with delayed time to viral resuppression beyond 12 weeks or did not achieve sustained viral suppression immediately after restarting ART

PID	TTVS	Viral blips or LLV after	Peak VL during ATI (copies/mL)	VL at ART restart (copies/mL)	ATI (weeks)	No. of visits after restarting ART till undetectable	INSTI based ART	Notes
P1	14	No	51789	892	11	7	No	
P2	24	No	3570000	3570000	5	12	Yes	
P3	16	No	6354	6354	12	12	No	
P4	5	Yes	344000	344000	5	5	Yes	Developed K65KE, D67DEG resistance, ART intensified.
P5	5	Yes	399000	399000	4	5	Yes	
P6	8	Yes	10000000	10000000	6	8	Yes	

PID: Participant ID number;;TTVS: Time to viral resuppression; LLV: Low Level Viraemia; ATI: Analytical

Treatment Interruption; ART: Antiretroviral therapy; INSTI: Integrase Strand Transfer Inhibitor

4.3.8 Adverse events in the RIO study

Of the 68 participants in RIO, 32/34 (94%) of participants in Arm A and 34/34 (100%) in Arm B had reported AEs throughout the study. There were nine SAEs reported in RIO, including one death 22 weeks after the initial bNAb dose. None of the SAEs were associated with the study drug, treatment interruption, or other study procedures. Apart from one participant who developed new drug resistance to ART as described above, none of the other 67 participants had new ART drug resistance reported at the time of restarting ART after their treatment interruption.

4.4 Discussion

This systematic review and meta-analysis is the first to evaluate the impact of ATIs and intervention therapeutics on time to viral suppression for people living with HIV enrolled in cure-related ATI studies. Overall, there was no difference in time to viral suppression after restarting ART in ATI studies regardless of whether an intervention drug was administered, and 87% of participants achieved viral suppression <50 copies/mL after 12 weeks (322). These findings provide reassurance for participants worried about the impact of ATI studies and novel therapeutics such as bNABs on their viral control after completion of the study, particularly with the concerns around onward HIV transmission as discussed previously.

However, 12.7% participants did not experience complete viral suppression by week 12 after restarting ART, and a longer time to viral suppression was associated with a greater plasma HIV viral load at ART restart, longer intervals between VL measurements, longer duration of HIV diagnosis, and the receipt of interventional drug in a multivariable cox regression model. Of note, a number of participants from the studies in the systematic review had extremely high plasma HIV viral loads greater than 10 million copies/mL, despite the international consensus guidelines for ATI studies which suggest ART is restarted when the viral load is confirmed above 100 000 copies/mL (219,220). One concern may be that repeat testing for confirmation of a VL above 100 000 copies/mL may not be required and future studies may consider recommending participants restart ART while awaiting repeat VL results when the initial VL measurement is crosses that threshold, to avoid unnecessary delay in restarting ART. This association between peak viraemia at ART restart with longer time to viral resuppression seems to be reversed by the use of integrase inhibitors containing ART regimes, resulting in no overall difference between the cohort who received interventional drugs, the majority being bNABs, and those undergoing an ATI alone. Integrase inhibitors have been demonstrated to result in more rapid viral suppression (58,343). In the RIO study, despite the longer ATI duration for participants in the interventional arm, there did not seem to be an impact on the time to viral

suppression overall, and the factors associated with a longer time to viral resuppression appeared to be consistent with the findings from the pooled systematic review and meta-analysis.

The association between the receipt of interventional drugs, in particular bNAbs, and a longer time to viral resuppression found during the multivariable analysis in the meta-analysis is novel and may generate possible hypotheses based on findings from previous studies. The clonal expansion of T cells and active replication of their associated HIV reservoir by these drugs may drive persistent viraemia, as seen with latency reversal agents such as romidepsin (344), immune checkpoint inhibitors which may also increase HIV-specific CD8 T cell activity and activate HIV transcription (345). I have previously discussed the ‘vaccinal’ effect, which may be elicited by bNAbs or their immune-complexes through the priming of HIV-specific cellular responses (243) resulting in the proliferation and expansion of stimulated T cells. However, a recent systematic review (221) did not identify any safety concerns for studies involving ATIs, particularly those involving bNAbs.

With regards to safety events, the overall high numbers of adverse events in the RIO study may be explained by the long duration of the study, where the median ATI duration for Arm A participants was 36.5 weeks, and in Arm B, although participants had much shorter durations of their first ATI, most remained in the study on ART for longer periods of time for the stage two open-bNAb labelled phase. However, there were no serious adverse events associated with the bNAbs, treatment interruption or other study procedures. The death of the participant during the study was not deemed to be associated with bNAbs due to the long duration after the initial dosing, and post-mortem findings suggesting long-standing atherosclerosis-driven cardiovascular disease.

There were limitations to this analysis. ART adherence, history of ART drug resistance at baseline or time of rebound, duration of ART use since diagnosis to ATI, were not available from all studies, and I was unable to include these factors in the multivariable model. The time to viral suppression may be overestimated with

longer intervals between viral monitoring after restarting ART, and thus this association may reflect a methodological bias rather than a true association.

In conclusion, LS-bNAbs appeared to be safe, and did not affect the time to viral resuppression compared to people who undertook ATIs without intervention. When designing future cure-related ATI studies, from these data I would recommend considering the use of integrase inhibitors, along with clear VL thresholds for restarting ART, with frequent plasma viral load monitoring during and after an ATI, and the regular reporting of sustained viral suppression (which may be defined as two or more undetectable readings after restarting ART, already in used in some studies (80)). Researchers should commit to documentation and sharing of these important safety data, for the purposes of transparency and to improve the safety profile of these interventional study drugs when used in future studies.

Chapter 5

Clinical outcomes of long-acting bNAbs in people diagnosed early in HIV

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5.1 Introduction

BNAbs have been associated with prolonged viral suppression in people with virus predicted or evaluated to be sensitive to bNAbs (80,132,175–177), however data from the use of multiple doses of long-acting LS-bNAbs in people without HIV are limited (346) and none have been reported for the use for the treatment of HIV at the time of writing. Pre-screening for bNAb resistance may improve outcomes, however existing screening assays may not reliably predict successful clinical response to bNAb therapy, as they are validated to in-vitro neutralisation assays (132,177,178). The results of previous therapeutic bNAbs studies since 2015 are presented in the Table 1.3.4, Chapter 1. The studies were varied in their study design, inclusion criteria, number of doses and inclusion of combination therapies. However, generally administration of the combination of dual bNAbs 3BNC117 and 10-1074 were associated with longer viral control off ART compared to placebo or historical controls, and bNAb monotherapy.

The RIO study will be the largest powered randomised placebo-controlled trial of the two LS-bNAbs 10-1074LS and 3BNC117LS to date (246). These LS-bNAbs may offer the advantage of longer dosing intervals, and the feasibility of a 6-monthly dosing strategy paired with other long-acting antiretroviral agents e.g. lenacapavir is currently under investigation, as shown in a proof-of-concept phase 1b study (347). However, the impacts of long-acting LS-bNAbs on the time to viral rebound and viral reservoirs have not been reported, although modest reductions in the intact proviral reservoir following bNAb therapy have been described in a single study (176). LS-bNAbs may also be detectable in blood beyond 24 weeks after a single infusion (133,348). In the randomised TITAN study of two bNAbs and Leftolimod, there was evidence that bNAbs were associated with slower viral doubling times, and initial viral doubling times were strongly correlated with time to loss of virological control (175). These findings suggest bNAbs may continue to influence partial control of viral recrudescence, even after loss of complete viral suppression occurs.

In this chapter, I analysed the clinical outcomes of the RIO to assess the impact of two doses of LS-bNAbs compared to placebo on viral rebound in people treated during primary or early HIV.

5.2 Methods

The RIO study design and methodology is described in full in Chapter 2. Briefly, the trial included 68 participants who were diagnosed with primary or early HIV who were virally suppressed on ART for at least a year. After being screened for 10-1074 resistance mutations using an in-silico resistance prediction algorithm from single genome sequencing of PBMC proviral *env* sequences, study participants were randomised in a 1:1 allocation to receive either a single dose of dual bNAbs or placebo infusion. This was followed by a closely monitored ATI initiated two days after randomisation, until they met the protocol-defined criteria for ART restart and unblinding. Participants were advised to restart ART during the ATI if they reached the following criteria:

1. Viral rebound confirmed by HIV VL measurement from venous blood:
 - a. Sustained HIV viral load ≥ 1000 copies/mL for 6 weeks $\pm$ 7 days.
 - b. HIV viral load $\geq 100\,000$ copies/mL for two readings, 7 days apart $\pm$ 7 days.
 - c. Instances outside of the above two categories but were close to these definitions and necessitate ART restart for clinical reasons. These instances will be individually reviewed by an independent panel (Endpoint Adjudication Committee) to confirm if they meet the definition for viral rebound.
2. CD4 count decrease to less than 350 cells/ μ L.
3. Clinical symptoms attributable to ATI.
4. Participant preference.
5. Major concerns over risk mitigation for HIV transmission.
6. New COVID-19 diagnosis (SARS-CoV-2 Polymerase Chain Reaction (PCR) positive) during ATI.

In addition,

- At any VL threshold, ART was restarted if the participant changed their mind during the treatment interruption.

- In cases when participants did not formally meet the criteria for viral rebound and ART restart, and yet have evidence of sustained viraemia (for example, viral loads >1000copies/mL for less than six weeks). In these cases, an independent Endpoint Adjudication Committee reviewed these cases to determine if viral rebound was reached for the purposes of the primary outcome analyses.
- If there was a confirmed drop in CD4 count to < 350cells/ μ L or in the presence of symptoms that were attributed to the ATI, ART was recommenced.
- After restarting ART
 - Participants were followed up at two-weekly intervals until they achieved undetectable plasma viral load measurements, followed by monthly visits until the end of the study.

Participants who did not experience HIV rebound viraemia off ART within 20 weeks continued to remain off therapy for the duration of the study or until viral rebound. At the end of the study, all participants were recommended to re-start ART regardless of HIV viral load in accordance with national UK guidelines.

Plasma HIV RNA was measured using validated clinical assays at the local laboratories at each study site. ART therapeutic drug monitoring was performed by Professor Saye Khoo, Centre for experimental Therapeutics, Liverpool, UK. Serum bNAb measurements were performed by Duke University as described in Chapter 2.

Participants demographics were described. Clinical data were locked and extracted from the study electronic data capture system Openclinica at the time of study unblinding in December 2024 and used in the analysis presented in this chapter. A time-to-event analysis was undertaken of the time to rebound stratified by study arm, and plotted as a Kaplan-Meier survival curve using the R packages 'Survival' and 'Survminer'. Differences in time to rebound by Kaplan-meier survival curves was compared using a long-rank test, and hazard ratios calculated using a cox proportional-hazards regression model. All participants were included in the stage 1 analysis. Stage 2 data from Arm B participants who received open-labelled bNAbs after completion of their 1st ATI were not included in this analysis. Due to the extended nature of the study, 16/34

the participants in stage 2 had not initiated or completed their 2nd ATI, thus the results available for the stage 2 outcomes were incomplete for analysis. Sensitivity analyses using the above survival analyses methods were also performed for different rebound thresholds, at 50 copies/mL and 200 copies/mL.

Plasma viral doubling times were analysed using the readings between and inclusive of the first detectable plasma HIV RNA measurement above 50 copies/mL since ATI, and the first peak plasma HIV RNA before ART restart. The last undetectable plasma HIV RNA measurement was used as the initial value if there were no other detectable results prior to the peak value. These timepoints were chosen, to capture the exponential phase of viral growth. The slope of this increase was calculated by a linear regression model. Differences in groups were compared using the Wilcoxon rank-sum test for non-parametric data. Participants who withdrew from the 1st ATI before week 20 and those that did not rebound >50 copies/mL were not included in this comparison.

All analyses were performed using R (R version 4.2.2 (2022-10-31))(327).

5.3 Results

5.3.1 Study demographics

Participants were recruited to the RIO study between May 2021 to July 2024, and the study was unblinded after the primary endpoint was met by the last participant in December 2024. In total, 68 participants were enrolled in the study, of whom 34 (50%) were randomly allocated to receiving dual bNAbs at randomisation (Arm A), and 34 (50%) were allocated to the placebo arm (Arm B). Three participants in Arm A and no participants in Arm B withdrew from the ATI before the primary endpoint at 20 weeks. One participant restarted ART due to severe symptoms secondary to multiple gastrointestinal infections at week 12 but remained blinded and recommenced his treatment interruption from week 20 after he recovered from his acute illness, and remained off ART until his subsequent virological rebound at week 33. He was deemed to have reached virological endpoint at week 12 due to the presence of plasma viraemia >1000 copies/mL at that visit. No other participants were lost to follow-up.

Overall, both arms were generally balanced on key demographics parameters (Table 5.3.1). Both arms were composed of all men, the majority were of white ethnicities with well controlled HIV, and a similar range of ages between groups, but the median age in arm B was 42.5 years compared to 36 years in Arm A. All participants were diagnosed in primary or early HIV infection.

Table 5.3.1. Table of RIO participant demographics presented as overall data and by arms.

	Overall N / Median (n=68)	% / Range	Arm A N / Median (n=34)	% / Range	Arm B N / Median (n=34)	% / Range
Age (years)	39.5	22 - 58	36	22 - 58	42.5	23 - 56
Male	68	100%	34	100%	34	100%
gender						
Ethnicities						
- White	58	85%	28	82%	30	88%
- Black or Black British	3	4%	1	3%	2	6%
- Asian or Asian British	3	4%	3	9%	0	0.0%
Baseline CD4 count (cells/μL)	722	314 - 1502	684	314 - 1502	762	535 - 1463
Duration on ART (years)	5	1 - 12	5	1 - 12	5	2 - 12
ART regimen						
- PI	6	9%	3	9%	3	9%
- INSTI	58	65%	27	79%	31	91%
- NNRTI (prior to ART switch)	5	7%	4	12%	1	3%
HIV Subtype						
- B	38	56%	19	56%	19	56%
- Non-B	16	24%	7	21%	9	26%
- Not available at screening	14	21%	8	24%	6	18%
Primary HIV infection	61	91%	32	94%	29	88%

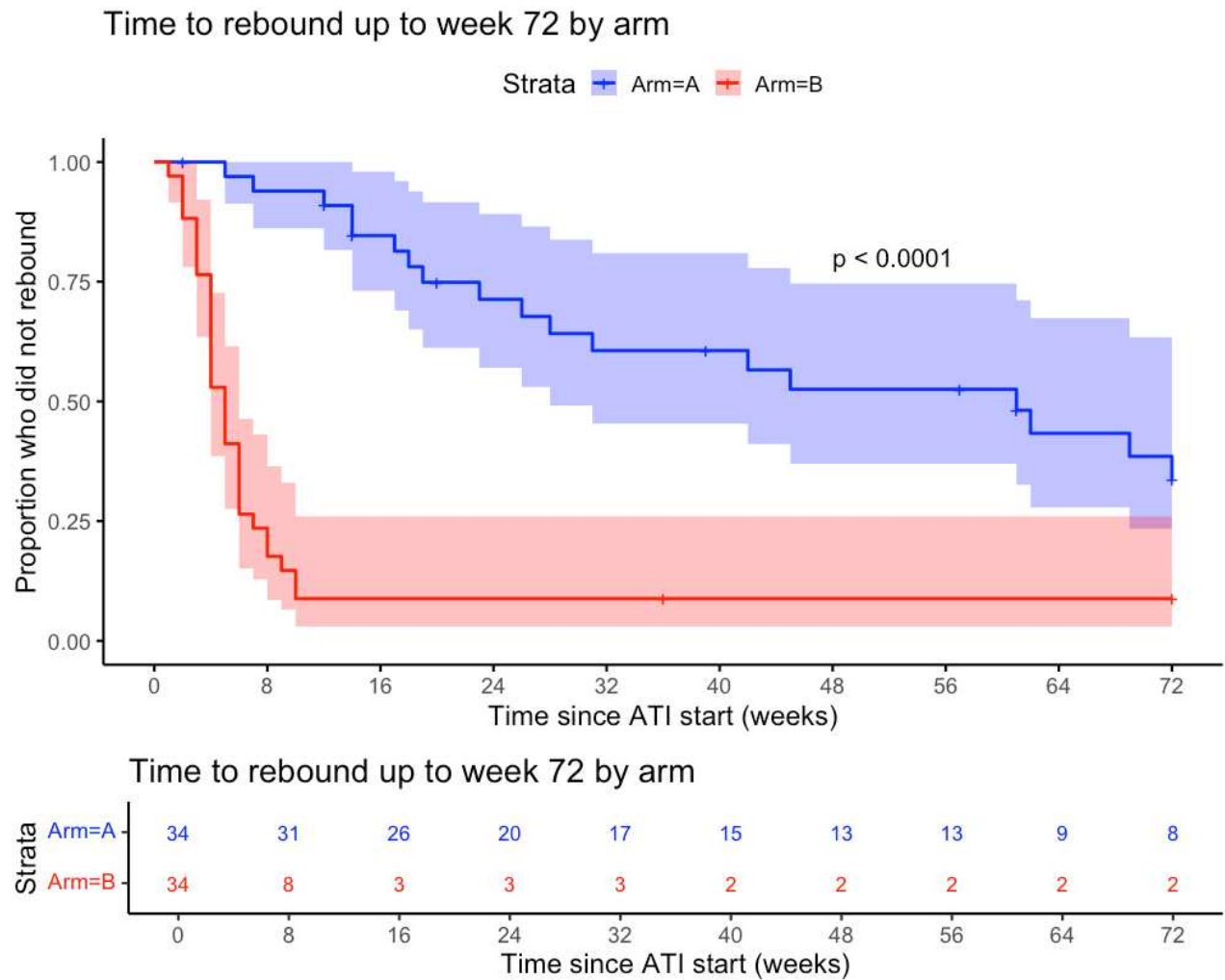
5.3.2 Time to viral rebound after ART interruption

All but two participants who received placebo (Arm B) experienced loss of virological control, at a median of 3 weeks (range 1 – 11 weeks). Two participants in Arm B remained with no HIV RNA detected in plasma up to week 124 and 128 respectively and continue to remain suppressed off ART, with no detectable ART drug levels present in their plasma samples. Of the participants who received LS-bNAbs in Arm A, three participants withdrew from the ATI, and one participant died after their week 20 visit, from causes unrelated to the study drug or procedures. These four participants ended their ATI before meeting the ART-restart criteria, and their data was censored at the time of their last visit. Nine participants (six in Arm A and three in Arm B) remained off ART without meeting the ART-restart criteria at the time of the interim data lock in December 2024, the shortest at 20 weeks and the longest achieving ART-free virological control for beyond 148 weeks. For the primary outcome of the study, participants in Arm A were significantly less likely to experience viral rebound compared to those receiving placebo (Arm B) by week 20 (HR 0.09, 95%CI 0.04 – 0.21, $p < 0.001$). By week 72, the result remained consistent with Arm A participants being 76% less likely to experience viral rebound compared to people in Arm B (HR 0.24, 95%CI 0.13 – 0.44, $p < 0.0001$) (Figure 5.3.2.1). The mean difference between the between the time to viral rebound outcomes between Arm A and Arm B participants 46.7 weeks (mean Arm A vs Arm B time to viral rebound 46.7 vs 20.3 weeks, 95%CI for mean difference 21.2 – 72.3 weeks) by week 148.

Of the 56 participants included in the analyses, 34 received a 2nd bNAb dose at least 20 weeks after their first dose (17 in Arm A). The median time of the 2nd dose for arm A participants was 28 weeks from the first dose (range 21 to 50 weeks). When outcomes of participants who received bNAbs in Arm A were stratified by number of doses and analysed by time from last dose, people receiving two doses were less likely to experience viral rebound over time within 72 weeks after their last dose compared to a single dose (HR 0.24, 95%CI 0.09 – 0.66, $p = 0.006$) (Figure 5.3.2.2). Again, the number of participants remaining in the study by weeks 48 and week 72 after the last dose were small, with an n of 9 and 3 respectively. The mean modelled

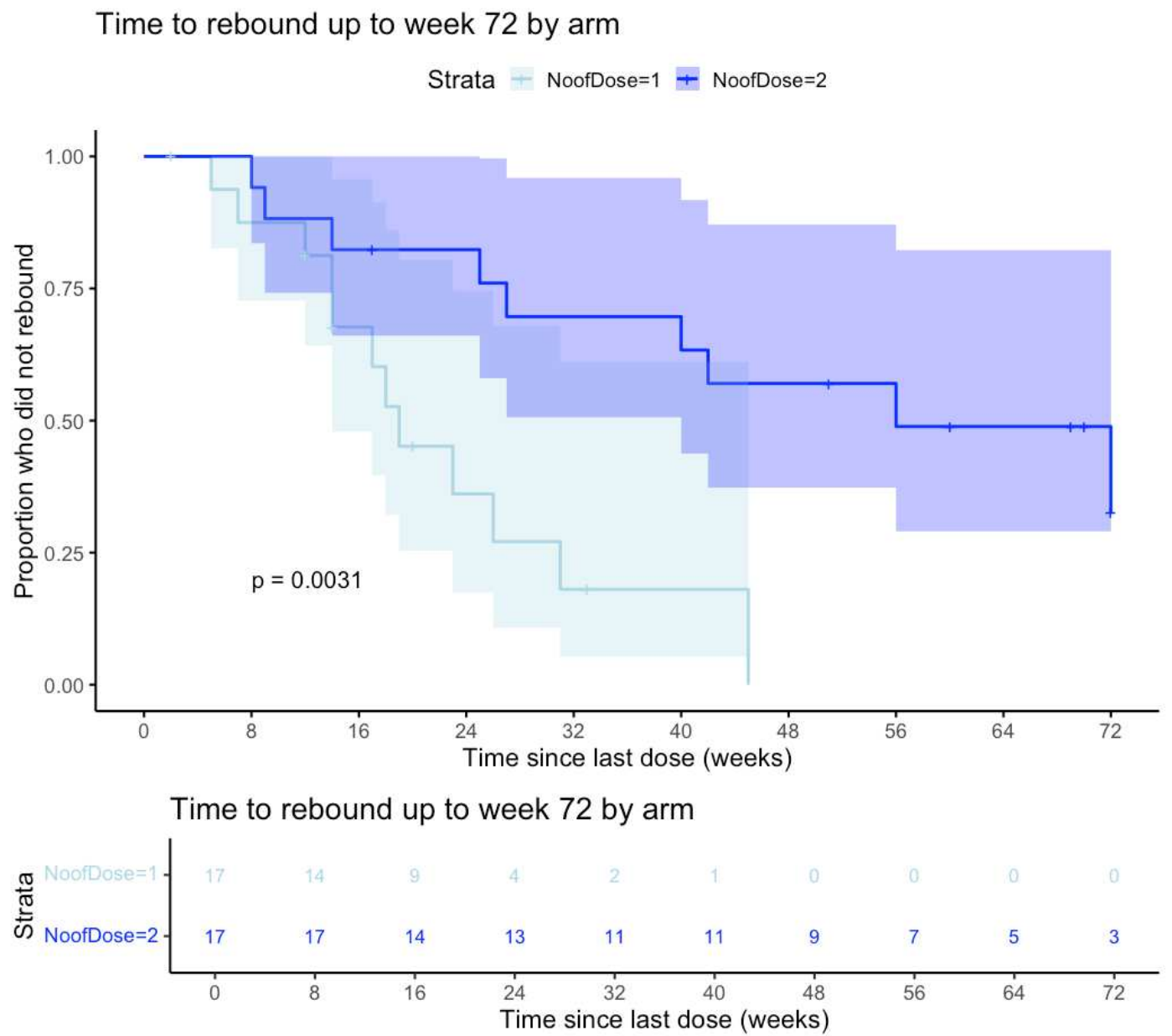
time to serum bNAbs concentrations falling below a threshold of 10 µg/mL was 48 weeks after a second dose for both LS-bNAbs, further details of the two-compartment pharmacokinetics model will be described in chapter 6. There were six participants in Arm A who remained virally suppressed off ART beyond 48 weeks after the time of their last dose, compared to two in Arm B, with three further Arm A participants who continue to remain off ART and have yet to meet this timepoint.

Figure 5.3.2.1. Survival curve of time to rebound during the 1st ATI (Stage 1) stratified by study arm



Statistical test: log-rank test.

Figure 5.3.2.2. Survival curve of time to rebound for Arm A participants during the 1st ATI (Stage 1) stratified by number of bNAb doses received

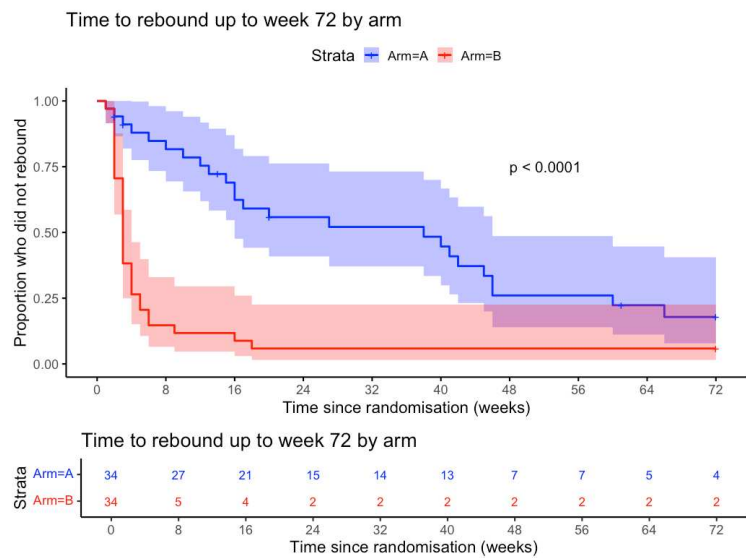


Statistical test: log-rank test.

Other viral load thresholds were compared in the sensitivity analyses. Arm A participants who received bNAbs demonstrated longer time to viral rebounds to above 50 copies/mL (HR 0.27 ,95%CI 0.15 – 0.47, P <0.001) (Figure 5.3.3.3), and above 200 copies/mL (HR 0.22, 95%CI 0.13 – 0.39, p<0.001) (Figure 5.3.3.4). Of the participants who experienced viral rebound >50 copies/mL by week 20, more participants achieved

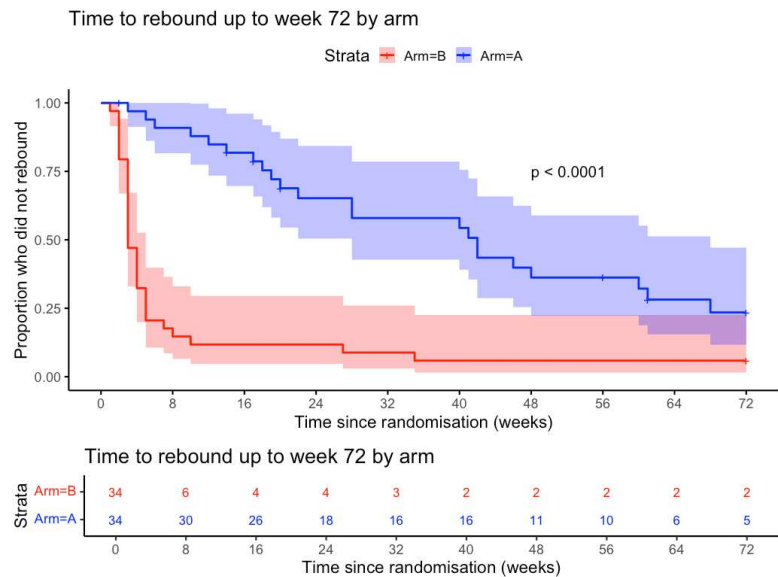
spontaneous re-suppression (defined as VL under 50 copies/mL for at least 4 weeks after the first VL reading above 50 copies/mL) in Arm A (4/16, 25%) compared to Arm B (0/31, 0%).

Figure 5.3.3.3. Survival curve of time to VL >50 copies/mL during the 1st ATI (Stage 1) stratified by study arm



Statistical test: log-rank test.

Figure 5.3.3.4. Survival curve of time to VL >200 copies/mL during the 1st ATI (Stage 1) stratified by study arm



Statistical test: log-rank test.

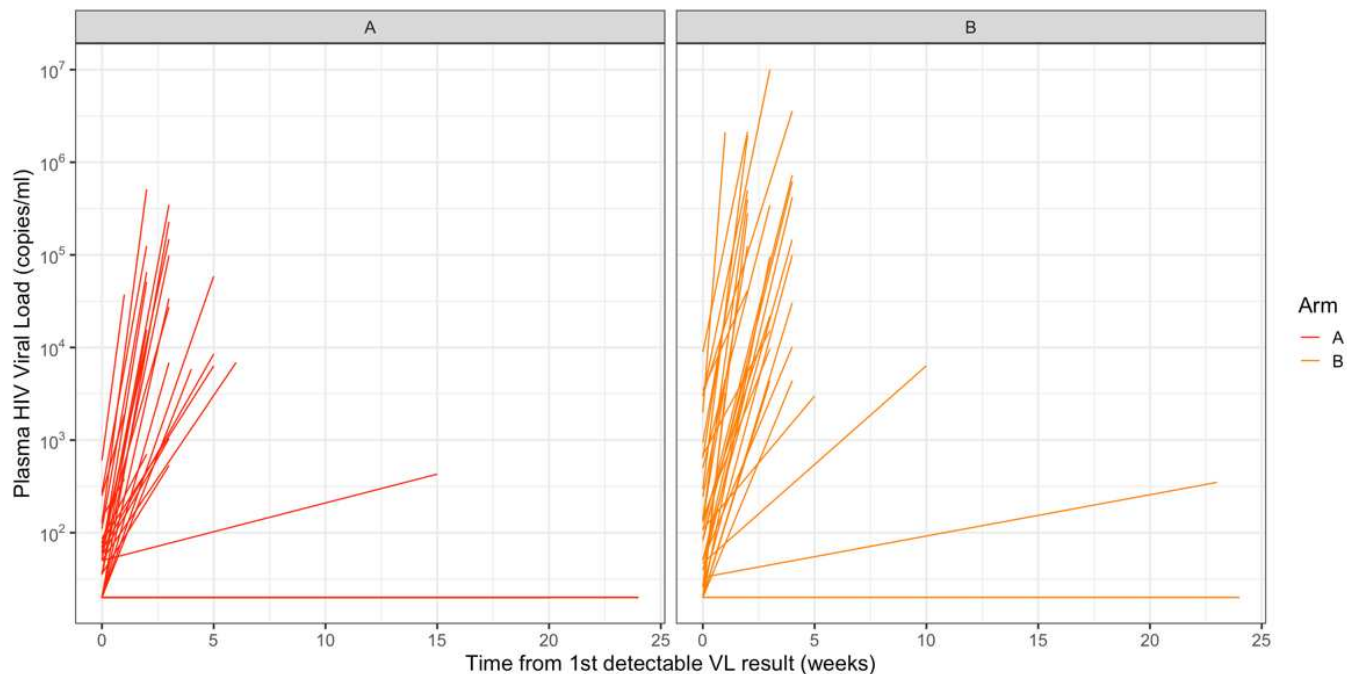
5.3.3 Viral rebound kinetics by doubling times

Viral doubling times were assessed to determine the impact of the intervention on the rebound viral kinetics even after loss of complete suppression occurred. There were 25/31 participants in Arm A and 32/34 participants in Arm B who experienced loss of viral suppression. 6 participants in Arm A and 2 participants in Arm B remained virally suppressed <50copies/mL at the time of the analysis (Figure 5.3.3a)

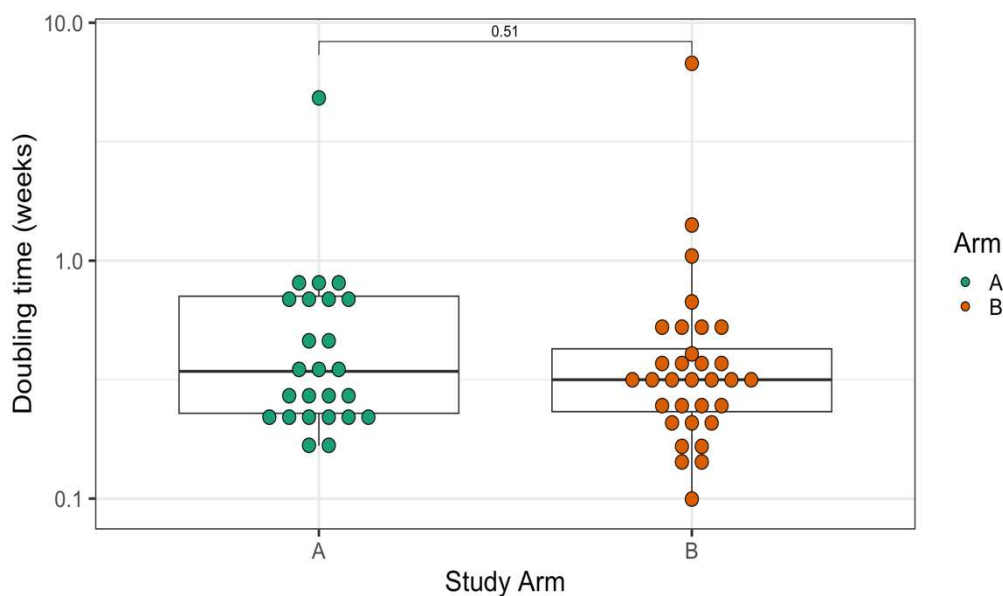
There was no difference in the doubling times between participants in Arms A and B who experienced viral rebound >50copies/mL (median doubling time 0.34 vs 0.32 weeks, $p=0.51$) (Figure 5.3.3b). However, Arm A participants who experienced viral rebound beyond 20 weeks after dosing, had a slower doubling time compared to those who rebound within 20 weeks (median doubling time 0.57 vs 0.23 weeks, $p = 0.00082$), and compared to the doubling times of participants in Arm B during their first ATI (median doubling time 0.57 vs 0.32 weeks, $p = 0.028$). Arm A Participants who rebound within 20 weeks after bNAb dosing demonstrated viral doubling times comparable to participants who received placebo (median doubling time 0.23 vs 0.32 weeks, $p = 0.07$). The differences in viral doubling times stratified by time to rebound by week 20 are presented in Figure 5.3.3c.

Figure 5.3.3 Analyses of viral rebound doubling times

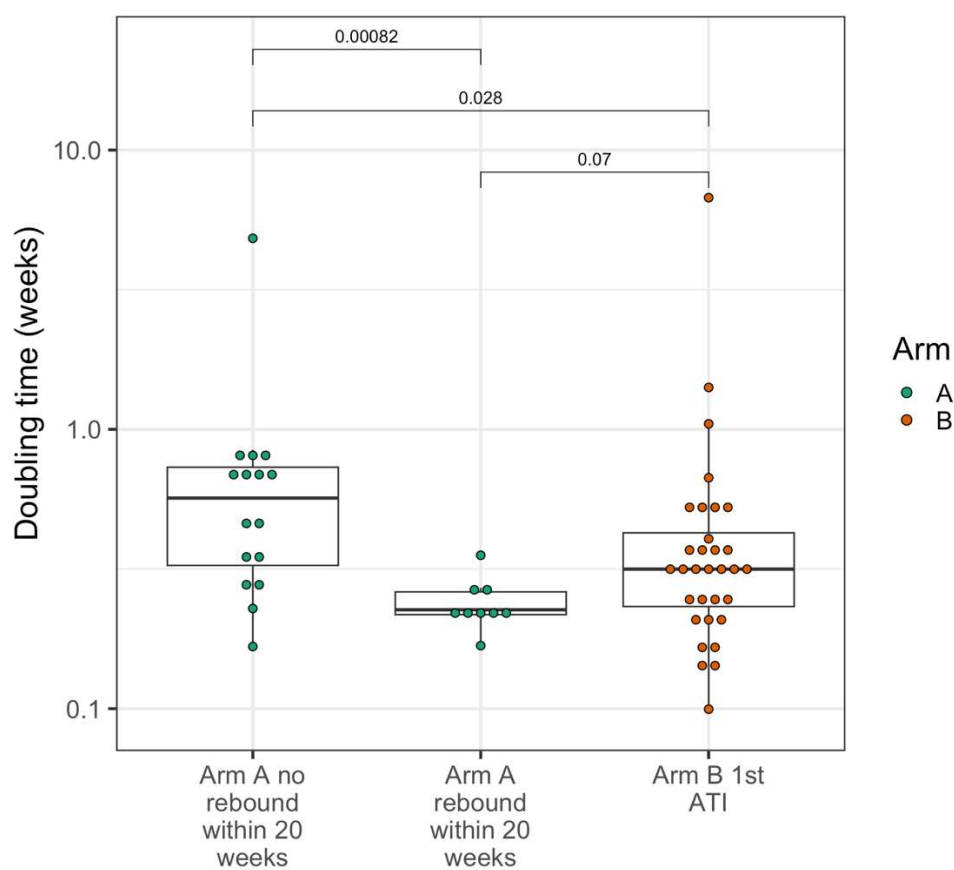
a) Line plots of viral rebound times by study arms, and stratified by time to viral rebound response compared to placebo group.



b) Plasma HIV viral doubling times by arms



c) Plasma HIV viral doubling times by time to rebound within 20 weeks



Statistical test: Mann Whitney U test

5.4 Discussion

The RIO trial is the first double-blinded, randomised, placebo-controlled study demonstrating two long-acting bNAbs 10-1074LS and 3BNC117LS are associated with a significant delay in viral rebound compared to placebo in people who were diagnosed in early HIV with no predicted resistance to 10-1074. There was a 91% reduction in the hazard risk of viral rebound by week 20 after a single dose of dual bNAbs. Participants who received two doses of bNAbs experienced a longer duration of virological control after receiving their last dose, and at the time of writing, six participants in Arm A and three in Arm B remain suppressed off ART in the stage 1 ATI. Previous smaller studies have shown that people living with HIV with bNAb-sensitive virus, who received 3-8 infusions of these bNAbs, have maintained prolonged viral suppression in the absence of ART (132,175–177) but none have used the long-acting LS-variant bNAbs to date. Recently, a study conducted in South Africa using different LS-bNAbs (CAP256V2LS and VRC07-523LS) in Black African women who were diagnosed in hyperacute HIV infection, reported 6 of 20 participants delayed viral rebound beyond 48 weeks (349).

More participants (17%) who received bNAbs were more likely to demonstrated prolonged viral suppression beyond 48 weeks after the last dose compared to those who received placebo (6%). These findings suggest the possibility of post-intervention control following administration of bNAbs that lasts beyond the presence of the antibodies, above and beyond the expected frequency of post treatment controllers from an early treated cohort alone. As previously described in the CHAMP study, 6 – 19% of a PHI cohort were estimated to be PTCs depending on the definitions used (131). A meta-analysis of 24 studies found that rates of sustained PTC was 4%, rising to 6% in those treated during early HIV (350). This adds to the growing literature of long-term viral control induced by bNAbs (80,132), and may be mediated by enhanced HIV-specific cellular responses that persist after bNAbs administration, a bNAb ‘vaccinal effect’ (178,245). This is further supported by the slower viral doubling times observed in those participants that experienced a longer time to viral rebound, suggesting that even when complete viral suppression is lost, there may be an ongoing

partial effect of viral control mediated by the bNAbs. However this effect was not seen in those who rebounded early before 20 weeks post-dose, suggesting that people with viruses who may have resistance to the bNAbs not detected on screening, may not experience this effect of partial control on their rebounding viral replication. These findings are consistent with a previous study which reported bNAbs were associated with slower doubling times when dual bNAbs were given with lefitolimod compared to those who did not receive bNAbs, and median viral doubling time was strongly correlated with time to loss of virological control (175).

Although LS-bNAbs may be a promising treatment for long-acting viral suppression, the rates of viral rebound above 50 copies/mL (16/34, 47%) observed by week 20 are still inferior to those achieved by existing long-acting ART such as injectable cabotegravir/rilpivirine (14,351), and may suggest for effective use to maintain viral suppression, administering these bNAbs in combination with other long-acting ART to prevent the development of resistance may be required, as seen in this phase 1 study of dual bNAbs with lenacapavir (347), particularly as the differences in pharmacokinetics of both bNAbs means a period of effective monotherapy. This will be discussed further in chapter 6 when the pharmacokinetics of the bNAbs are examined.

In conclusion, bNAbs are a promising intervention associated with prolonged viral control with both duration of viral suppression, and slower doubling times on rebound when given to individuals who had evidence of residual viraemia. Participants were motivated to join ATI studies for altruistic reasons, but concerns around viral transmission should be addressed through careful counselling prior to enrolment and access to HIV prevention strategies such as PrEP be incorporated as part of ATI study protocols, and regaining virological control after restarting ART should be addressed through the use of integrase inhibitors-containing ART regimens and regular viral load monitoring in ATI studies.

Chapter 6

Defining the serum pharmacokinetics profile and rebound thresholds of HIV-specific long-acting broadly neutralising antibodies

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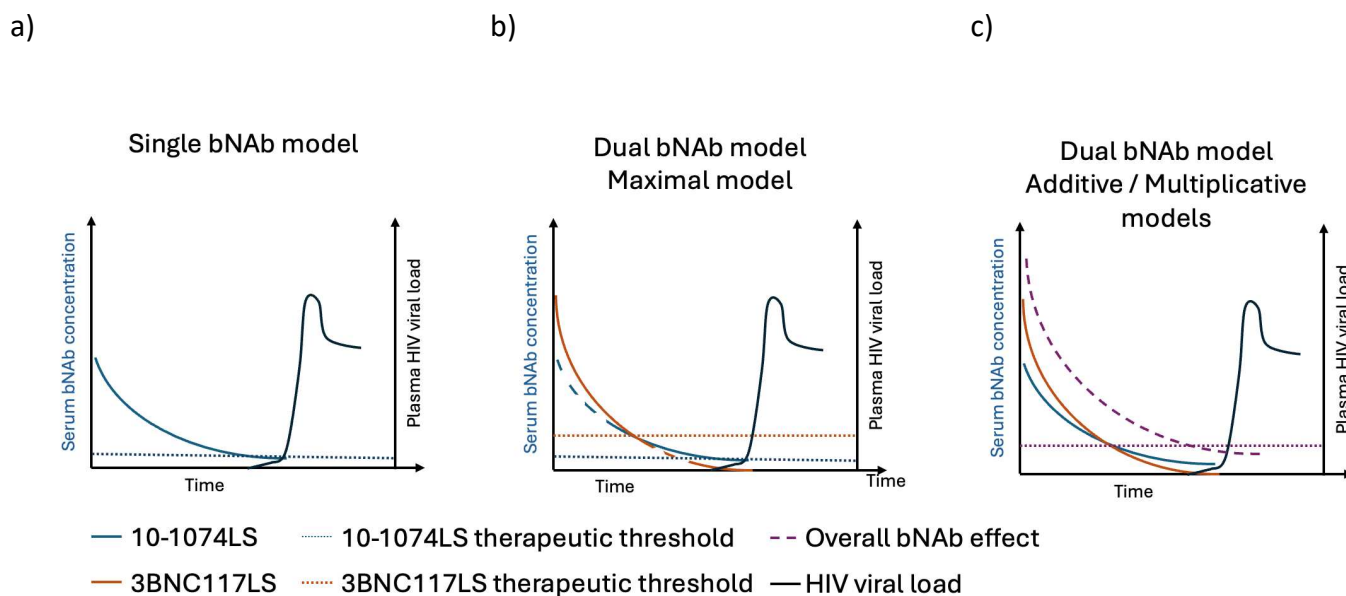
6.1 Introduction

The latest generations of bNAbs have been shown to be promising agents in the treatment of HIV, particularly with the use of the LS mutation (M428L/N434S) in the Fc region of bNAbs which substantially prolong their half-lives (227,352) and potentially improve in vivo activity (353). The long-acting bNAbs 1074LS and 3BNC117LS have demonstrated extended half-lives of nearly 3-4 times longer than their parental antibodies (133). The LS mutation enhances the affinity of the Fc region of the bNAbs to the neonatal Fc receptor (FcRn) at low pH, which rescues IgG from lysosomal degradation and recycles them back into circulation(354).

There has been limited data examining the pharmacokinetic profiles of these long-acting bNAbs in people living with HIV, including multiple dosing strategies and how they impact the maximum concentrations and clearance rates. In people living with HIV, the presence of virus may result in lower bNAb concentrations due to the 'antigen sink' effect, with increased clearance of antibodies after binding to viral antigen present (355). Studies of bNAbs in people with HIV who were viraemic generally had lower bNAb half-lives compared to those with suppressed viral loads(230,231,356). There is even less data for the impact of bNAb concentrations on viral dynamics, due to the small numbers and fixed-duration treatment interruption periods present in most ATI study designs. Previous studies have suggested for people living with HIV with no pre-existing baseline bNAb resistance, a therapeutic serum threshold of 10 µg/mL above which viral rebound was not observed in a small study of nine participants (132), however, this has yet to be validated in larger studies and the assays for predicting resistance remains unreliable particularly for predicting clinical outcomes (237). The RIO study, powered to detect a difference in clinical outcomes of an open-ended ATI with predefined ART restart criteria in people living with HIV who were randomised to receiving either bNAbs or placebo, provided a unique opportunity to understand the pharmacokinetics of these long-acting bNAbs in people living with HIV.

To model the pharmacokinetic-pharmacodynamic (PKPD) interaction of bNAbs, one could assume a direct inhibitory model using the bNAb concentrations and the outcome being the viral load measures. In this model, the effect (viral load) is directly inhibited by the bNAb concentration in a sigmoidal dose-response curve relationship (Figure 6.1a), and viral rebound occurs when the corresponding effect from the falling bNAb concentration rises above the rebound threshold (assumed to be 50 copies/mL). The bNAb concentration at which viral rebound occurs would be considered the bNAb rebound concentration. To account for bNAbs given in combination, several bNAb interaction models have been proposed (357). The simplest may be the maximum interaction model, which assumes only the most potent bNAb neutralises a given virus. Once the first bNAb drops below the rebound threshold, the second bNAb may take over as the more potent bNAb effect until it too crosses the threshold and viral rebound occurs (Figure 6.1b). Another is the additive model, where antibody effects may be expressed as a sum of individual antibody effects (358). Finally, there is the multiplicative effect, described by Wagh et al. (359) as the Bliss-Hill (BH) independence model. The BH describes a 'best-case' scenario where bNAbs cover missing breadth of one another to co-neutralise strains, expressed as an multiplicative effect of each bNAb. This has been supported by invitro studies (358,359), however, it is unclear if this also applies in the outcomes from a therapeutic study. The synergistic effect of the additive and multiplicative models is summarised in Figure 6.1c.

Figure 6.1. Proposed bNAb PKPD models



In this chapter, I aimed to estimate the population parameters, including the maximum serum concentrations and clearance rate half-lives, of the long-acting bNAbs 10-1074LS and 3BNC117LS, from serum measurements taken from participants in the RIO study. To understand the therapeutic thresholds of the bNAbs, I developed a direct inhibitory pharmacodynamics model combining the effects of both bNAbs in a Bayesian approach, to estimate the time of viral rebound, predicted rebound bNAb concentration, and the rebound IC80 values from the observed viral load measurements of participants who experienced viral rebound.

6.2 Methods

6.2.1 Serum sample collection and measurements

Serial serum samples were collected from participants in the RIO study which was described in Chapter 2. In summary, 68 participants were recruited and randomised in a 1:1 allocation to receive either a single dose of dual bNAbs (Arm A) or placebo infusion (Arm B), and subsequently underwent a closely monitored ATI. When they experienced viral rebound meeting the protocol definition for restarting ART (Chapter 2, section 2.1.5), they were advised to restart ART and had their study arm allocation unblinded. bNAbs were administered in weight-adjusted doses as follow: 10-1074LS 10mg/kg and 3BNC117LS 30mg/kg, based on findings from earlier phase 1 pharmacokinetic studies to address the differences in half-lives of the long-acting bNAbs. Participants who were initially randomised to receive the placebo infusion were invited to receive an open labelled infusion of the dual bNAbs at the same weight-adjusted dosing as the arm A participants, after they restarted ART. A study amendment was introduced midway during the study following approval by the study funders and received REC approval on the 22nd June 2022, offering an optional second dose of the blinded IMP at least 20 weeks after the first dose during the first ATI, or a second open-labelled dose of bNAbs offered at least 20 weeks after the first bNAb dose for the Arm B participants. Arm B participants remained on ART for at least 24 weeks after the last bNAb dose, and underwent a second ATI. The monitoring schedule and criteria to restart ART was identical to that of the first ATI. Once ART was restarted after the final ATI, participants in both arms were followed up until the end of study 16 weeks after restarting ART.

During the first treatment interruption, serum samples were collected at baseline, weeks 2,4,8,12,24,38,48, and every four weekly thereafter. For Arm B participants who received the open-labelled bNAbs, serum samples were collected before infusion, weeks 2, 4, 8, 20 after 1st bNAb dose, weeks 2,4 after 2nd bNAb dose, and weeks 0, 2,4,8,12,24,38,48, and every four weeks thereafter after initiating the 2nd ATI. Serum samples were also collected at the time of ART restart visit, and at the end of the study.

Serum samples were measured by a validated anti-idiotypic ELISA by Kelly Seaton, Tomaras Lab, Duke University. Briefly, high-bind 384 well plates were coated overnight with anti-idiotypic antibody specific to 10-1074 or 3BNC117. Plates were then washed, blocked, and washed again and diluted samples, standards, and controls were added. Plates were then washed and incubated with an anti-human IgG-HRP detection antibody, followed by washing, addition of tetramethylbenzidine substrate and then stop solution. Plates were then read at 450 nm to obtain an OD reading. Infused mAb concentration in each sample was determined based on a standard curve.

Several criteria were used to determine if data from an assay were acceptable and could be statistically analyzed. Any positive sample with coefficient of variation between replicates greater than 20% was repeated. If the blank well on the plate exceeded 0.1 OD, the plate was repeated. Standard curve EC50 also had to be within 3 standard deviations of the historical mean EC50 as determined by optimization assays. 10-1074-LS and 3BNC117-LS standard curve concentrations were determined based on A280 absorbance using the manufacturer's IgG extinction coefficients of 1.68 and 1.69, respectively. Samples with optical density less than the LOD are negative and samples with optical density greater than the LOD are considered positive. For 3BNC117-LS, the LOD in HIV seropositive serum is 1265 ng/mL. For 10-1074-LS, the LOD is 197 ng/mL.

Anti-drug antibodies (ADA) measurements of serum samples pre-and post bNAb infusion from 29 RIO participants were performed and reported by the Dartmouth GCLP antibodies laboratory, USA.

6.2.2. Population parameters estimation of serum bNAb concentrations using one and two compartment models

Population and individual PK parameter estimates of serum bNAb measurements were modelled using both one and two-compartment models with the MonolixTM software as described in chapter 2. Models tested included the following models (Figures 6.2.2a, b, c): A) One compartment model with a single compartment (V) and a constant rate of elimination (k) with the equation $C_p(t) = D \cdot e^{(k \cdot t)}$ where D is the intravenous dose,

and t is a particular timepoint; B) Two compartment VK model with a central compartment (V), a peripheral compartment (V2), rate of transfer to and from the central to peripheral compartments (k_{12} and k_{21} respectively), and a linear elimination rate (k); C) Two compartment AB model with the previous parameters transformed into the input parameters A , B , α , β using the equations below.

Equations:

$$V = 1/(A+B)$$

$$k_{21} = \alpha - A \cdot V \cdot (\alpha - \beta)$$

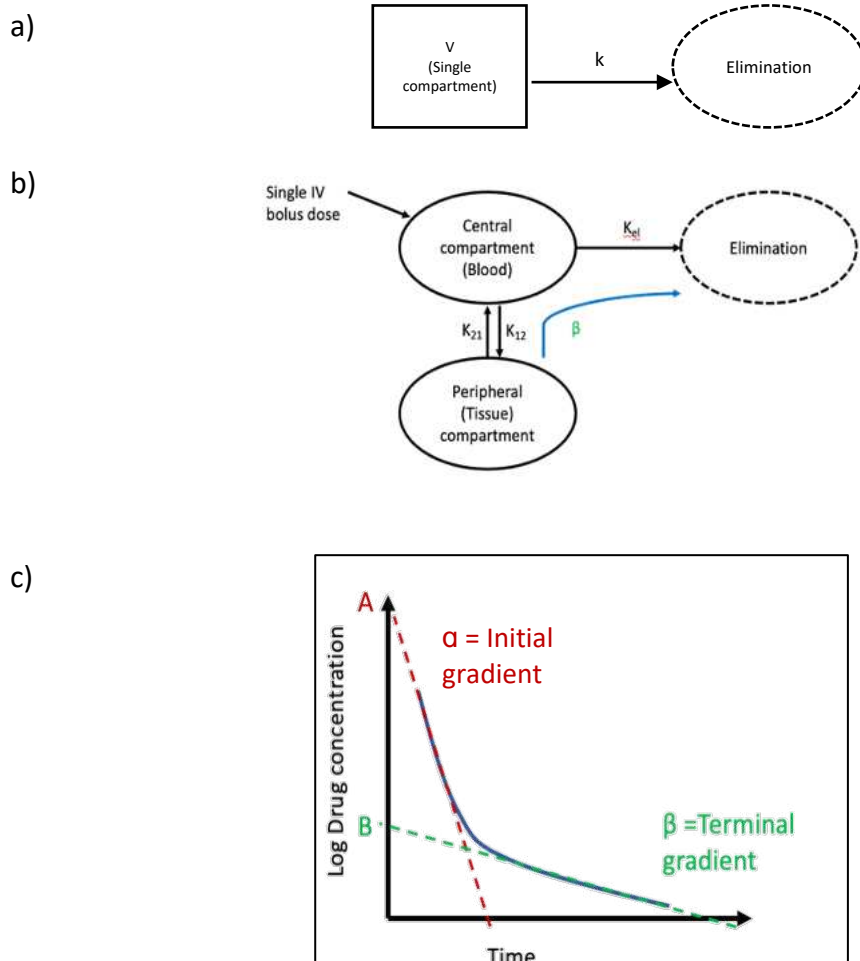
$$k = (\alpha \cdot \beta) / k_{21}$$

$$k_{12} = \alpha + \beta - k_{21} - k$$

Figure 6.2.2 Graphical representation of the models tested for best fit to the RIO serum

pharmacokinetics data a) 1 compartment VK model b) 2 compartment VK model c) 2 compartment

hybrid AB model



An iterative non-linear mixed effects model with a SAEM algorithm was built using the SAMB algorithm (294), with the criteria for final model acceptance based on the lowest BIC score, and the AIC score was also reported. The following covariates were tested: Age, Log(Weight), Virus clade (B vs non-B or unknown clade), study arm, and presence of predicted baseline bNAb resistance mutations using a validated algorithm (295). Broadly, each model was run with initial parameter estimates generated through automated initialisation of parameters function in monolix, and adjusted manually for improved fit to the observed values. The models were run unadjusted for covariates, and subsequent adjustment was made using the p_{50} posteriori conditional distribution to calculate the correlation between the random effects and covariates. Covariate models are chosen with the lowest BIC criterion value are evaluated using linear regression, error models are selected through calculation of the criterion for each possible residual error model (constant, proportional, combined1 and combined2), and a correlation model is selected through calculating the correlations between each parameter with the lowest BIC criterion. A proposed model is suggested based on the combination of the best error, covariate and correlation models calculated. The proposed model is applied and re-run using the same initial values as the base model, and is rapid as it does not require estimation of new parameters with SAEM for all evaluated models. The algorithm stops when there is no improvement proposed with further models, and the final model is re-run to estimate using standard error and maximum likelihood, and subsequently without an simulated annealing step to decrease the standard deviation of the conditional distribution if required. 90% prediction intervals for the final model parameters were calculated using the bootstrap method with the R package RsmLx, with 1000 bootstrap replicates.

6.2.3 Analyses of single and multiple doses of long-acting bNAbs through simulations

Due to the subsequent introduction of the second dose study amendment and study protocol design, there was substantial heterogeneity in the timing of the second dose administration. To address this, a simulation of the bNAb serum concentrations using the actual times observed in the study, and another simulation if all participants received the second dose at the same time (week 21 after first bNAb dose) were run. The structural model and population parameters estimated from the final two compartment model was imported from Monolix and run in the associated simulation software Simulx by Lixsoft. Conditional mean values of the parameters for each individual were entered into the simulation, and 1000 replicates were run with the same number of individuals (n=56). Simulation outputs were serum 10-1074LS and 3BNC117LS concentrations, time when serum bNAb concentrations remained above 10µg/mL and 50µg/mL, and maximum serum bNAb concentrations. Results are reported as median values (90% prediction interval (PredInt)) or mean (± 2 standard deviations(SD)).

6.2.4 Modelling the dynamics of bNAbs concentration on HIV viral rebound dynamics using a direct inhibitory model of dual bNAbs.

A direct inhibitory pharmacodynamic (PD) model was used to investigate the effect of direct neutralisation of the bNAbs effect on the viral load (E), to estimate the time of rebound (defined as plasma HIV RNA >50copies/mL) when the bNAb suppression effect drops below a specific threshold in relation to the IC50. The ratio of the bNAb concentration to the IC50, also known as the inhibitory quotient, has been well described in previous pharmacodynamics models for bNAbs (357,360). In the model, the action of inhibition is defined by the following parameters: The baseline viral load effect without bNAbs effect (E0), maximal fraction of inhibition (Imax), the half maximal inhibitory concentration (IC50), and sigmoidicity of the curve (gamma). The equation for both bNAbs are thus defined as:

For 10-1074LS:

$$E1 = E0 * (1 - (Imax1 * \max(Cc1,0)^{\gamma_1})/(\max(Cc1,0)^{\gamma_1} + IC50_1^{\gamma_1}))$$

For 3BNC117LS:

$$E2 = E0 * (1 - (Imax2 * \max(Cc2,0)^{\gamma_2})/(\max(Cc2,0)^{\gamma_2} + IC50_2^{\gamma_2}))$$

The IC80 value is calculated from the IC50, based on the slope of the neutralisation curve, assuming the slope (also known as the hill coefficient, n) is 1, thus the IC80 is assumed to be four-fold higher than the IC50 under the equation $IC80 = IC50 * (80/(1-80)^{1/n})$. This has been consistently shown in previous studies of different antibody-virus combinations in the CATNAP database (357).

The population parameters from the best fit pharmacokinetics model were fixed, and plasma viral load data from participants in Arm A who experienced viral rebound were included. Initially individual bNAbs PD models were built using the SAMBA method for the best fit model. Subsequently both bNAbs were modelled in a combined model using a Bayesian approach to determine the overall effect based on the lowest resultant effect (viral load).

To account for the mutation rate that may be driven by the selection pressure of the bNAbs, a further variable, the exponential mutation rate (MR) was introduced in subsequent models. The PD equations were therefore modified to the following:

For 10-1074LS:

$$E1 = E0 * (1 - (Imax1 * \max(Cc1,0)^{\gamma_1})/(\max(Cc1,0)^{\gamma_1} + (IC50_1 * \exp(MR * t))^{\gamma_1}))$$

For 3BNC117LS

$$E2 = E0 * (1 - (Imax2 * \max(Cc2,0)^{\gamma_2})/(\max(Cc2,0)^{\gamma_2} + (IC50_2 * \exp(MR * t))^{\gamma_2}))$$

To account for interaction models, I assumed each bNAb operates as a single product, and in a maximum interaction model, only the most bNAb with the most potent effect on viral suppression for a given individual's virus population, and when the bNAb concentrations drops such that, it is no longer effective at

viral suppression, the other bNAb's effect is considered the predominant effect until it's concentrations drop to levels which lead to loss of viral control.

Therefore the overall equation for the viral load (E) is $E = \min(E1, E2)$.

Other models for interactions between bNAbs were tested.

An additive model under the equation

$$E = E0 * ((1 - (Imax1 * \max(Cc1,0)^{\gamma_1}) / (\max(Cc1,0)^{\gamma_1} + (IC50_1 * \exp(MR * t))^{\gamma_1})) + (1 - (Imax2 * \max(Cc2,0)^{\gamma_2}) / (\max(Cc2,0)^{\gamma_2} + (IC50_2 * \exp(MR * t))^{\gamma_2})))$$

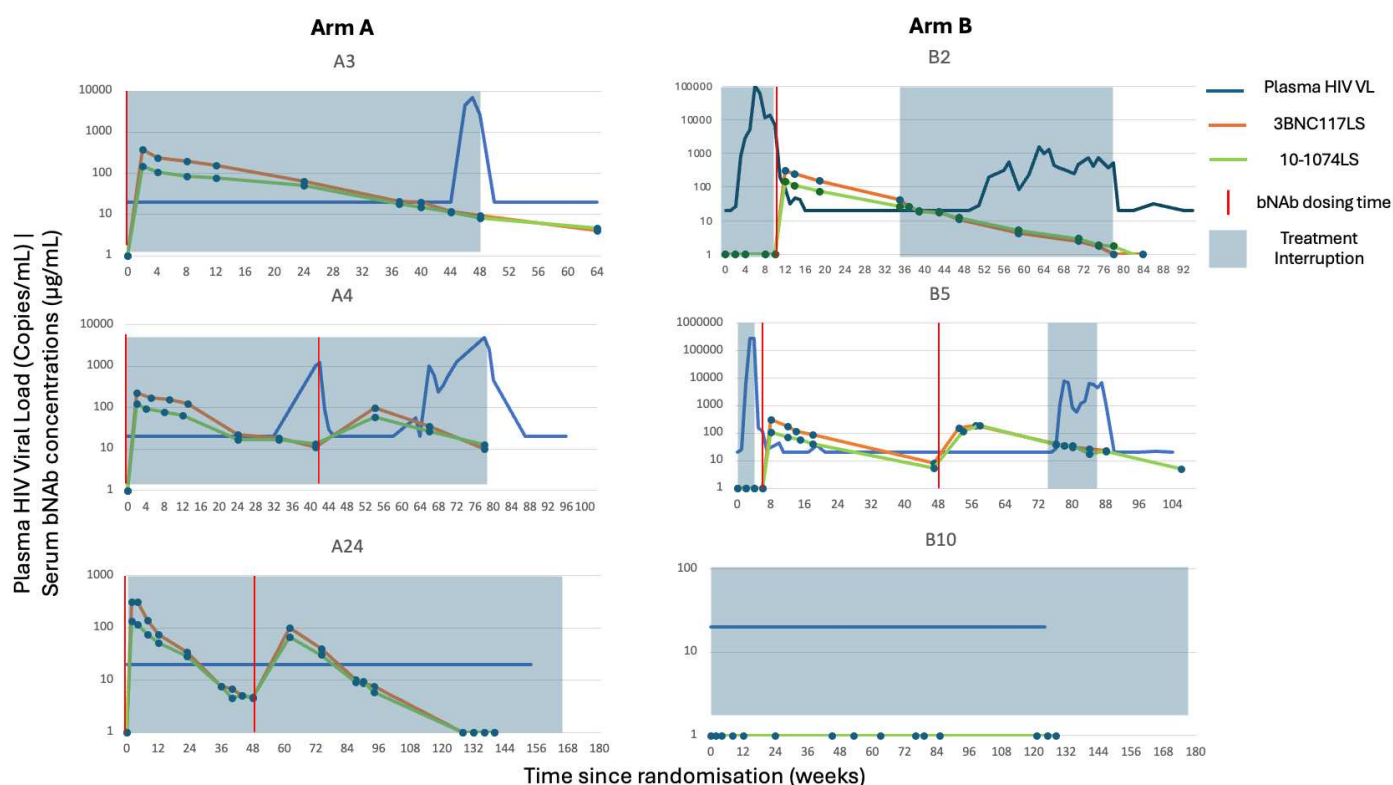
and a multiplicative interaction of both bNAbs under the equation

$$E = E0 * ((1 - (Imax1 * \max(Cc1,0)^{\gamma_1}) / (\max(Cc1,0)^{\gamma_1} + (IC50_1 * \exp(MR * t))^{\gamma_1})) * (1 - (Imax2 * \max(Cc2,0)^{\gamma_2}) / (\max(Cc2,0)^{\gamma_2} + (IC50_2 * \exp(MR * t))^{\gamma_2})))$$

6.3 Results

745 serum samples were sent from 65 participants from the RIO study across both study arms for serum bNAb measurements. bNAbs were detected in 56 participants: 31 from arm A after randomisation and 25 from arm B after open labelled bNAb dosing. Results were normalised to the time of the last dose for the PK parameters estimation. Demographics of the participants in the RIO study are described in Chapter 4. ADA measurements on serum samples from 29 RIO participants performed by Dartmouth university, USA, showed no evidence of post-infusion ADAs. Figure 6.3 shows serum bNAb measurements for selected RIO participants in relation to their viral load trajectories and dosing times during the course of the study

Figure 6.3 Serum bNAb measurements in relation to viral load trajectories and dosing times for selected Arm A participants and Arm B participants.



6.3.1 Population parameters estimation using a two-compartment AB model

Both bNAbs were best described by a two-compartment AB model demonstrating bi-phasic clearance following administration. Of the covariates that were tested for association in the model, only the log-transformed weight (LogWt) required adjusting for with parameter B for both bNAbs. The AIC and BIC values for the final model for 10-1074LS was 2854 and 2874 respectively, and the values for the 3BNC117LS final model was 3056 and 3077 respectively. The population parameters for both bNAbs and their 90% prediction intervals are presented in Table 6.3.1.

Table 6.3.1. Population parameters and 90% prediction intervals

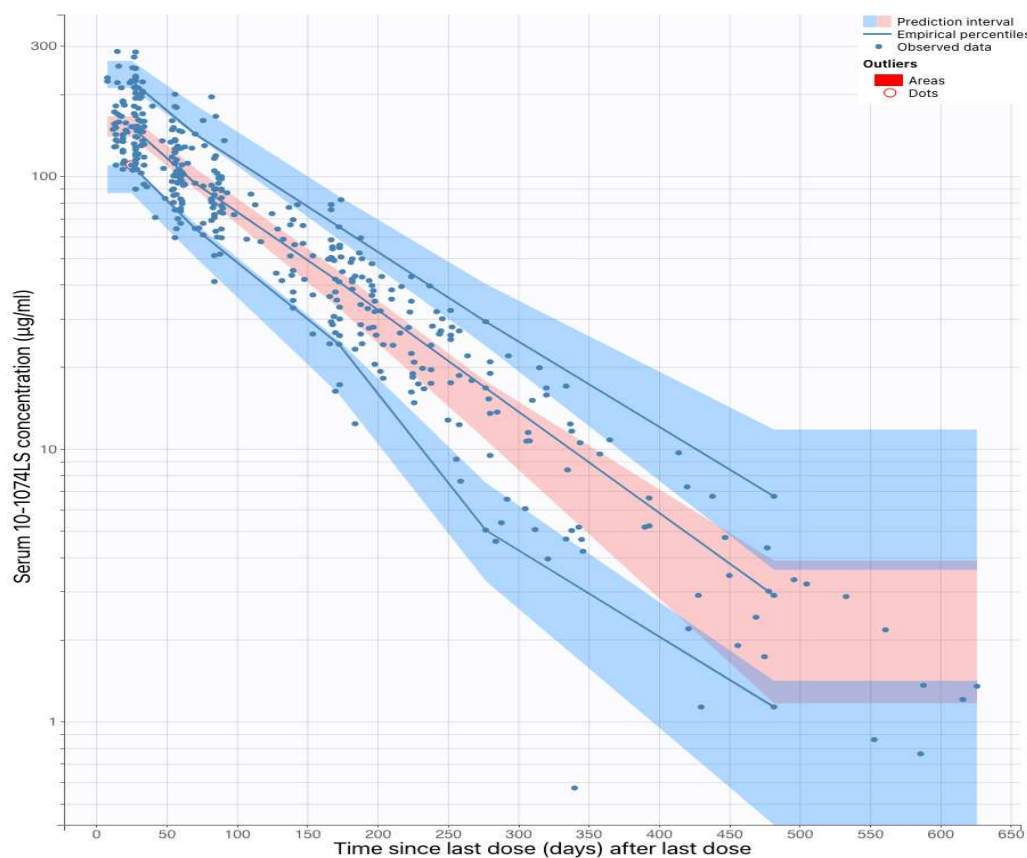
POPULATION PARAMETERS	10-1047LS	10-1074LS 90% PRED INTERVALS	3BNC117LS	3BNC117LS 90% PRED INTERVALS
A_POP	4.00×10^{-15}	$3.99 \times 10^{-15} - 4.01 \times 10^{-15}$	7.86×10^{-5}	$7.86 \times 10^{-5} - 7.86 \times 10^{-5}$
B_POP	2.51×10^{-4}	$2.38 \times 10^{-4} - 2.63 \times 10^{-4}$	1.19×10^{-4}	$1.14 \times 10^{-4} - 1.28 \times 10^{-4}$
α_POP	1.01×10^9	$9.78 \times 10^8 - 1.01 \times 10^8$	5.23×10^{-2}	$5.23 \times 10^{-2} - 5.23 \times 10^{-2}$
β_POP	9.65×10^{-3}	$9.09 \times 10^{-3} - 1.00 \times 10^{-2}$	1.07×10^{-2}	$1.04 \times 10^{-2} - 1.12 \times 10^{-2}$
β_B_LOGWT	0.00	-0.91 – 0.00	0.00	-1.10 – 0.00
Ω_A	3.07×10^{-8}	$2.30 \times 10^{-8} - 1.05 \times 10^{-5}$	1.71×10^{-8}	$2.47 \times 10^{-8} - 3.08 \times 10^{-8}$
Ω_B	1.86×10^{-1}	$5.37 \times 10^{-8} - 2.00 \times 10^{-1}$	1.12×10^{-1}	$7.51 \times 10^{-2} - 1.55 \times 10^{-1}$
Ω_α	6.68×10^{-8}	$2.50 \times 10^{-8} - 5.58 \times 10^{-3}$	6.84×10^{-8}	$2.23 \times 10^{-8} - 1.87 \times 10^{-7}$
Ω_β	1.55×10^{-1}	$8.94 \times 10^{-2} - 1.96 \times 10^{-1}$	2.29×10^{-1}	$1.19 \times 10^{-1} - 2.59 \times 10^{-1}$
BNAB CLEARANCE HALF-LIFE	71.8 days	69.1 – 76.2 days	64.6 days	61.8 – 66.6 days

Visual predictive charts for the models for 10-1074LS and 3BNC117LS are displayed in Figures 6.3.1 a and b respectively, showing a good fit with rare outliers within the 90% prediction intervals. Each dot represents an observed serum measurement value from the time of the most recent bNAb dose, The bottom and top blue shaded areas represent the 90% prediction interval for the 10th and 90th percentiles and the pink shaded

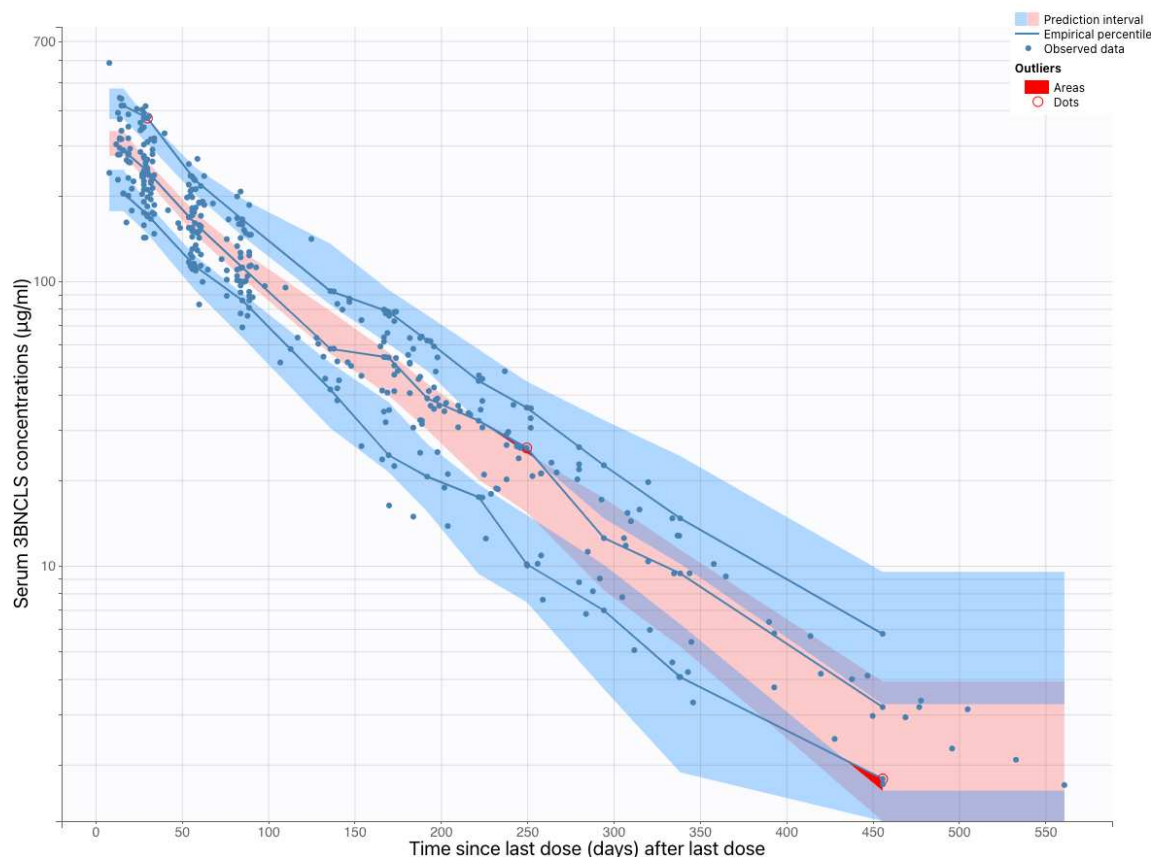
area represent the 90% prediction interval for the median. Outlier data points beyond the prediction intervals are circled in red, and outlier observed 10th, 50th and 90th percentile areas under the curve beyond the prediction intervals are highlighted in red.

Figure 6.3.1 Visual predictive charts for the final two-compartment PK models for a) 10-1074LS and b) 3BNC117LS.

a)



b)



6.3.2 Selection of the best fit pharmacokinetics model

As described above, the serum concentrations were best described using a two-compartment AB model. Other models were trialled using the iterative SAMBA methodology. Both one and two compartment VK models include an association between $\log_{10}wt$ and V , and the one compartment model included a correlation between V and k parameters. The two compartment AB final model included an association between B and $\log_{10}wt$. All models used a proportional error model for the random error effects. No other covariates, including age, viral clade, predicted baseline resistance, or study arm, met the threshold for proposed inclusion in any of the final models. The model with the lowest BIC value was selected, which was the two compartment AB model for both bNAbs. The maximum likelihood AIC and BIC values of all models trialled are reported in Table 6.3.2.

Table 6.3.2. Maximum likelihood values of tested pharmacokinetic models

Model	AIC	BIC
10-1074LS		
1 compartment VK model	3148	3162
2 compartment VK model	3157	3178
2 compartment AB model	2854	2874
3BNC117LS		
1 compartment VK model	3241	3252
2 compartment VK model	3087	3108
2 compartment AB model	3056	3077

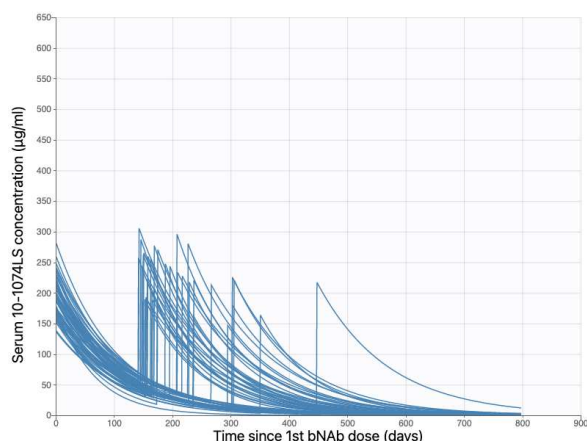
6.3.3 Pharmacokinetics of single and multiple bNAb doses

Of the 56 participants included in the analyses, 34 received a 2nd bNAb dose at least 20 weeks after their first dose. The median time of the 2nd dose was 174 days (25 weeks) from the first dose (range 141 to 447 days) (Figure 5).

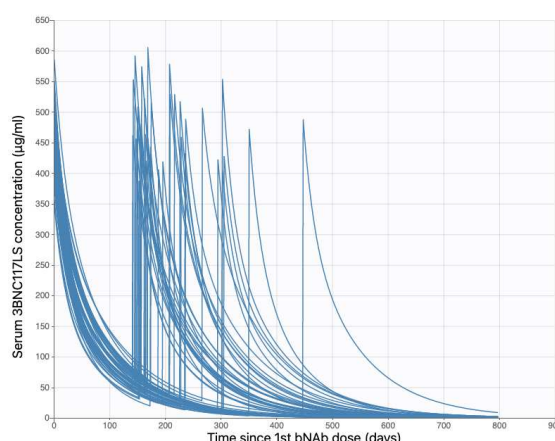
The mean maximum serum bNAb concentrations after the first dose for 10-1074LS, dosed at 10mg/kg, was 195 µg/mL (± 62.8 µg/mL 2SD), and 3BNC117LS, dosed at 30mg/kg, was 454 µg/mL (± 110 µg/mL). After the 2nd dose, the mean maximum serum bNAb concentrations for 10-1074LS was 229 µg/mL (± 82.8 µg/mL) and 3BNC117LS was 484 µg/mL (± 123.2 µg/mL). The serum bNAb concentrations given at the actual dosing times during the RIO study, simulated through to 800 days after the 1st bNAb dose, are shown in Figures 6.3.3.1 a and b for 10-1074LS and 3BNC117LS respectively.

Figure 6.3.3.1. Modelled serum bNAb concentrations of individual participants simulated up to 800 days after the first bNAb dose, with actual dosing times within the RIO study.

a) 10-1074LS



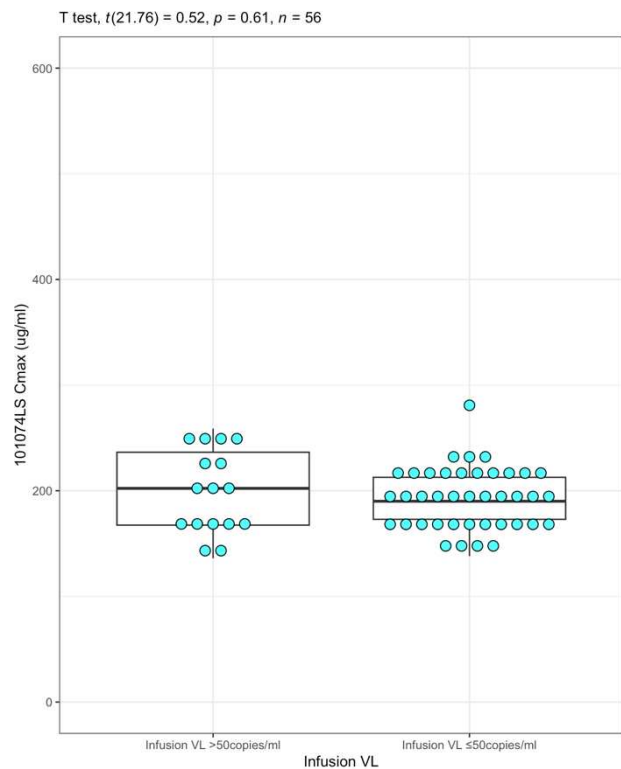
b) 3BNC117LS



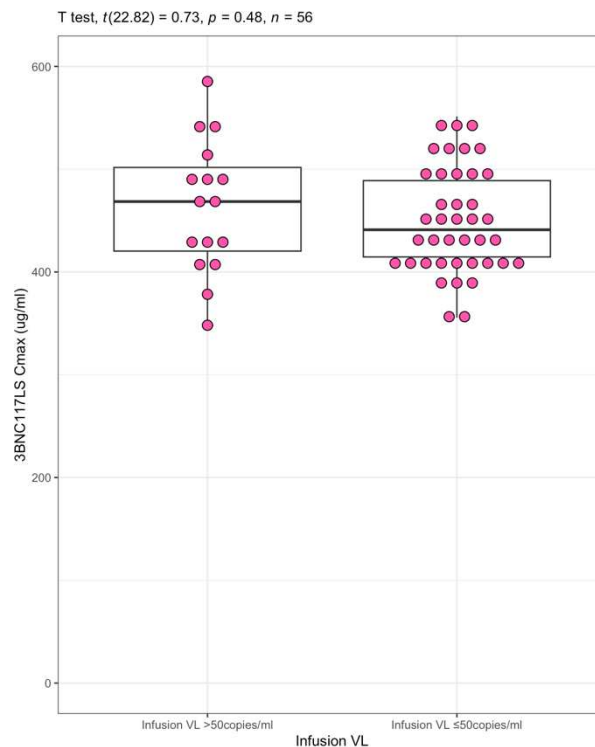
16/25 (64%) of participants in Arm B had a detectable VL >50 copies/mL at the time of their first bNAb infusion and overall 40 participants in both study arms had a plasma HIV VL $\leq$ 50 copies/mL at the time of bNAb infusion. When maximum serum bNAb concentrations after the 1st dose were compared between participants who had a detectable HIV VL at the time of infusion against those who were undetectable (VL $\leq$ 50copies/mL), there was no difference between the two groups for either bNAb (10-1074LS 202 vs 190 $\mu\text{g/mL}$, $p = 0.61$; 3BNC117LS 469 vs 441 $\mu\text{g/mL}$, $p = 0.48$) (Figures 6.3.3.2 a & b). The median VL in the detectable group was 229 copies/mL (range 54 – 32800 copies/mL).

Figure 6.3.3.2 First maximum serum bNAb concentrations (Cmax) stratified by detectable HIV VL at time of infusion for a) 10-1074LS and b) 3BNC117LS.

a) 10-1074LS



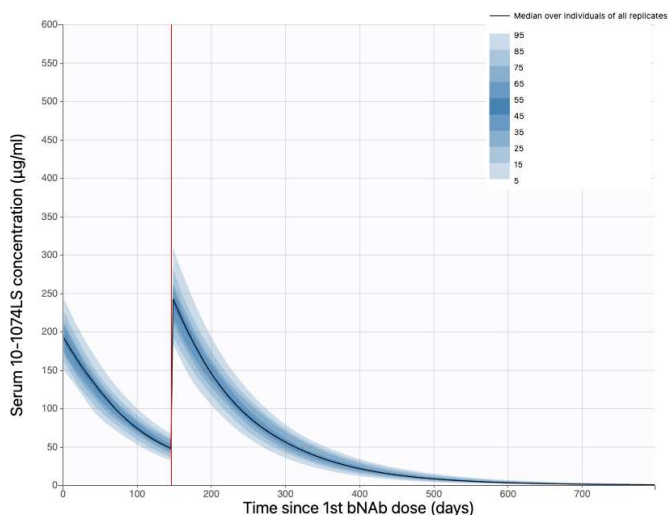
b) 3BNC117LS



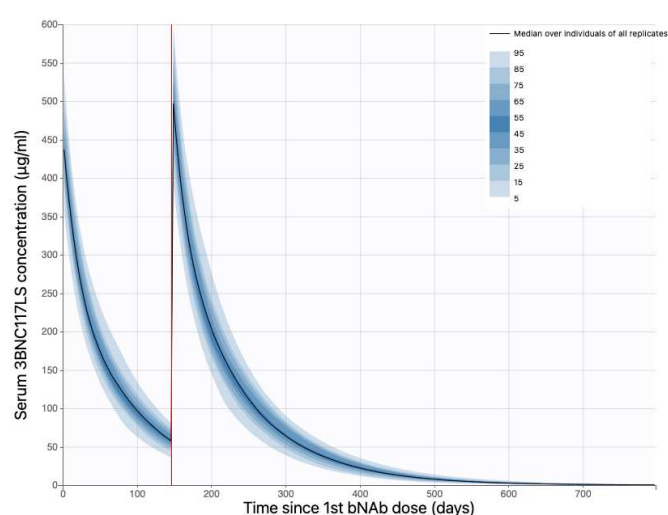
To compare bNAb exposures between the first and second doses and estimate the time of bNAb serum concentrations crosses certain thresholds, simulations of both bNAb were run with a new treatment definition, with the assumption that all participants received another dose equal to the first, at the same time, 147 days or 21 weeks after the first bNAb dose. The prediction distribution charts in figures 6.3.3.3 a & b show the simulated serum bNAb concentrations after two doses 147 days apart.

Figure 6.3.3.3. Simulations of pharmacokinetic profiles after two bNAbs doses 147 days apart a) serum 10-1074LS concentration and b) serum 3BNC117LS concentrations, c) serum 10-1074LS area under the curve d) serum 3BNC117LS area under the curve.

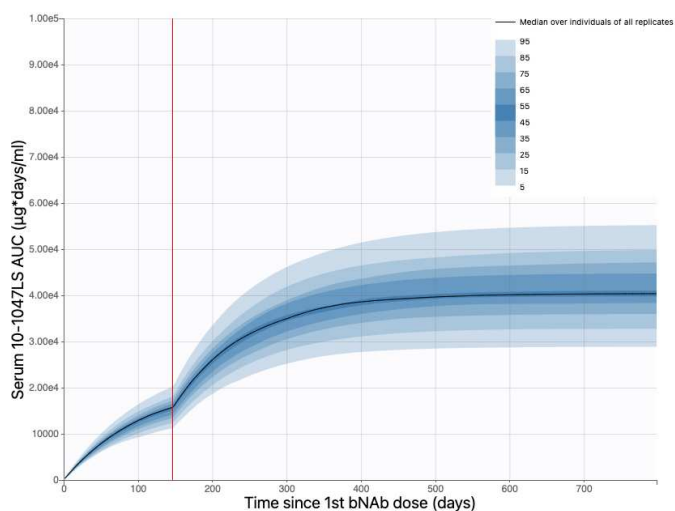
a) Serum 10-1074LS concentrations



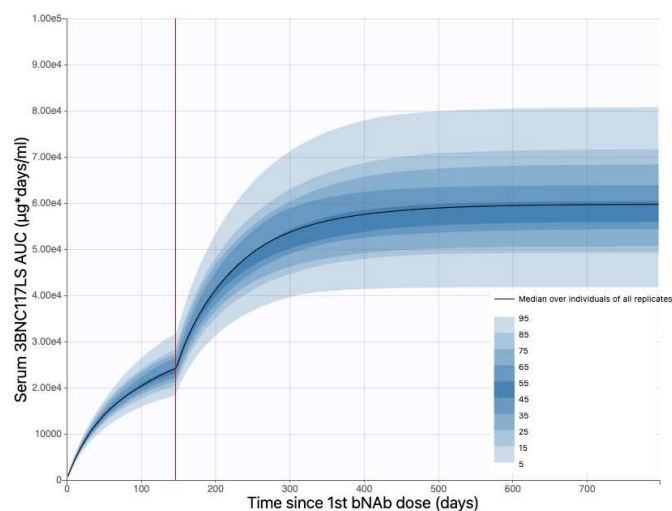
b) Serum 3BNC117LS concentrations



c) Serum 10-1074LS AUC



d) Serum 3BNC117LS AUC



The vertical red line indicates the time of the simulated 2nd dose at day 147 after first dose. Shaded areas represent the 90th prediction interval around the median of all replicates (n=1000).

Under the simulated dosing conditions, the mean maximum serum bNAb concentrations after the 2nd dose given at day 147 for 10-1074LS was 242 µg/mL (± 79.6 µg/mL) and 3BNC117LS was 512 µg/mL (± 130.6 µg/mL). The mean area under the curve (AUC) within 147 days after the 1st and 2nd bNAb dose for 10-1074LS was 15463 µg*days/mL (± 5380 µg*days/mL) and 19248 µg*days/mL (± 7184 µg*days/mL) respectively, and for 3BNC117LS the mean AUC within 147 days after the 1st and 2nd bNAb dose was 24316 µg*days/mL (± 7676 µg*days/mL) and 28682 µg*days/mL (± 9922 µg*days/mL) respectively.

6.3.4 Predicted bNAb rebound concentrations and IC80 values

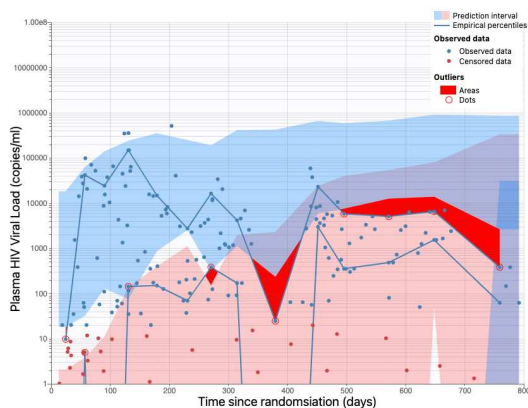
To estimate the time of viral rebound, I tested the direct inhibitory pharmacodynamics model of both bNAbs individually and subsequently in combination, using the equation . Modelling 10-1074LS alone on the viral rebound dynamics resulted in a final model, with a proportional error model, correlations between gamma and IC50, and the baseline resistance and gamma. The final model showed limited fit with multiple outliers (Figures 6.3.4.1a, b), and AIC and BIC values of 4164 and 4178 respectively.

Modelling 3BNC117LS alone resulted similarly in a limited fitted model. The final model described a correlation between E0 and IC50, baseline resistance and gamma, and a proportional error model for the viral loads. The AIC and BIC values were 4329 and 4343 respectively. (Figures 6.3.4.1c, d)

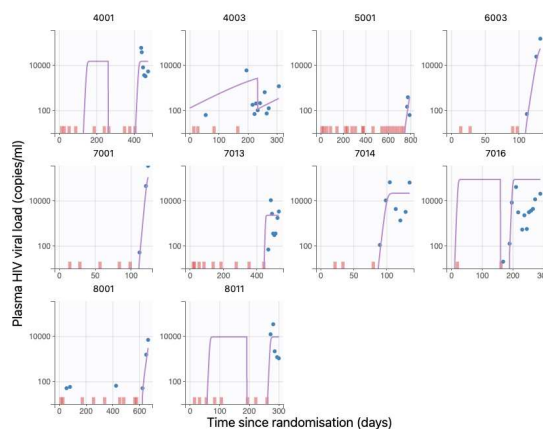
Both bNAbs were modelled together in a combined model. The combined model demonstrated an improved fit compared to the previous individual bNAbs models (Figures 6.3.4.1e, f). The model described a correlation between beta1, B2, and beta 2, and a second correlation between E0 and IC501. The AIC and BIC values were 5422 and 5477 respectively.

Figure 6.3.4.1. a) Visual predictive chart and b) individual fits of selected participants for the final 10-1074LS pharmacodynamic model. c) Visual predictive chart and d) individual fits of selected participants for the final 3BNC117LS pharmacodynamic model. e) Visual predictive chart and f) individual fits of selected participants for the final combined bNAbs pharmacodynamic model.

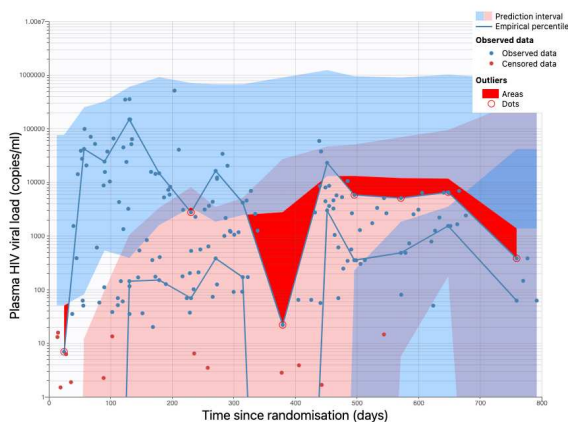
a)



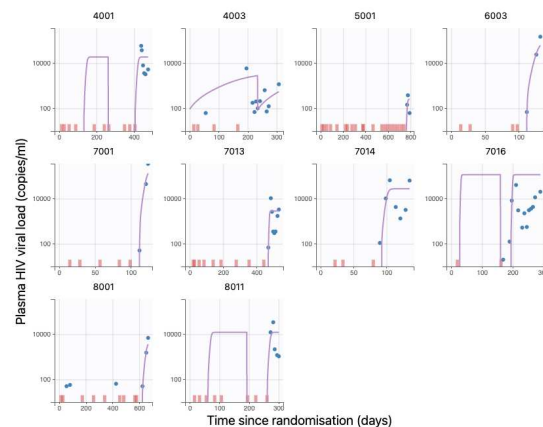
b)



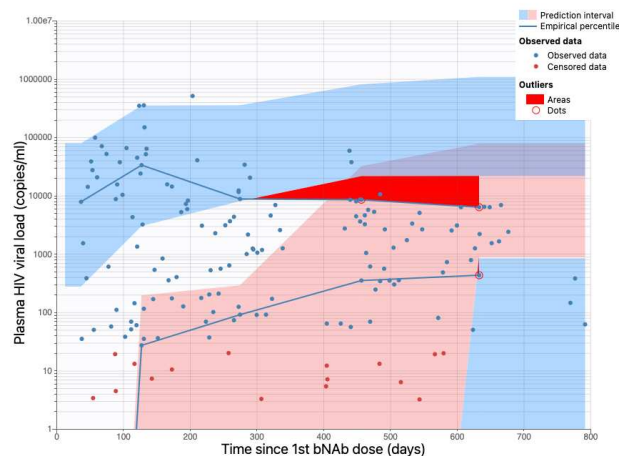
c)



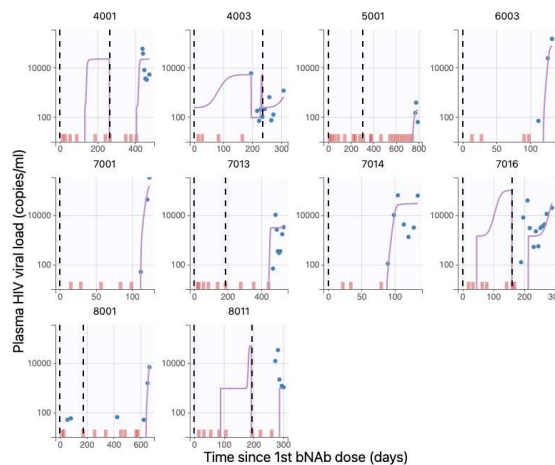
d)



e)



f)



Shaded dark red areas highlighting the outliers to the model, and lighter blue and pink areas representing the 90th prediction intervals of the 90th and 50th percentiles respectively. Imputed censored values are represented by red dots, and uncensored observed viral load values are represented by blue dots. The censored (pink tags) and observed viral load results (blue dots), and the predicted viral load (purple line) for individual participants are shown for individual fits.

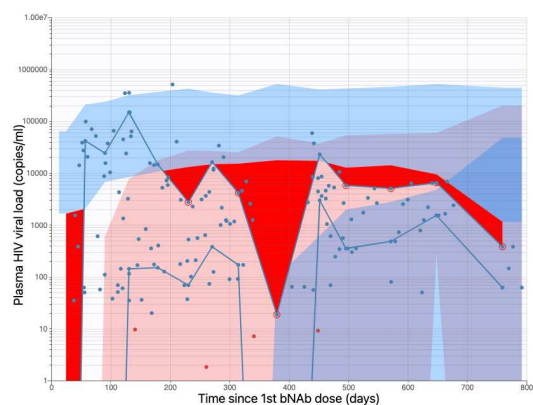
However, the limitation of the above model was such that the IC50s were assumed to be constant throughout the time off treatment. This is unlikely to be true due to the selection pressures exerted by bNAbs on the virus, and earlier studies of bNAbs have shown viral RNA *env* sequence changes with greater predicted resistance to bNAbs at the time of rebound (132,183,231). This may explain the earlier modelled rebound in a number of participants who remained undetectable between the first and second rebound e.g. 4003, 7016, 8011.

Thus, an exponential mutation rate (MR) was introduced in the model equation. The results are shown in Figure 6.3.4.2 a – f. The models for the individual bNAbs alone showed poor fits in the VPCs (AIC were 4214 and 4475 respectively, BIC 4228 and 4494 respectively for 10-1074LS and 3BNC117LS), however, when both bNAbs were combined, the model showed an improved fit and AIC/BIC compared to the earlier combined bNAbs model (AIC 5266 and BIC 5293 vs AIC 5422 and BIC 5477). In this model, there was a correlation between beta 1 and beta 2. The addition of a correlation between the predicted baseline resistance and the IC50s of both bNAbs did not improve the fit of the model (BIC 5650 vs 5293).

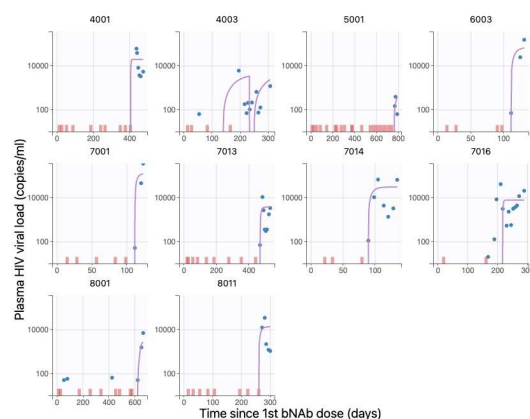
The addition of baseline CD4 proliferation T cell data did not improve the fit of the modelled viral load rebound curves (5427 vs 5293), nor did changing the models from a maximum interaction model to additive or multiplicative models (Additive model BIC 5450, Multiplicative model BIC 5492). The predicted values for the rebound concentrations, IC50 and IC80s, and predicted baseline resistance are shown in Table 6.3.4 (Predicted sensitivity data provided by Penny Zacharopoulou, Frater Lab, University of Oxford, and Marcilio Fumagalli, Nussenzweig group, Rockefeller University). The median modelled bNAbs IC80 at viral rebound >50copies/mL was 106 (interquartile range 27 – 174) $\mu\text{g/mL}$ and 80.9(67.7 – 103) $\mu\text{g/mL}$ for 10-1074LS and 3BNC117LS respectively. Of note, 5/10 (50%) of participants who rebounded were predicted to have developed resistance to 10-1074 from their rebound sequences despite being screened to not have resistance mutations at baseline, but there was no new 3BNC117 predicted resistance at rebound compared to baseline.

Figure 6.4.3.2 a) Visual predictive chart and b) individual fits of selected participants for the 10-1074LS pharmacodynamic model with the addition of a mutation rate for the IC50. c) Visual predictive chart and d) individual fits of selected participants for the modified 3BNC117LS pharmacodynamic model. e) Visual predictive chart and f) individual fits of selected participants for the modified combined bNAbs pharmacodynamic model.

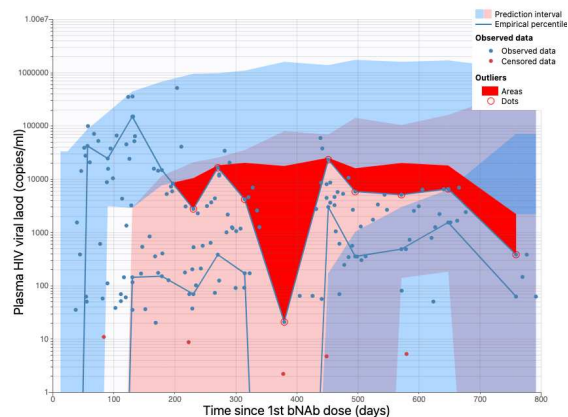
a)



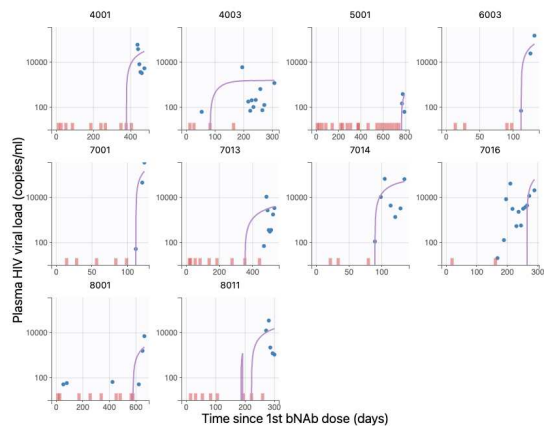
b)



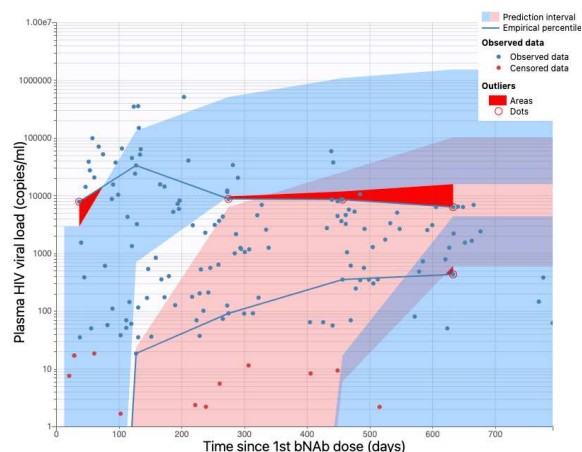
c)



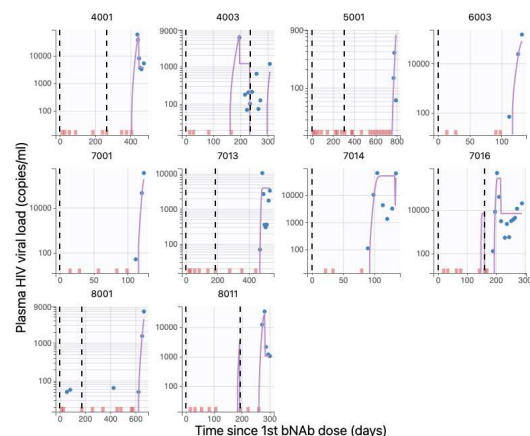
d)



e)



f)



Shaded dark red areas highlighting the outliers to the model, and lighter blue and pink areas representing the 90th prediction intervals of the 90th and 50th percentiles respectively. Imputed censored values are represented by red dots, and uncensored observed viral load values are represented by blue dots. The censored (pink tags) and observed viral load results (blue dots), and the predicted viral load (purple line) for individual participants are shown for individual fits.

Table 6.3.4 Modelled IC50, IC80 values, rebound times and bNAb concentrations, and predicted bNAb sequence resistance.

BASELINE									
PARTICIPANT ID	Modelled 10-1074LS IC50	Modelled 3BNC117LS IC50	Modelled Baseline 10-1074LS IC80	Modelled Baseline 3BNC117LS IC80	Baseline predicted 10-1074LS sensitivity	Baseline predicted 3BNC117LS sensitivity			
A1	1.46269	29.9339	5.85076	119.736	Sensitive	Sensitive			
A2	0.973015	32.3226	3.89206	129.29	Sensitive	Sensitive			
A3	0.676843	13.8836	2.70737	55.5344	Sensitive	Sensitive			
A4	0.113623	13.7435	0.45449	54.974	Unamplifiable	Unamplifiable			
A5	1.0077	13.6729	4.0308	54.6916	Sensitive	Sensitive			
A6	3.23185	14.9759	12.9274	59.9036	Sensitive	Sensitive			
A7	1.11012	24.9162	4.44048	99.6648	Unamplifiable	Unamplifiable			
A8	1.27211	18.148	5.08844	72.592	Sensitive	Sensitive			
A9	1.80415	18.1018	7.2166	72.4072	Sensitive	Resistant			
A10	0.330043	20.5992	1.32017	82.3968	Sensitive	Sensitive			
A11	5.08228	14.4318	20.3291	57.7272	Sensitive	Sensitive			
A12	0.65749	16.3322	2.62996	65.3288	Sensitive	Sensitive			
A13	0.654133	18.1387	2.61653	72.5548	Sensitive	Sensitive			
A14	1.66765	5.7543	6.6706	23.0172	Sensitive	Sensitive			
A15	0.155994	17.2675	0.62398	69.07	Sensitive	Sensitive			
A16	0.957613	14.4517	3.83045	57.8068	Sensitive	Sensitive			
A17	1.93003	15.793	7.72012	63.172	Sensitive	Sensitive			
A18	0.676608	14.5997	2.70643	58.3988	Mixed	Sensitive			
A19	4.48908	18.8497	17.9563	75.3988	Sensitive	Resistant			
A20	0.323969	16.0193	1.29588	64.0772	Sensitive	Sensitive			
A21	0.208435	13.5165	0.83374	54.066	Sensitive	Sensitive			
A22	0.593922	15.6177	2.37569	62.4708	Sensitive	Resistant			
AT THE TIME OF REBOUND									
	Modelled 10-1074LS IC50	Modelled 3BNC117LS IC50	Modelled 10-1074LS IC80	Modelled 3BNC117LS IC80	Rebound predicted 10-1074 sensitivity	Rebound predicted 3BNC117 sensitivity	Modelled rebound time since 1st bNAb dose(days)	Modelled rebound 10-1074LS concentration (µg/mL)	Modelled rebound 3BNC117LS concentration (µg/mL)
A1	24.0937	32.2093	96.3748	128.837	Sensitive	Sensitive	144	39.7703	56.5198
A2	22.5452	37.7966	90.1808	151.186	Sensitive	Sensitive	115	62.5312	90.824
A3	9.86178	26.2124	39.4471	104.85	Resistant	Sensitive	310	11.7984	13.8169
A4	1.33699	16.8438	5.34796	67.3752	Not available	Not available	451	34.2595	43.5241
A5	91.3967	15.493	365.587	61.972	Not available	Not available	214	144.484	163.228
A6	67.8802	15.5149	271.521	62.0596	Sensitive	Sensitive	45	122.04	193.71
A7	30.1643	26.7911	120.657	107.164	Resistant	Sensitive	84	48.4885	44.2497
A8	34.3697	19.9229	137.479	79.6916	Not available	Not available	122	52.7212	74.647
A9	27.6794	18.4486	110.718	73.7944	Sensitive	Resistant	35	113.616	225.205
A10	7.9927	32.5988	31.9708	130.395	Not available	Not available	570	11.0693	14.897
A11	25.4348	15.7105	101.739	62.842	Sensitive	Sensitive	77	90.5062	111.003
A12	22.8958	22.7077	91.5832	90.8308	Not available	Not available	320	56.5599	70.5549
A13	36.0624	23.6575	144.25	94.63	Not available	Not available	407	69.1472	99.7232
A14	6.76374	6.69167	27.055	26.7667	Not available	Not available	162	27.7601	32.3819
A15	2.32753	32.5935	9.31012	130.374	Not available	Not available	763	4.13366	3.23994
A16	45.6821	15.4302	182.728	61.7208	Resistant	Sensitive	117	77.7254	94.2062
A17	52.3064	17.2039	209.226	68.8156	Resistant	Sensitive	116	67.5821	87.5448
A18	9.202	21.7622	36.808	87.0488	Not available	Not available	470	10.5208	7.1712
A19	36.525	20.5316	146.1	82.1264	Resistant	Resistant	93	51.9271	47.6755
A20	121.819	18.5762	487.276	74.3048	Not available	Not available	195	158.944	235.083
A21	1.92263	23.9954	7.69052	95.9816	Not available	Not available	626	4.05988	3.53457
A22	79.3785	19.2082	317.514	76.8328	Not available	Not available	262	120.082	118.194

Under this model, rebound times and serum rebound concentrations could be predicted for participants who experienced viral rebound. However, there the predicted bNAb IC50s poorly correlated with predicted rebound sensitivities from the envelope sequences (Figures 6.3.4.3 a - d). The modelled 10-1074LS baseline IC50s were predictive of the protocol-defined rebound times, but not the rebound 3BNC117LS IC50 values (Figure 6.3.4.4 a, b).

Figure 6.3.4.3 Comparisons between the modelled IC50 values and predicted bNAb envelope resistance using the Rockefeller algorithm. a) Baseline 10-1074LS IC50 b) Rebound 10-1074LS IC50 c) Baseline 3BNC117LS IC50 d) Rebound 3BNC117LS IC50

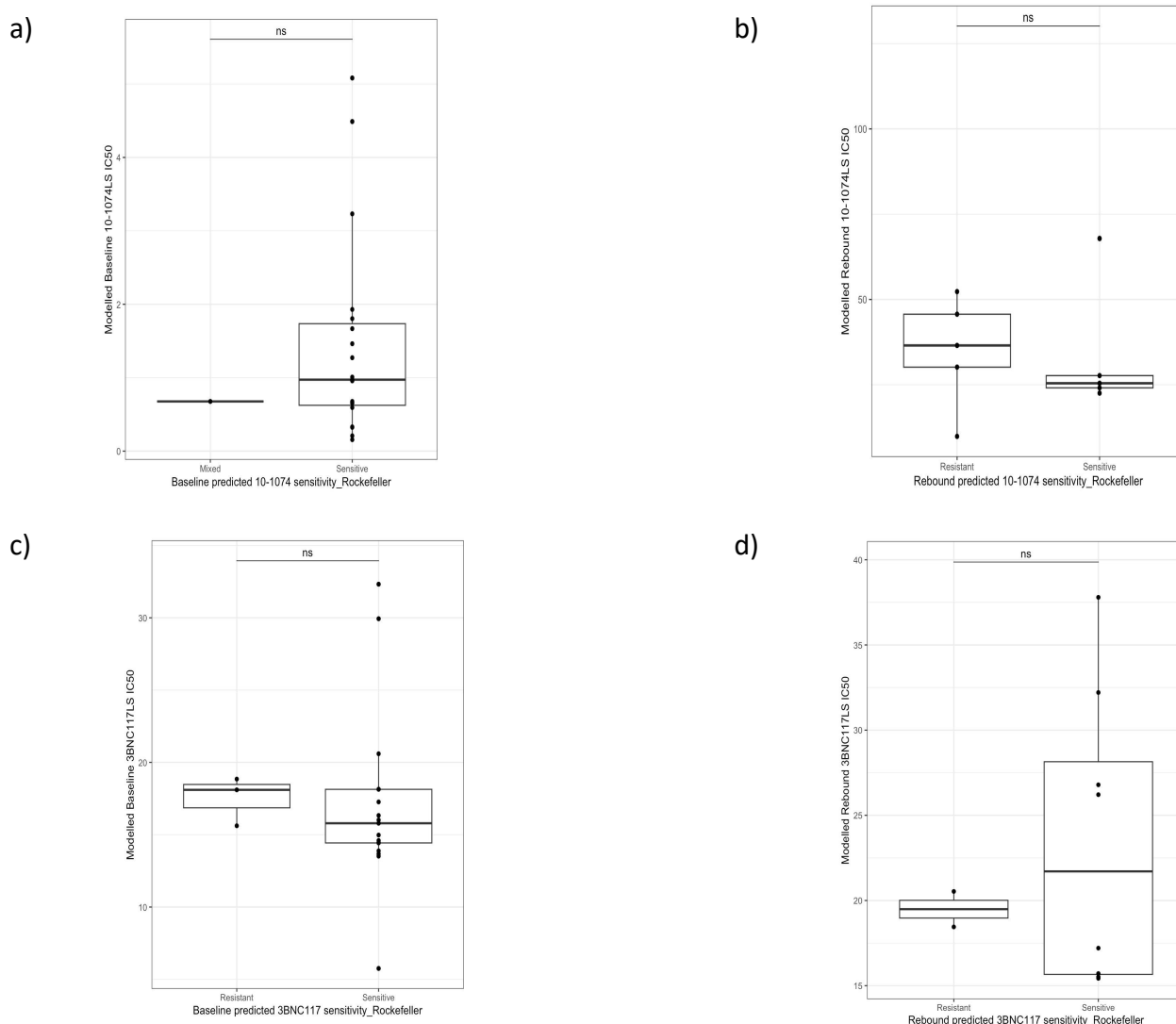
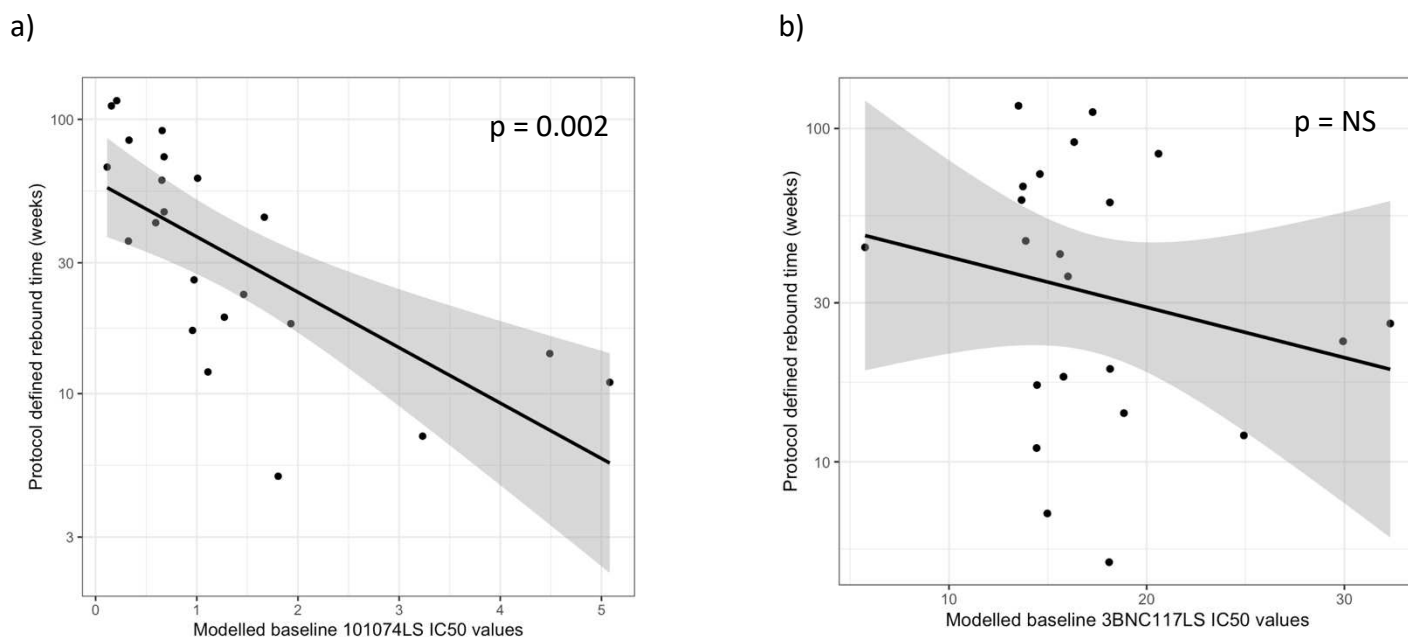
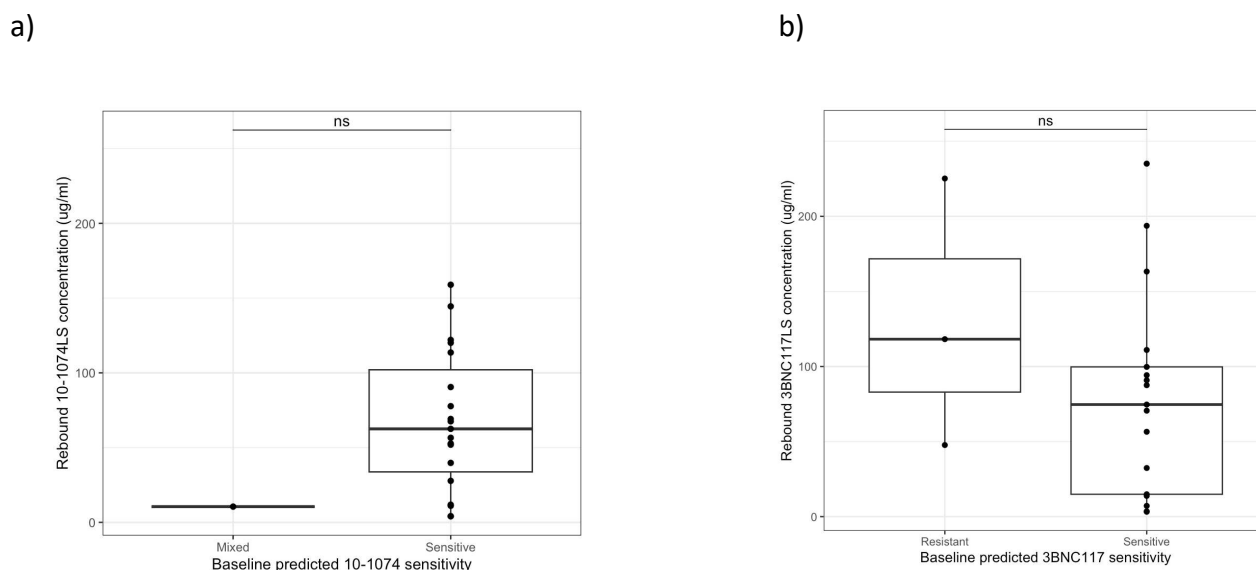


Figure 6.3.4.4 Comparison of modelled baseline IC50 values to protocol-defined rebound times for a) 10-1074LS and b) 3BNC117LS



The median rebound concentrations for 10-1074LS and 3BNC117LS in the participants who rebounded in the RIO intervention arm were 54 $\mu\text{g/mL}$ (range 4 - 159 $\mu\text{g/mL}$) and 73 $\mu\text{g/mL}$ (range 3 – 235 $\mu\text{g/mL}$) respectively. The rebound concentrations did not differ by sequence-predicted bNAb sequence sensitivities (Figure 6.3.4.5 a, b).

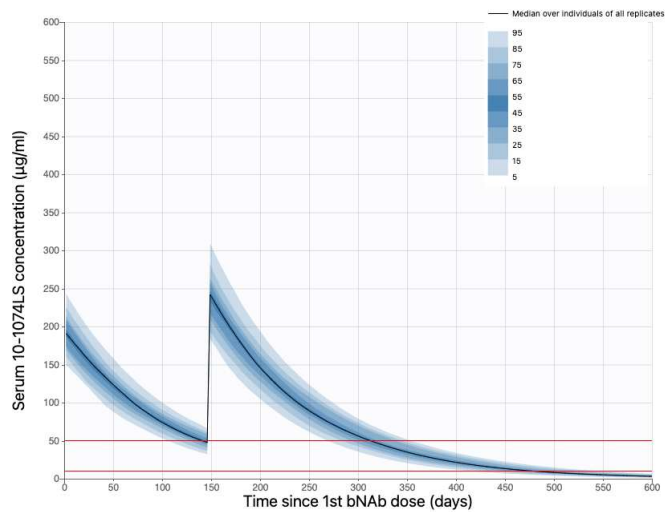
Figure 6.3.4.5. Comparison of modelled rebound bNAb sensitivities to sequence-predicted bNAb sensitivities for a) 10-1074LS and b) 3BNC117LS



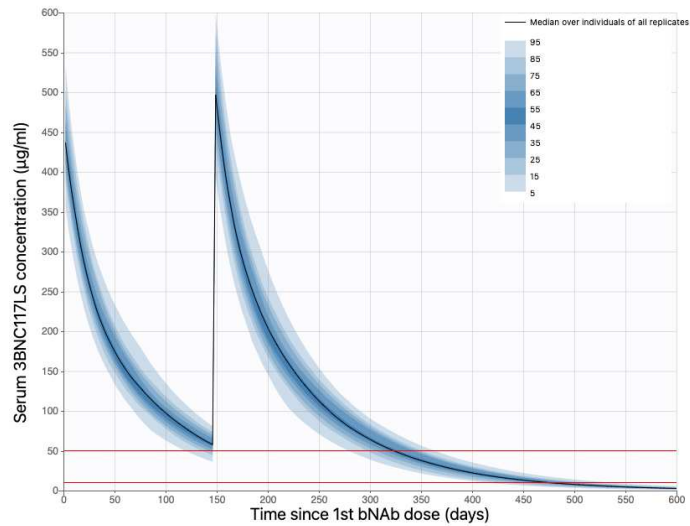
To understand the duration of effect of the bNAbs, the data from the prior PK simulations were used to determine the time from 1st dose to 50 µg/mL or 10 µg/mL thresholds. The median time after two doses 147 days apart which the serum bNAb concentrations dropped below 10 µg/mL was 340 days (90% PredInt 260 - 413 days) and 334 days (90% PredInt 270 - 400 days) after the 2nd dose for 10-1074LS and 3BNC117LS respectively. However, when a threshold of 50 µg/mL was used, the median time to dropping below the threshold was 161 days (90% PredInt 86 - 208 days) and 182 days (90% PredInt 104 - 223 days) for 10-1074LS and 3BNC117LS respectively. Between the first and second dose, the mean time below the 50 µg/mL threshold for 10-1074LS was 12.1 days ($\pm 2SD$ 31 days) compared to 4.4 days ($\pm 2SD$ 17.2 days) for 3BNC117LS (Figures 6.3.4.6 a & b). Neither bNAbs dropped below 10 µg/mL between the first and the second doses.

Figure 6.3.4.6. Simulated serum bNAbs concentrations with serum concentrations thresholds (red lines) at 10 and 50 $\mu\text{g}/\text{mL}$ for a) 10-1074LS and b) 3BNC117LS.

a) 10-1074LS



b) 3BNC117LS



6.4 Discussion

In this chapter, I report the pharmacokinetics profile of two LS-bNAbs, 10-1074LS and 3BNC117LS, when used in combination in participants in a large randomised controlled trial (the RIO study) after a two-dose schedule to determine population parameter estimates, elimination half-lives, maximum serum concentrations, and areas under the curve using a two-compartment model. I also described a direct inhibitory model to explain the effect of bNAbs on viral rebound dynamics and estimate rebound serum bNAb concentrations, as a measure of the therapeutic threshold of bNAbs, from the participants who experience viral rebound after receiving bNAbs.

The population parameters of the two bNAbs in this study are mostly consistent with previously reported parameters in earlier studies (133), and the kinetics of a second dose of bNAbs are similar to a single dose, apart from the half-life of 10-1074LS being slightly lower than previously reported in a pooled analysis of people without HIV receiving LS-bNAbs. (71 days vs 81 days). The shortened half-lives seen in people living with HIV is consistent with previous reports of the lower half-lives of bNAbs in people living with HIV compared to people without HIV (230,231,356). The extended half-lives of the bNAbs and residual serum bNAb exposure at the time of the second dose results in the higher second maximum serum bNAb concentrations after the second dose, and under the simulated dosing at day 147, the mean serum AUC was 24% and 18% higher in the 147 days after the 2nd dose compared to the 1st dose for 10-1074LS and 3BNC117LS respectively. The presence of viraemia at the time of bNAb infusion did not appear to impact the maximum serum bNAb concentrations alleviating concerns of a substantial ‘antigen sink’ effect where the viral antigens present lowered the amount of bNAbs in the serum due to formation of immune complexes. However the caveat remains that the median viraemia was low at 229 copies/mL as most participants had restarted ART prior to receiving bNAbs as per the study protocol. Reassuringly, there have been no ADAs detected post-infusion, which ameliorate concerns that multiple doses of LS-bNAbs may induce ADAs, increasing the clearance and shorten the half-lives of these LS-bNAbs, limiting their effectiveness.

The dosing strategy of the RIO study was determined based on the differences in elimination half-lives of the two bNAbs, to minimise the duration of a monotherapy tail under the assumption that the bNAbs remain effective at concentrations above 10 µg/mL. At this threshold, this strategy has been successful as ensuring the mean time both bNAbs cross the threshold is 48 weeks after the last dose. However, for those people who reached viral rebound after bNAb dosing in the intervention arm ($n = 22$), viral rebound has been observed at much higher mean serum bNAb concentrations of 54 to 73 µg/mL for 10-1074LS and 3BNC117LS respectively in this study. These findings reflect the ‘therapeutic thresholds’ associated with loss of viral control below these levels for either bNAbs are likely to be higher than 10 µg/mL as suggested from a previous study (132). There was a greater frequency of 10-1074 resistance-associated *env* mutations compared to 3BNC117 observed in the viral sequences at the time of rebound despite pre-screening for predicted 10-1074 resistance, suggesting that under this dosing strategy, despite the higher levels of 3BNC117LS at the time of rebound, 3BNC117LS did not prevent the selection of 10-1074 resistance. Although it remains unclear what the true therapeutic threshold of the bNAbs are, if a higher threshold of 50 µg/mL is used, the mean duration 10-1074LS drops below the threshold was 25 days earlier than 3BNC117LS after the second dose given at day 147, however even between the two doses, 10-1074LS dropped below the threshold for 8 days longer than 3BNC117LS. Dosing strategies for future studies should be modified to keep the trough serum bNAb concentrations above the threshold which bNAb resistance is observed.

When modelling the dynamics of bNAbs concentrations on the viral rebound effect, I was able to build a model incorporating both bNAbs serum concentrations, and using the time to rebound data to fit a direct inhibitory model to estimate the time of viral rebound >50copies/mL.

There have been previous attempts to model the pharmacokinetics and pharmacodynamics of bNAbs, however, most of these studies have been in the context of HIV-prevention studies (357,359,360), with estimation of inhibitory quotients and ID50 values for the neutralisation of a known panel of virus variants. However the extent of existing virus panels are unable to cover all possible viral variants that participants in

the HIV prevention setting may be exposed to (360). Due to the limited numbers of LS-bNAb studies including people living with HIV with adequate time-to-rebound data, there has been few studies interrogating the therapeutic thresholds of these LS-bNAbs when used for treatment. In therapeutic trials such as the RIO study, where longitudinal serum bNAb concentrations and plasma viral load measurements are available, this allows the modelling of the inhibitory effect of the bNAbs on viral replication and the estimated therapeutic thresholds below which bNAbs are no longer effective in maintaining viral suppression. The median serum bNAb concentrations at which rebound is observed in the RIO study has been shown to be substantially higher than the previously suggested 10 µg/mL threshold, with implications for future bNAb dosing strategies, however, there was a wide range of bNAb concentrations at which viral rebound was observed. This may be explained by rising overall IC80 values over time. Some participants experienced viral rebound early after a second dose even if they had remained virally suppressed for a longer period of time between the first and second dose. This may be due to selection of more resistant HIV variants. This is consistent with observed data, where half the participants with no evidence of baseline 10-1074 predicted resistance, were predicted to have resistant 10-1074 in all sequences when plasma HIV RNA was sequenced from timepoints after they rebounded. Whether this was due to *de novo* mutations, or selection of previously missed minority resistant strains, the overall effect is an increase in resistance and therefore higher IC80 bNAb values over time. One implication is there is likely ongoing viral replication and selection, despite the apparent undetectable status of many participants after bNAb dosing. Although bNAbs are increasingly understood to inhibit multiple steps in the viral replication pathway, not just through neutralisation of virus particles and blocking cell entry, but also intracellularly at sites of endosomes, and prevention of viral budding(361,362). However, viral replication may still be possible through mechanisms such as cell to cell transmission, which provide a pathway of resistance to bNAb-inhibition of viral replication (363).

The evolution of bNAb IC80 over time may also explain the higher bNAb concentrations observed at the time of viral rebound, compared to a previous report suggesting no viral rebound was seen until bNAb concentrations dropped below 10 µg/mL (132). In that study, non-LS 10-1074 and 3BNC117 were used and the protocol defined ATI was up to 30 weeks, a shorter duration compared to the RIO study, where the open-ended ATI meant participants could potentially continue remaining off treatment for the duration of the study, with some participants having remained off ART for nearly three years at the time of writing. Further in vitro studies to confirm the presence of ongoing viral replication in participants who remain suppressed after bNAb dosing, however, if viral replication is present, the implications for the development of resistance, and estimations of effective bNAb therapeutic thresholds.

However, the modelled IC50 and IC80 values do not appear to correlate with in-silico bNAb predictions from the viral RNA or proviral DNA *env* sequences. There may several explanations: there is still no gold standard algorithm or assay for predicting bNAb sensitivity, and algorithms for predicting 10-1074 sensitivity may be more reliable than 3BNC117 (237). The modelled IC80 values were based on the clinical outcomes of viral rebound, which may not be reflective of the IC80 values measured in vitro through neutralisation assays, as the effect of bNAbs may not be explained by neutralisation alone, but other non-neutralising binding or Fc-mediated effector functions such as ADCC, or priming of cellular responses such as HIV-specific CD8 responses (364). These findings further emphasises that the mechanisms of bNAbs as an immunotherapeutic agent may not depend on a direct neutralisation or inhibition of viral replication effect in the same manner as traditional small-molecule ART drugs, but further analyses of the dynamics of bNAbs on viral outcomes have to consider additional effects such as the enhancement of HIV-specific cellular responses.

There were limitations to this analysis. Firstly, the analysis was only limited to participants who experienced viral rebound, as participants who did not experience rebound at the time of the analysis may have different mechanistic factors explaining viral control not accounted for in this model, such as other non-neutralising

effects of the bNAbs (364). However, this inhibitory model appears to explain the viral dynamics of those people who rebound due to the loss of inhibitory effect with decreasing concentrations of the bNAbs. The bNAb interaction model may not fully account for other interactions between the two bNAbs. However, although other models have been described, including the 'Bliss-Hill' model by Mayer et al. (357), a 'best-case' multiplicative model where bNAbs provide complimentary coverage to cover the missing breadth of a single bNAb, when this was modelled under the inhibitory model described, there was no improved model fit to the observed data. There is also a lack of neutralisation data and limited rebound HIV sequence-predicted bNAb sensitivity data to validate this model and the modelled IC80 values.

The analyses also only included people who experienced viral rebound, and as discussed in the previous chapter, there were a sub group of participants ($n = 6$) who received bNAbs remained virally suppressed off ART even beyond the predicted duration of bNAb in serum. These participants may be demonstrating a post-bNAb 'vaccinal' effect as postulated by previous studies (178,179,243). Consideration of other non-bNAb factors in addition to the bNAb pharmacokinetics may be required to build a model to explain the mechanism of action of bNAbs in these individuals. These may include HIV-specific cellular responses, presence of HIV resistance-associated mutations, HIV latent reservoir size, viral fitness and replication rates. More in-depth mechanistic models may consider factors such as antibody-antigen binding and dissociation rates, target saturation, rates of viral replication and reactivation of HIV-containing latent reservoir cells. Different models of clearance of virus-antibody immune complexes have been described using data from a study of VRC01 monotherapy in people living with HIV (365), and these models may be further investigated with the two LS-bNAbs used in RIO.

In summary, bNAbs given in combination to virally suppressed people diagnosed early in HIV infection in the RIO study exhibit extended pharmacokinetics profiles similar to previously described. Of those participants in the RIO who experienced viral rebound, this occurred at serum bNAb concentrations higher than 10 $\mu\text{g/mL}$, suggesting that the true therapeutic concentrations is higher than previously suggested, and may vary

depending on an individual's baseline viral resistance profile as well as immune factors. The effectiveness of bNAbs may also depend on penetration into tissue reservoirs at adequate levels to prevent viral replications, and this will be explored in the next chapter describing the pharmacokinetics and impact of bNAbs within rectal tissue.

Chapter 7.

Understanding the pharmacokinetics and impact of bNAbs in gut tissue compartments

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7.1 Introduction

The potential of the LS-bNABs due to their extended half-lives and serum pharmacokinetics have been discussed in the previous chapter. However, the LS-bNABs may have further benefit with regards to tissue penetration as well. The tissue distribution of FcRn have been postulated enhance delivery of these LS-bNABs to increase concentrations in tissue compared to their non-LS variants (352) . A study of the non-LS bNAB VRC01 was shown to be 10-fold lower in tissue compared to paired serum samples in HIV-negative individuals (229), but it has not been proven that LS antibodies are present at higher concentrations in tissue, for the use of bNABs to target tissue-associated HIV reservoirs.

However, as discussed in chapter 1, antiretrovirals and immunotherapeutics such as bNABs may penetrate tissue sites at different levels compared to blood (229,279), with implications for using these interventions to target the tissue-associated HIV reservoir. Compartmentalisation of viral replication and development of resistance in tissue sites may be a concern, for example in the gut. If bNABs exposure in tissue do not reach adequate levels to prevent viral replication or eliminate reservoir cells.

In HIV-negative individuals receiving the non-LS variant bNAB VRC01, bNAB tissue concentrations in vaginal and rectal tissues were 10-fold lower than in paired serum samples, and breakthrough infections were observed in an ex-vivo challenge study of tissue explants due to sub-protective bNAB concentrations. However, data around tissue bNAB penetration from people living with HIV receiving bNABs for HIV cure-related studies are lacking.

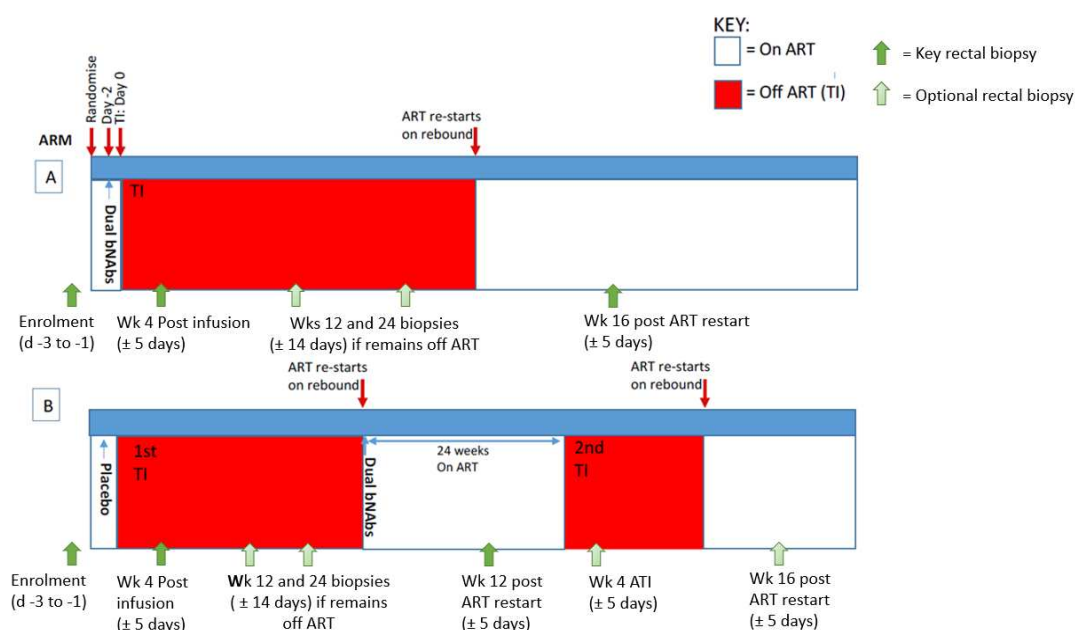
To understand the pharmacokinetics and impact of bNABs within gut tissue, I compared the serum and gut tissue bNAB exposures from rectal tissue, using two-compartment pharmacokinetic modelling, and described changes in rectal tissue-associated HIV reservoir between baseline and at least 12 weeks after receiving bNABs.

7.2 Methods

7.2.1 Rectal tissue sample collection and measurements

Longitudinal rectal biopsies and paired serum samples were collected as part of a Gut sub-study from participants in the RIO study. Both the RIO study and the Gut sub-study protocol are described in chapter 2. Briefly, participants who were enrolled in the RIO study, were invited for up to seven rectal biopsies over the duration of their study participation – three key biopsies taken at baseline, week 4 after dosing, and at the end of the study for Arm A participants, or 12 weeks after restarting ART for arm B participants. Participants were also invited for biopsies at weeks 12, 24 after dosing during the first ATI if they remained off ART, and week 4 during the 2nd ATI and at the end of study after the 2nd ATI for arm B participants (Figure 7.2.1).

Figure 7.2.1 The RIO Gut sub-study design and sampling schedule.



Blood samples were centrifuged and the supernatant collected for serum samples, and tissue samples were snap frozen in liquid nitrogen without cryopreservative media for measurement of tissue bNAbs, or with cryopreservative media, for cell sorting, and both types of samples stored frozen at -150°C until analysis.

Tissue and serum bNAb measurements: Frozen serum and tissue samples were sent to the Tomaras lab, Duke University, for bNAb measurements, performed by Dr Kelly Seaton. The bNAbs were measured by anti-

idiotype ELISA specific for both bNAbs in serum, and ultra-sensitive SMCxPro immunoassay in homogenised tissue. The protocol for both assays are described in chapter 2.

Tissue bNAb concentrations were normalised to total protein concentrations measured using the QuantIT kit (Thermofisher), as well as total tissue IgG antibodies measured using a custom binding antibody multiplex assay using rabbit anti-human IgG light chain capture, and anti-human IgG detection. Serum bNAb concentrations were normalised to a reference mean serum total protein concentrations as well as reference mean serum IgG concentrations obtained from the clinical biochemistry lab. Friedman's and Dunn's tests was used to compare specific activity values normalised to total protein or total IgG.

7.2.2 Population parameters estimation and comparison of paired serum and rectal bNAb exposures using two compartment modelling

Population and individual PK analyses of serum and tissue bNAb measurements were estimated using two-compartment modelling with the MonolixTM software. As described in the previous chapter, the model included a central compartment (V1) which were mapped onto the serum bNAb measurements, a peripheral compartment (V2) which were mapped onto the rectal tissue bNAb measurements, with a rate of transfer to and from represented by the constants k12 and k21, and a linear elimination with a rate constant k. Ac is the amount of bNAb in the central compartment, Ap is the amount of drug in the periphery (tissue), Cc is the serum bNAb concentration, and Ct is the tissue bNAb concentration.

Equations:

$$dAc = \text{input} - k \cdot Ac - k_{12} \cdot Ac + k_{21} \cdot Ap$$

$$ddt_Ac = dAc$$

$$dAp = k_{12} \cdot Ac - k_{21} \cdot Ap$$

$$ddt_Ap = dAp$$

$$Cc = Ac/V1$$

$$Ct = Ap/V2$$

To compare bNAb exposure between compartments and describe mean cohort values, simulations with 1000 replicates were run using Simulx™ of the conditional means values of each individual participants. Two doses given 147 days (21 weeks) apart were simulated under these conditions. Mean peak serum and tissue bNAb were reported as bNAb specific activity (SA) normalised to both total protein (TP) and IgG, and the mean serum to tissue bNAb AUC ratios at week 147 prior to the 2nd dose, were reported, with two standard deviations (2SD). Values normalised to total protein represent the fraction of bNAbs compared to all proteins that cross the basement membrane from blood to tissue and may reflect the overall tissue transfer of proteins through both passive hydrostatic pressure, and active transcytosis. SA values normalised to IgG represent the fraction of bNAbs that penetrate the tissue compared to other IgG antibodies, and may measure the degree of affinity of the LS-bNAb towards tissue compared to autologous antibodies.

7.2.3 Measurement of tissue total and intact HIV proviral reservoir

The homogenisation, sorting and measurements of tissue-associated HIV proviral reservoir was done in collaboration with Dr Marion Pardons and Professor Linos Vanderkerckhove at the University of Ghent. Protocols for the isolation and FACS of mucosal CD45+ cells from rectal biopsies, and the measurement of the total and intact HIV proviral reservoir are described fully in chapter 2. Briefly, pooled frozen rectal tissue biopsies were thawed at 37 °C in a water bath, and washed before being digested in a two-step process. Firstly, the biopsies were incubated in 1% EDTA/HBSS with a magnetic stirrer set on 350 RPM, at 37 °C for 15 minutes. Subsequently, the biopsies were then washed with 2% FBS/PBS, before being transferred to 4mL of RPMI-1640 media containing 25mM Hepes solution, 500µL of collagenase IV (Sigma C5138) and 500µL of 10mg/mL collagenase VIII (Sigma C2139). The biopsies were then incubated with a magnetic stirrer set at 500RPM, at 37 °C for 30 minutes.

After digestion, the contents were passed through a 40 µm filter into a 50mL falcon tube on ice, and the container washed with cold 2% FBS in PBS. The falcon tube was then centrifuged at 1700rpm for 7 minutes at 4 °C, followed by the removal of the supernatant. The remaining cell pellet resuspended in 400µL of 2%PBS in FBS and further centrifuged at 2700RPM for 5 minutes at 4 °C, before removing the supernatant, and

resuspending the cell pellet in 200 μ L of 1 in 1000 LiveDead dye in PBS solution and incubated under aluminium foil for 20 minutes.

The sample was then washed with 800 μ L 2%FBS/PBS, centrifuged at 2700RPM for 5 minutes at 4°C, before removing the supernatant. The cell pellet was incubated with the 200 μ L of solution containing the remaining staining panel (see Table 2.4.2, Chapter 2) under aluminum foil for 25 minutes at 4°C. The cells were subsequently washed with 800 μ L of 2%FBS/PBS, centrifuged at 2700RPM for 5 minutes to remove the supernatant, and resuspended in 100 μ L of 4% PFA for 15minutes at RT for fixation. The samples were then washed with 800 μ L of 2%FBS/PBS, centrifuged at 3500RPM for 5 minutes to remove the supernatant, and transferred to a FACS tube for cell sorting. All samples were acquired on the BD FACSAria™ Fusion Flow cytometer with a 100 μ M nozzle in yield mode at the Vandekerckhove lab. Samples were sorted for live CD45+ cells based on the following gating strategy: Total cells by forward and side scatter -> CD45+ cells -> Single cells by width against area of forward scatter -> live cells (Figure 2.4.2, Chapter 2). Data were analysed using FlowJo Software v10.10.1 (BD Life Sciences). The sorted cells were centrifuged at 140000RPM and the dry pellet was incubated in DirectPCR lysis reagent at 45 minutes at 85°C.

Total and intact proviral DNA reservoir was measured from the direct cell lysate by a multiplex 5-probe digital PCR assay described by Delporte et al. (86). Probes targeting the LTR, , *gag*, *pol*, *env*, and packaging site (ψ) regions of the HIV genome, and linked to the ROX, Cy5, ATTO550, HEX, and FAM fluorophores respectively were used to quantify the amount of total and intact HIV provirus present in each sample. The assay was performed on the QIAcuity Four platform, and participant samples were run in triplicates. Measurements were normalised to RPP30, a human housekeeping gene, as a marker of the total number of human cells in each sample. Results were analysed using a R shiny app software developed by Delporte et al. (86), and thresholds adjusted manually. Intact HIV DNA was quantified based on either two regions (ψ , *env*) from the classical IPDA (81) or all five regions from the multiplex assay. Total HIV DNA was quantified based on the presence of LTR regions detected. Intact HIV DNA levels were adjusted for DNA shearing and normalised per

million cells using the RPP30 measurements. Peripheral blood mononuclear cells (PBMC) HIV reservoir measures using the classical 2 probe IPDA were provided by Dr Marcilio Fumagili, Rockefeller University, for comparison with the tissue measurements. Total HIV DNA from the 2 probe (*env* and ψ) IPDA results in PBMCs and tissue were compared by calculating total HIV DNA using the formula:

Total HIV DNA = (0.5 x total number of single probe defective copies/ 10^6 cells) + (number of 2-probe intact copies/ 10^6 cells).

7.3 Results

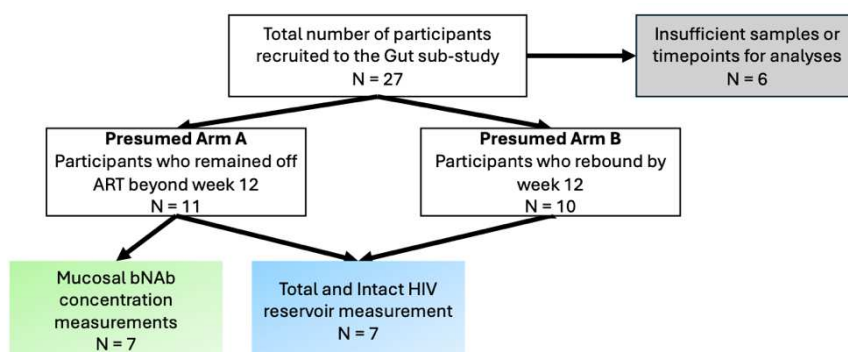
27 out of 68 participants in the RIO study consented to one or more rectal biopsies in the Gut sub-study. The demographics and allocation by main study arm of the sub-study participants are presented in Table 7.3. There were no serious adverse events associated with the sub-study procedures, and 21 participants had adequate sampling timepoints for further analyses.

Figure 7.3 shows the flowchart of the assays which samples from each participants were used for. Six of the 27 participants were not included in further analyses due to insufficient samples or timepoints for analyses. Of the remaining 21 participants, blinded samples from seven participants who remained off ART by week 12 of the ATI were sent for mucosal tissue bNAb measurements, with the assumption they were likely to be participants who had received bNAbs. However one participant was subsequently revealed to have received placebo and was a likely post-treatment controller who remained undetectable beyond week 12. Samples from another seven (4 from Arm A and 3 from Arm B) participants were sent for total and intact HIV reservoir measurements. The remaining seven participants had their samples used for HIV *env* sequencing for phylogenetics and resistance analyses.

Table 7.3. Demographics of participants in the RIO Gut sub-study (n=27)

Demographics	N / Median	% (Range)
Age	41	27 - 56
Male gender	27	100%
Ethnicity		
- White or Caucasian	17	63%
- Black African	3	11%
- Asian	2	7%
- Other Ethnicity not listed	5	19%
HIV Clade		
- B	15	56%
- non-B or unknown	12	44%
Study arm		
- A (bNAbs)	12	44.4%
- B (placebo)	15	55.6%

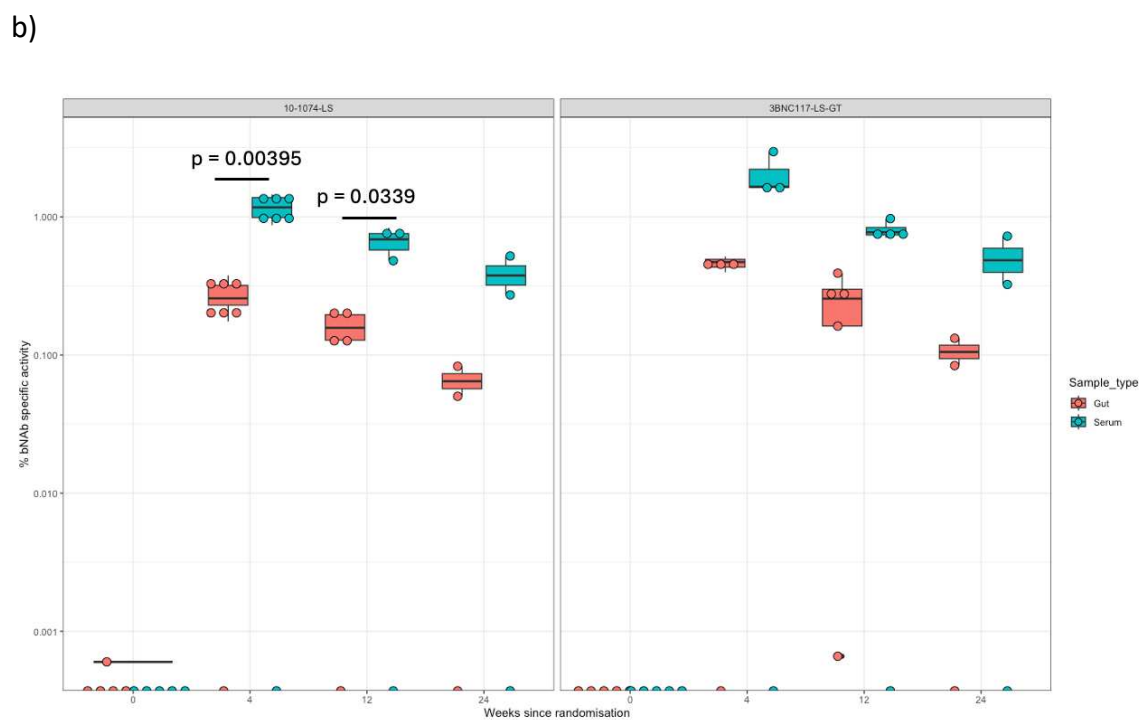
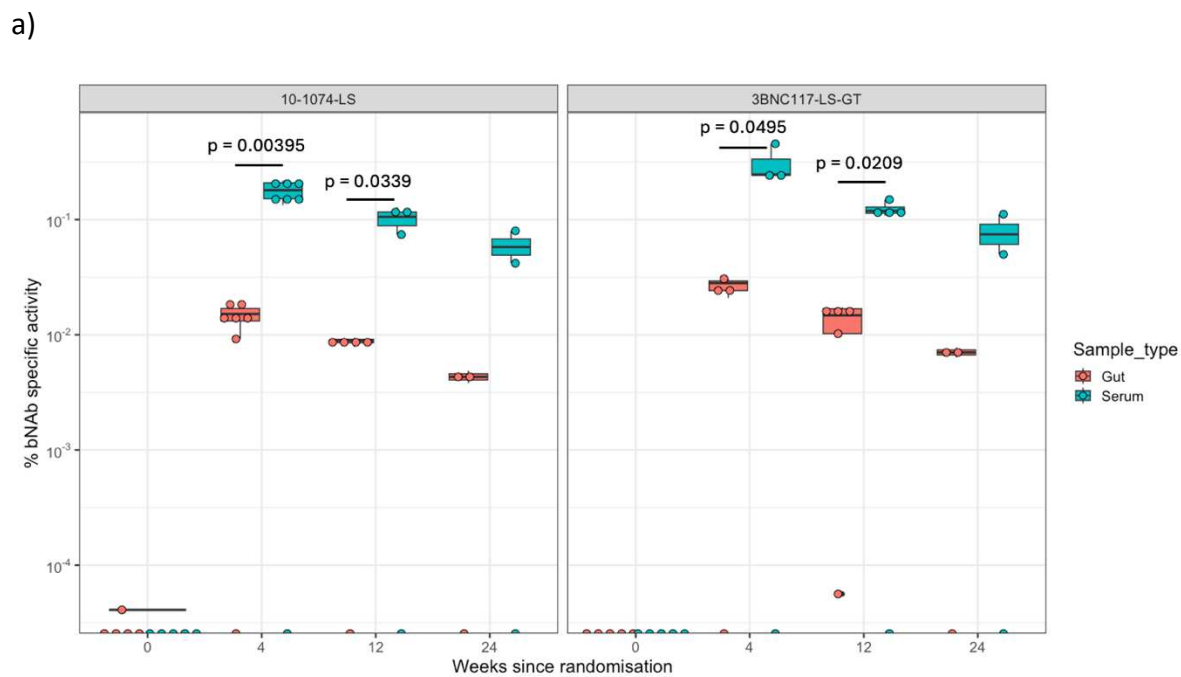
Figure 7.3 Flowchart of which assays were samples from each participant used



7.3.1 Comparison of paired serum and rectal tissue bNAb exposures

Both bNAbs were detectable in rectal biopsy lysates up to week 24 post bNAb dosing in 6 out of 7 participants. One participant was unblinded to have received a placebo infusion, and his samples were consistently negative for bNAbs. For the remaining participants, at 4/12/24 weeks, median tissue 10-1074LS SA were 0.000151/ 0.000089/ 0.000043 $\mu\text{g}/\mu\text{g}$ protein; paired serum 10-1074LS values were 0.00180/ 0.00106/ 0.00061 $\mu\text{g}/\mu\text{g}$ protein; median tissue 3BNC117LS SA were 0.000281/ 0.000158/ 0.000071 $\mu\text{g}/\mu\text{g}$ protein, paired serum 3BNC117LS values were 0.00246/ 0.00119/ 0.000806 $\mu\text{g}/\mu\text{g}$ protein (Figure 7.3.1.1a). There was a similar trend with values normalised to IgG, where rectal bNAb SA were approximately a fifth lower compared to serum (Figure 7.3.1.1b).

Figure 7.3.1.1. Serum and Tissue bNAb concentrations measurements normalized by a) total protein, and
b) total IgG.



Statistical test: Dunn's test

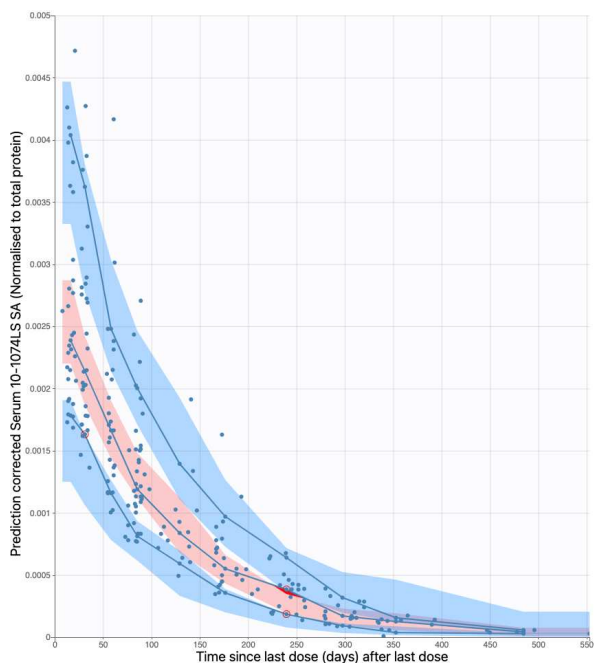
Using two-compartment modelling, the population parameters of both bNABs in serum and tissue were estimated (Table 7.3.1). BNAbs serum to tissue exposure (AUC) ratios were 7.79-fold ± 4.46 (TP) (2.65-fold ± 2.00 (IgG)) for 10-1074LS and 8.75-fold ± 4.36 (TP) (3.84-fold ± 2.38 (IgG)) for 3BNC117LS respectively. Visual predictive charts displaying the fits of individual models for 10-1074LS and 3BNC117LS, normalised to TP or IgG, are shown in Figures 7.3.1.2 a-f.

Table 7.3.1. Two-compartment parameters of serum and rectal bNAB pharmacokinetics

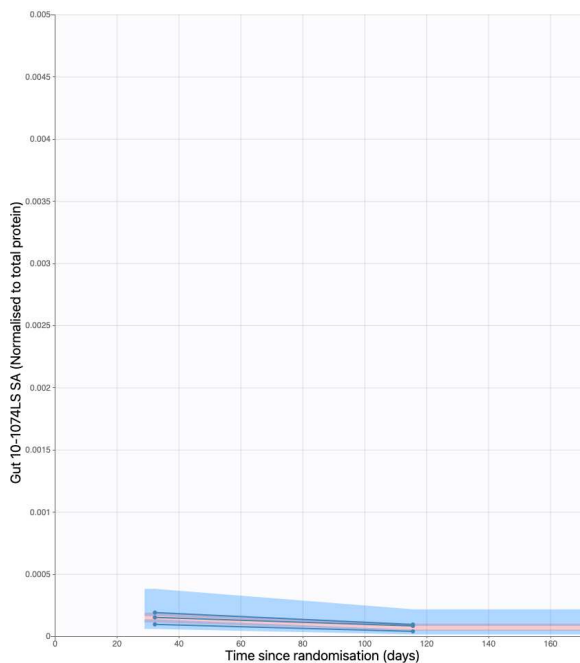
	10-1074LS	90% Interval / 2SD	3BNC117LS	90% Interval / 2SD	10-1074LS	90% Interval / 2SD	3BNC117LS	90% Interval / 2SD
Parameter	Normalised to Total Protein				Normalised to Total IgG			
V_{pop}	4.27 x10 ⁸	3.86 – 4.73 x10 ⁸	7.66 x10 ⁸	6.88 – 8.30 x10 ⁸	5.82 x10 ⁷	4.75 – 6.92 x10 ⁷	1.12 x10 ⁸	1.01 – 1.28 x10 ⁸
B_{V_logwt}	0.72	0.20 – 1.17	-	-	0.72	0.15 – 1.13	-	-
V_{2_pop}	5.11 x10 ⁹	1.95 – 6.92 x10 ⁹	9.89 x10 ⁹	6.29 – 14.7 x10 ⁹	4.16 x10 ⁷	1.71 – 7.67 x10 ⁷	5.57 x10 ⁷	5.11 – 6.25 x10 ⁷
K_{pop}	1.13 x10 ⁻²	1.02 – 1.24 x10 ⁻²	1.35 x10 ⁻²	1.29 – 1.45 x10 ⁻²	1.26 x10 ⁻²	1.07 – 1.52 x10 ⁻²	1.42 x10 ⁻²	1.30 – 1.49 x10 ⁻²
K_{12_pop}	0.29	0.12 – 0.49	1.00	0.97 – 1.00	0.63	0.24 – 0.98	0.53	0.30 – 0.66
K_{21_pop}	1.96	1.76 – 2.00	6.38	4.60 – 6.38	2.00	0.94 – 4.58	2.35	1.95 – 2.97
Mean C_{max} (Serum) µg/µg TP or IgG	1.26 x10 ⁻³	1.28 x10 ⁻³	2.70 x10 ⁻³	1.60 x10 ⁻³	1.21 x10 ⁻²	0.50 x10 ⁻²	1.82 x10 ⁻²	1.08 x10 ⁻²
Mean C_{max} (Gut) µg/µg protein or IgG	3.61 x10 ⁻⁴	2.16 x10 ⁻⁴	6.05 x10 ⁻⁴	2.42 x10 ⁻⁴	8.08 x10 ⁻³	3.80 x10 ⁻³	9.14 x10 ⁻³	5.88 x10 ⁻³
Mean Serum : Gut AUC ratio	7.79	4.46	8.75	4.36	2.65	2.00	3.84	2.38

Figure 7.3.1.2. Visual predictive charts of 10-1074LS and 3BNC117LS serum and rectal tissue concentrations, normalized to total protein (a-d) and IgG (e-h). Serum results are shown as time after last dose and tissue bNAb results are shown as time since randomisation in days.

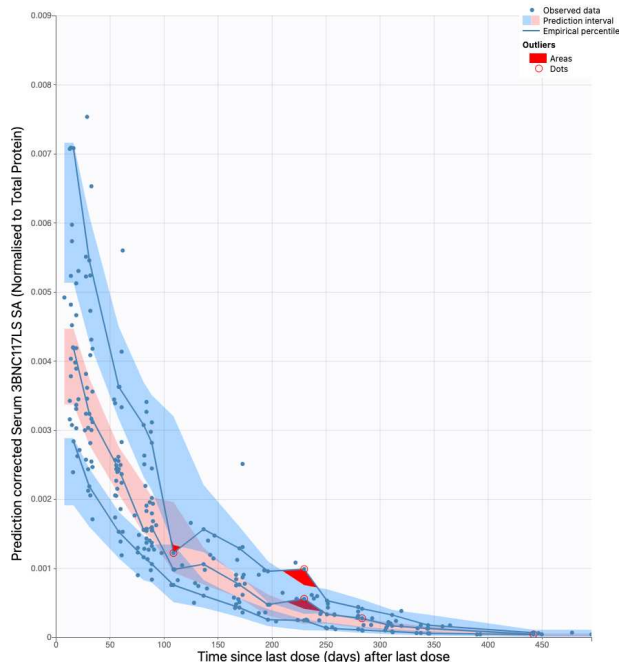
a) Serum 10-1074LS (TP)



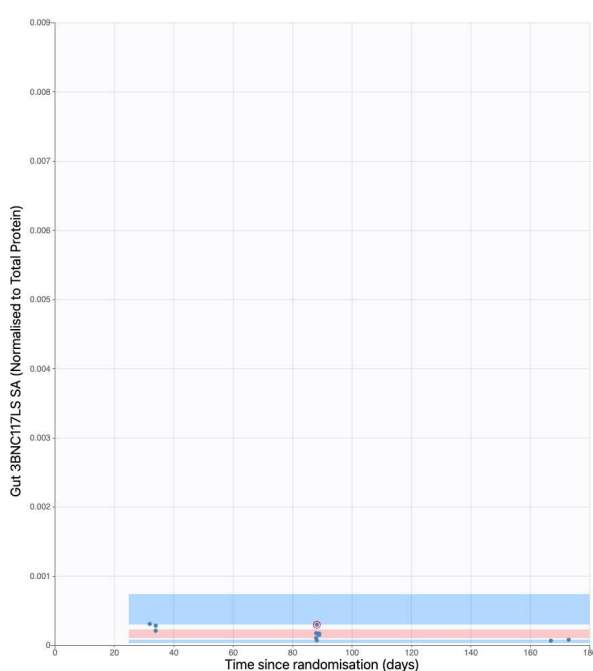
b) Rectal tissue 10-1074LS (TP)



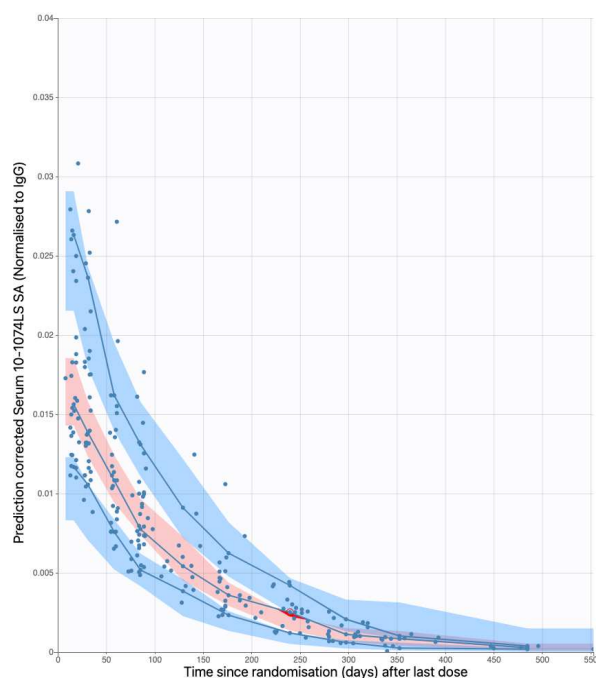
c) Serum 3BNC117LS (TP)



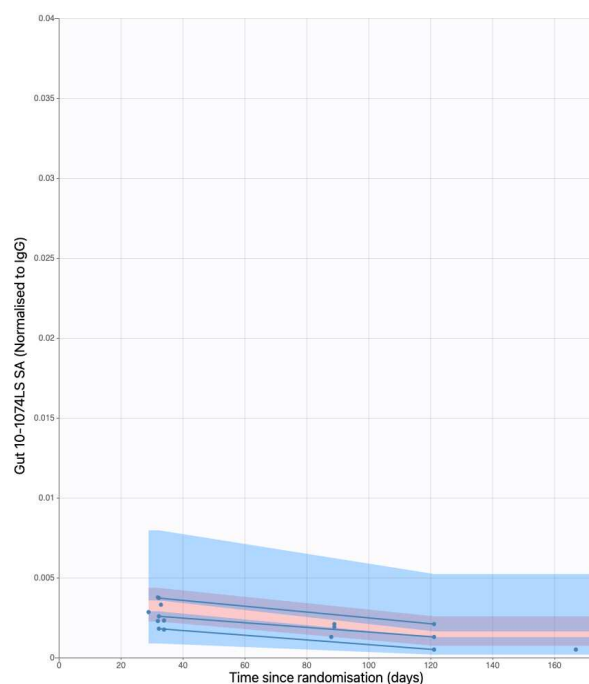
d) Rectal tissue 3BNC117LS (TP)



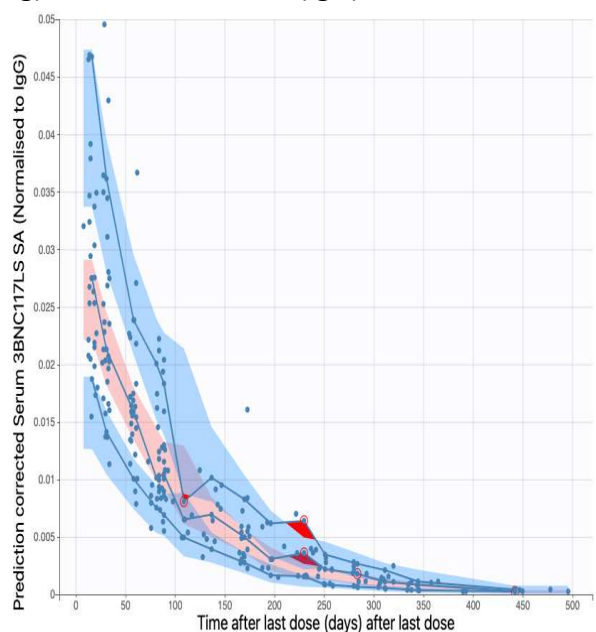
e) Serum 10-1074LS (IgG)



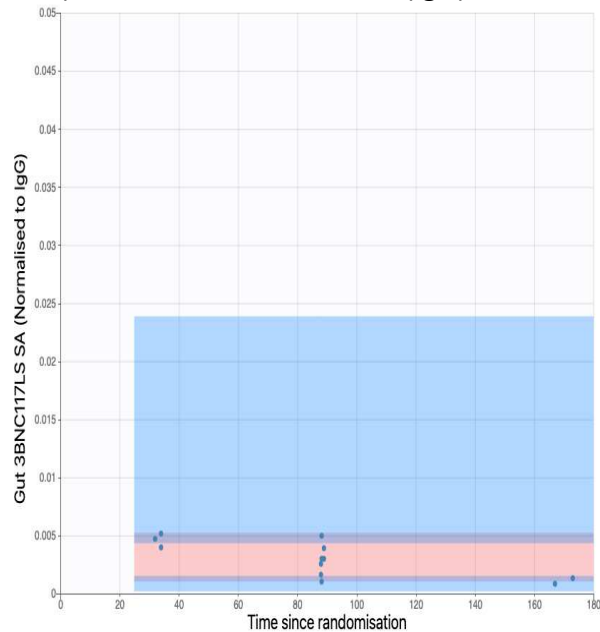
f) Rectal tissue 10-1074LS (IgG)



g) Serum 3BNC117LS (IgG)



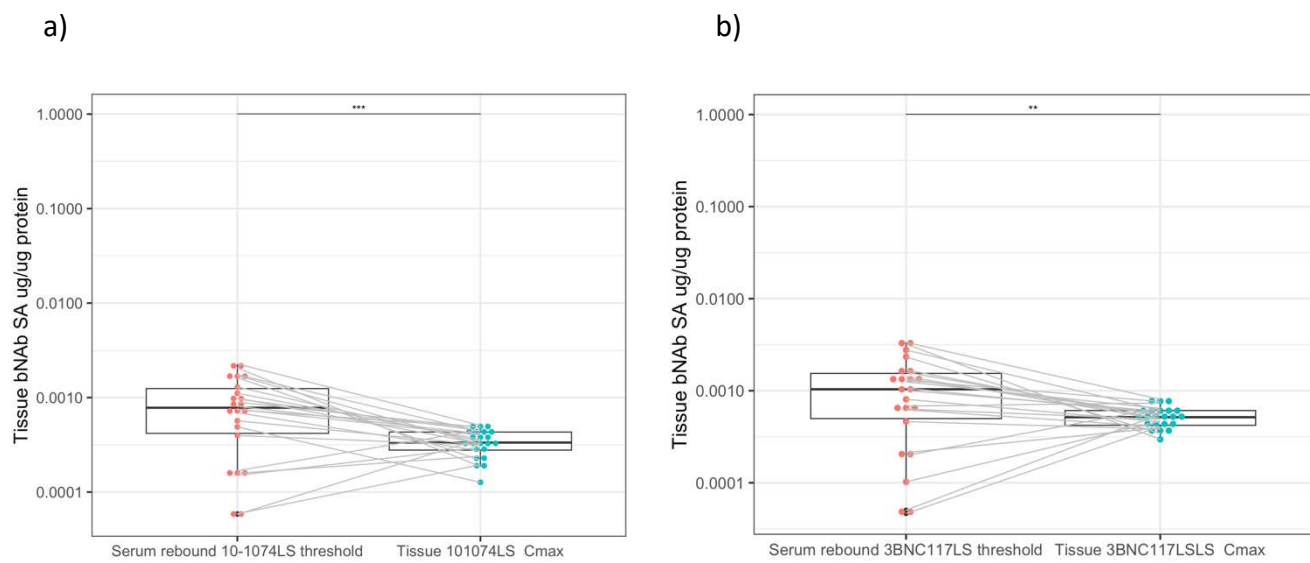
h) Rectal tissue 3BNC117LS (IgG)



The bottom and top blue shaded areas represent the 90% prediction interval for the 10th and 90th percentiles and the pink shaded area represent the 90% prediction interval for the median. Outlier data points beyond the prediction intervals are circled in red, and outlier observed 10th, 50th and 90th percentile areas under the curve beyond the prediction intervals are highlighted in red.

Based on the serum rebound bNAbs concentrations at the time of viral rebound >50copies/mL estimated from the serum pharmacokinetics described in the previous chapter, the estimated median serum rebound bNAbs SA normalised to total protein were 7.81×10^{-4} and 1.04×10^{-3} $\mu\text{g}/\mu\text{g}$ protein for 10-1074LS and 3BNC117LS respectively. Cmax values in tissue were significantly lower than the rebound bNAbs protein-normalised serum SA thresholds for both bNAbs (median 10-1074LS tissue Cmax 0.000334 vs rebound bNAbs SA 0.000078 $\mu\text{g}/\mu\text{g}$ protein, $p = 0.0003$, median 3BNC117LS tissue Cmax 0.000516 vs rebound bNAbs SA 0.00104 $\mu\text{g}/\mu\text{g}$ protein, $p = 0.005$) (Figure 7.3.1.3a-b).

Figure 7.3.1.3. Comparison of rebound bNAb protein-normalised specific activity to maximum protein-normalised tissue bNAb specific activity for a) 10-1074LS and b) 3BNC117LS respectively.



*Paired Wilcoxon signed-rank test

7.3.2 Impact of bNABs on gut associated HIV proviral reservoir after 12 weeks

Seven participants had rectal biopsies for gut HIV proviral reservoir measurements, of whom one participant who was unblinded to Arm A experienced viral rebound at the time of the 12-week biopsy and was excluded due to the inability to accurately measure the latent HIV reservoir during viral suppression. Of the remaining six participants, three received bNABs (Arm A) and had the second rectal biopsy 12 weeks after dosing. The remaining three were in Arm B, where they received a placebo infusion before starting an ATI, and upon viral rebound and restarting ART, received an open-labelled bNAB infusion. They subsequently had a rectal biopsy at least 12 weeks after their bNAB dose whilst virally suppressed back on ART. Demographics and details of HIV duration and antiretroviral therapy are presented in Table 7.3.2.1.

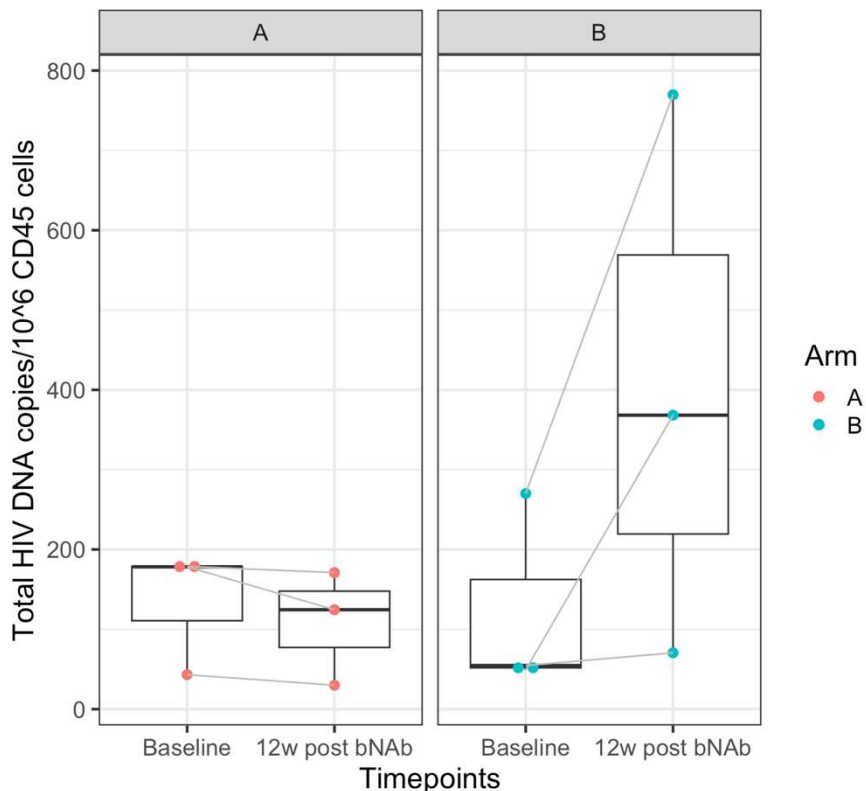
Table 7.3.2.1: Demographics and details of HIV duration and antiretroviral therapy of participants who had their tissue HIV reservoirs measured

	Arm A (bNAB)			Arm B (placebo)		
Participant	P1	P2	P3	P4	P5	P6
Age (years)	38	42	36	56	31	52
Gender	Male	Male	Male	Male	Male	Male
Duration since HIV diagnosis (years)	12	5	7	11	6	Not available
HIV clade	B	B	B	B	Not available	A
ART at enrollment	Dovato	Triumeq	TDF/FTC, Raltegravir	TDF/FTC, Dolutegravir	Biktarvy	Biktarvy
Time of 2 nd biopsy (weeks since bNAB dose)	12	12	12	12	14	12

TDF/FTC: Tenofovir Disoproxil Fumerate / Emtricitabine

HIV DNA (as measured by the LTR probe) was detectable in all samples. However, intact provirus (whether measured by 2 probes to *env* and ψ , or all 5 probes) was only detected in samples from two participants. Shearing was low, the mean digital shearing index (DSI) was 9.3% ($\pm 2SD$ 3.7%). Median pre-enrollment baseline total HIV DNA was 116 (range 43 – 270) copies/ 10^6 CD45+ cells. In the Arm A participants there was a small decrease in total HIV DNA from baseline to post bNAbs (median 178 vs 124 copies/ 10^6 CD45+ cells), and in Arm B participants there was an increase in total HIV DNA between timepoints (median 55 vs 368 copies/ 10^6 CD45+ cells) after their first ATI, restarting ART and receiving a dose of bNAbs (Figure 7.3.2.1). However, due to small numbers, it was not possible to determine the statistical significance of these differences between timepoints.

Figure 7.3.2.1. Box plot comparison of tissue associated total HIV DNA from baseline to 2nd biopsy timepoint, stratified by study arm.



For the Arm B participants, there did not appear to be a trend between the peak plasma HIV viral load, duration of detectable viraemia, during the first ATI and the increase in total HIV DNA. The duration of viral suppression after restarting ART prior to the 2nd biopsy timepoint was similar across all three participants (Table 7.3.2.2).

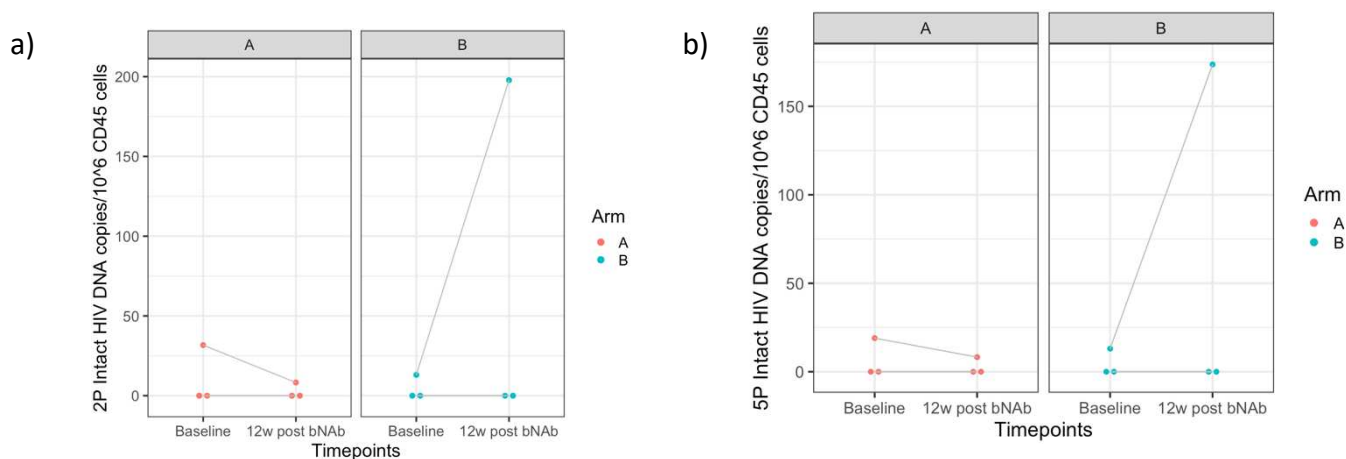
Table 7.3.2.2. Total HIV DNA measurements and factors associated with 1st ATI in Arm B participants

Participant	P4	P5	P6
Baseline total HIV DNA (copies/10 ⁶ CD45+ cells)	55	270	49
2 nd Timepoint total HIV DNA (copies/10 ⁶ CD45+ cells)	71	770	368
Difference in total HIV DNA (copies/10 ⁶ CD45+ cells)	+21	+500	+319
Peak plasma HIV viral load prior to 2 nd timepoint (copies/mL)	302000	125000	15246
Duration of detectable viraemia in 1 st ATI (weeks)	5	3	8
Duration since last detectable result till 2 nd timepoint (weeks)	12	13	12

Intact HIV DNA was only detected in participant 003-010 from Arm A and 001-033 from Arm B. At baseline, 003-010 had 32 and 19 intact HIV copies/10⁶ CD45+ cells using the 2 probes or 5 probes measurements, and 12 weeks after bNAbs, he had 8 and 8 intact HIV copies/10⁶ CD45+ cells respectively. At baseline, 001-033 had 13 and 13 intact HIV copies/10⁶ CD45+ cells using the 2 probes or 5 probes measurements, and 12 weeks after restarting ART and receiving bNAbs, he had 198 and 174 intact HIV copies/10⁶ CD45+ cells respectively (Figure 7.3.2.2. a,b).

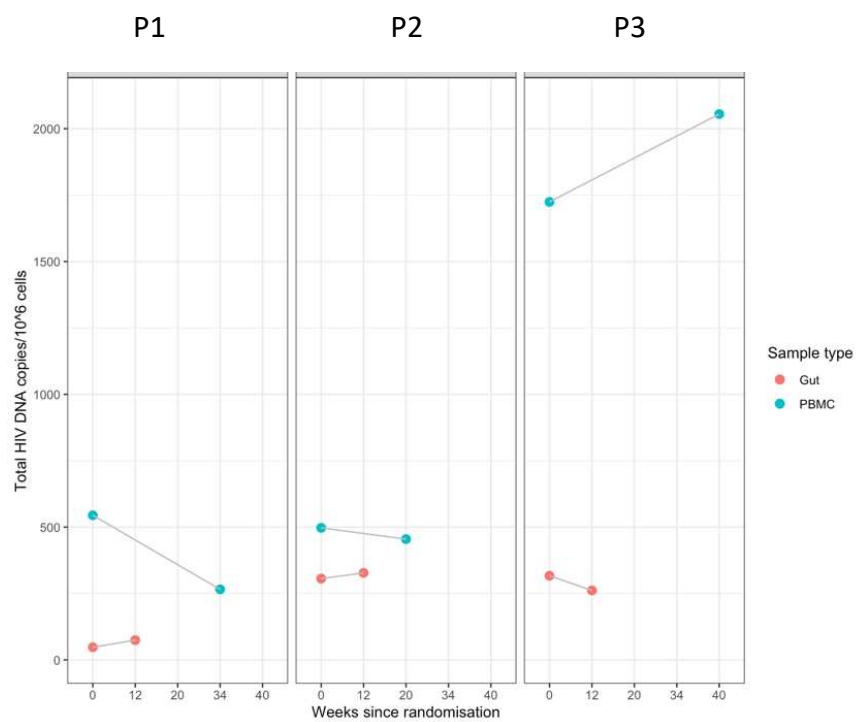
Of note, all three Arm A participants eventually received two doses of bNAbs, however 003-010 experienced viral rebound at 48 weeks from his first bNAb dose and restarted ART due to an episode of acute illness, while the remaining two Arm A participants who did not have any detectable intact HIV reservoir in gut tissue present, remained undetectable off ART beyond 72 weeks during their ongoing ATI.

Figure 7.3.2.2. Comparison of tissue associated intact HIV DNA from baseline to 2nd biopsy timepoint, stratified by study arm, using the a) 2 probes or b) 5 probes measurements.



Comparing the differences in Total HIV DNA between PBMC and tissue cells, PBMC results across two timepoints were available for the three Arm A participants with tissue biopsies. There did not appear to be a clear trend between the week 12 results and the corresponding blood reservoir measures, with the caveat that the second timepoint was taken at different times for blood and gut, and PBMC results were from unsorted cells. The results are displayed in Figure 7.3.2.3. Intact proviral DNA was detectable from PBMCs from all three Arm A participants at baseline (001-031: 16 copies/10⁶ cells; 003-010 44 copies/10⁶ cells; 003-012 22 copies/10⁶ cells).

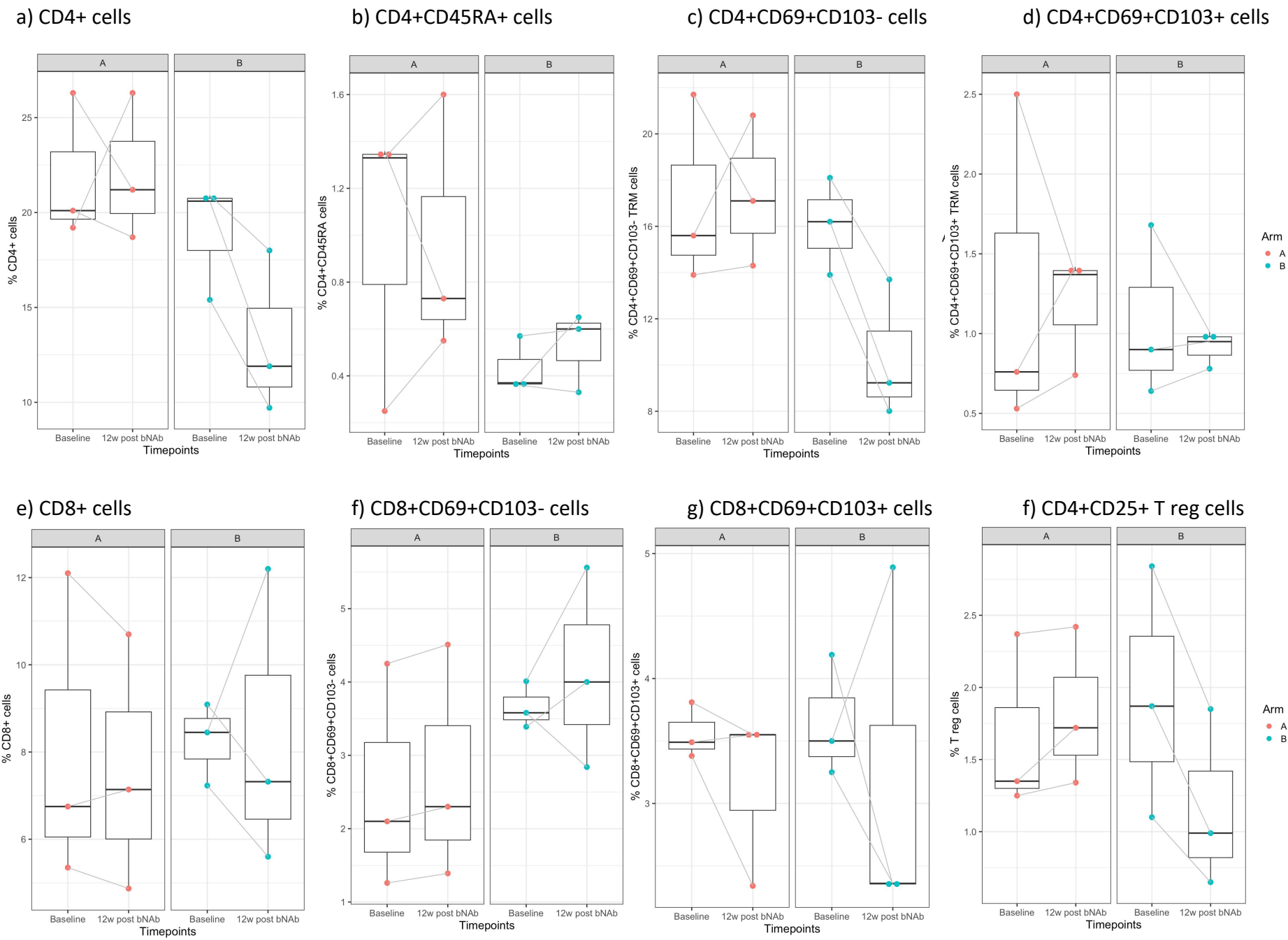
Figure 7.3.2.3. Comparison of blood and gut tissue total HIV reservoir measures.



7.3.3 Changes in the rectal mucosal immunity after bNAb administration and ATI

Phenotyping of the CD45+ cells within rectal biopsy samples were performed prior to sorting and HIV DNA quantification using FACS. The results are presented in Figure 8 a-h. Compared to the virally suppressed Arm A participants, participants in Arm B demonstrated a greater reduction in proportions of tissue-associated CD4+ cells amongst total CD45+ cells (median % change -1.40 vs -5.69), CD25+ T regulatory cells (median % change +0.09 vs -0.88) and CD69+ CD103- CD4+ cells (median% change +0.4 vs -5.89), however, there was no substantial changes between study arms observed in other CD4+ T cell populations including CD69+CD103+CD4+ tissue resident memory cells, or CD4+CD45RA+ T cells including naïve T cell populations. Neither were there substantial differences in the changes between study arms seen in CD8+, CD69+103+CD8+ , CD69+CD103-CD8+ , or CD3+ gamma delta T cell populations. These results are presented as box plots stratified by study arms in Figure 7.3.3. However, the sample sizes were small and therefore presented as descriptive changes in cell populations only.

Figure 7.3.3. Changes in rectal tissue T cell populations (% of total CD45+ cells) between baseline and post bNAbs infusion, stratified by study arms.



7.4 Discussion

An effective immunotherapeutic intervention towards a HIV cure ideally crosses from blood to tissue at adequate levels to impact the tissue-associated latent HIV reservoir, where most of the body's latent HIV reservoir is sited. This is the first study to define the pharmacokinetics profile of LS-bNAbs in tissue from in people living with HIV when administered for therapeutic intervention. Despite the hypothesised advantages of the LS mutations towards enhancing tissue penetration, I have demonstrated that LS-bNAbs are still present in lower concentrations in rectal tissues compared to serum when dosed at 10mg/kg and 30mg/kg for 10-1074LS and 3BNC117LS respectively in virally suppressed people living with HIV.

Protein-normalised LS-bNAb tissue exposure were eight to nine-fold lower compared to serum up to 21 weeks after dosing, and suggest that despite the rectal mucosa being extensively vascularised (366), only a fraction of the antibodies crosses from serum into tissue, mediated by both convective transport down the blood-tissue hydrostatic gradient (284), and active transcytosis or uptake of the antibodies intracellularly across vascular epithelial cells, mediated by the FcRn (285). It has been hypothesised that modification of the Fc portion of bNAbs may enhance FcRn binding, improving tissue transport (229,352). However, despite the lack of data for the tissue penetration of non-LS antibodies, these findings suggest that any additional benefit of the LS-mutation for tissue penetration, is likely to be small, as these findings are similar to the results of a previous study of rectal and vaginal tissue penetration of non-LS bNAb VRC01 in people living without HIV (229).

The true therapeutic thresholds at which a bNAb is effective is still unclear, and previous reports have suggested differing thresholds. One report suggests 70% of HIV strains in the Americas and South Africa have a neutralisation IC₈₀ of >1µg/mL (367), while others have observed that in therapeutic studies, participants living with HIV who received combination bNAb therapy did not experience viral rebound off ART until serum bNAb thresholds fell below 10 µg/mL. However, our findings from the previous chapter suggest a higher bNAb serum concentration threshold at which viral rebound is observed for those who experienced rebound.

To understand if the lower levels of tissue bNAb penetration are still effective, I used data from the PKPD model to estimate rebound serum bNAb thresholds, and comparing it to the estimated maximum tissue concentrations. Even at maximum tissue concentrations after dosing, neither bNAbs appeared to penetrate tissue above the levels associated with rebound in serum, for the participants who experienced viral rebound in the study. However, it is still uncertain if the tissue thresholds for the therapeutic effect of bNAbs are the same as the levels seen in serum. The activity of monoclonal antibodies in tissue depend not just on drug levels, but also the amount and turnover of target antigen, antibody-antigen binding kinetics, and other indirect cellular effects such as cell-to-cell viral transmission (363,368). The amount of HIV in the ileum is 5-6 times higher than blood (264), and it may be postulated that a lower bNAb concentration in tissue is adequate to create immune complexes and target virus-containing reservoir cells.

Although the number of participants who had their rectal tissue-associated HIV reservoirs measured after bNAb dosing were small, these results represent the first evaluation of the impact of bNAbs on the tissue reservoir, and provides important hypothesis generating data for future studies. Reassuringly, there was no increase in tissue associated HIV reservoirs in those who remained virally suppressed 12 weeks after receiving bNAbs, and the two participants with levels of tissue-associated intact HIV reservoir below the levels of detection at baseline, remained suppressed beyond 72 weeks, while the single participant with a detectable tissue-associated intact HIV reservoir experienced viral rebound.

A marked increase in tissue-associated total HIV DNA was observed in participants who experienced viral rebound during their treatment interruption after receiving placebo followed by restarting ART and receiving a dose of LS-bNAbs in Arm B. Although reassuringly, intact proviral DNA was not detected in two of the three Arm B participants, 001-033 who had the highest measures of total HIV DNA, showed a corresponding increase in intact proviral HIV reservoir even after achieving viral suppression on restarting ART and receiving bNAbs. Previous studies have suggested that there is a transient increase in total HIV DNA in the blood during an ATI, with a return to baseline levels of total or integrated HIV DNA reservoir measures 6-12 months after

restarting ART (320,369,370). It may be that the higher tissue HIV reservoir measures seen in Arm B participants who experienced viral rebound may reflect residual active HIV reservoir due to the earlier sampling time, and not a true reflection of the latent HIV reservoir. However, further samples and timepoints taken from these participants will be required to confirm this hypothesis.

These results are the first analyses of LS-bNAb effect in gut tissue, a key site of the latent HIV reservoir and serves as the basis for future tissue studies to inform the mechanistic actions of LS-bNAb and other immunotherapeutic agents targeting the tissue HIV reservoir. I have shown that longitudinal sampling of rectal biopsies from trial participants is safe and had high uptake with more than a third of RIO study participants agreeing to the Gut sub-study. By using data from the ultrasensitive SMCxPro immunoassay, I was able to model tissue LS-bNAb concentrations and compare them to serum to estimate generalisable population parameters to inform future LS-bNAb studies.

The changes in mucosal immunity seen were primarily in participants who received placebo initially (Arm B). The decrease in CD4 cells and CD25 T reg cells are consistent with HIV-associated immune dysfunction (371). Participants in Arm B experienced during their 1st ATI prior to restarting ART and receiving bNAbs. In contrast, at 12 weeks, there did not appear to be a noticeable change in T cell populations for Arm A participants who remained virally suppressed after receiving bNAbs.

There were limitations to this study, including the small numbers of participants, particularly for the measures of tissue HIV reservoir and mucosal immunity, which only allowed for descriptive analyses. The comparison between serum and gut bNAb specific activity relied on a mean reference value to normalise serum bNAb results to protein and IgG, due to actual serum total protein and IgG results being unavailable. While the total serum protein in people with HIV have been reported to be comparable to people without HIV (372,373), however serum IgG levels have been reported to be higher in people with HIV compared to those without (374,375). This may be related to abnormal or non-specific B cell activation associated with HIV (376). While this pooled reference approach has been described (377), it may result in small variations

in the normalised values, particularly for the IgG results. However, the resultant models remain tightly fitted in comparison with the observed data. Similarly, the comparison between serum and gut reservoir measures used different assay platforms and different denominator cell populations, which may contribute to the differences observed in the results. It remains uncertain what the therapeutic threshold at which bNAbs are effective, and the serum concentration at rebound presented here may not reflect the tissue concentrations required to prevent viral replication and thus may not be the ideal threshold for comparison, however in the absence of other data such as neutralisation IC80s or immune T cell-killing assays to determine the HIV-specific effects induced by bNAbs, the use of plasma viral load results may offer an insight into the mechanistic thresholds effect of bNAbs.

In conclusion, LS-bNAbs were safe and effective and maintaining long term viral suppression. LS-bNAb concentrations were lower in rectal tissue compared to blood, and may be an important consideration for the use of LS-bNAbs for in HIV cure interventional studies for targeting the latent tissue HIV reservoir. Despite the lower levels of bNAbs in gut tissue, early gut tissue reservoir data suggest participants diagnosed during early HIV who receive bNAbs who remain virally suppressed 12 weeks after dosing during their treatment interruption maintained low tissue reservoir measures and long-term viral suppression outcomes.

Chapter 8.

Discussion

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8.1 Main findings

An effective immunotherapeutic cure for HIV has to target the tissue-associated latent HIV reservoir. HIV-specific long-acting LS-bNAbs have shown promise in maintaining long-term viral suppression off ART for people living with HIV and has been associated with small decreases in the latent intact HIV reservoir in blood. The overall aims of this thesis were to investigate if LS-bNAbs were able to penetrate the gut tissue, the largest site of the HIV reservoir in the body, and their impact on clinical outcomes, the tissue associated HIV reservoir, and mucosal immunity.

The main findings from this thesis are:

1. LS-BNAbs are associated with prolonged viral suppression off ART, with a sub-group of participants who received bNAbs achieving viral suppression beyond duration of bNAbs in blood suggesting a post-bNAb viral control effect.
2. BNABs and ATIs are safe and do not impact time to viral resuppression in HIV cure-related ATI studies, with frequent monitoring and the use of potent integrase inhibitor containing ART regimens on restarting ART.
3. Two doses of dual LS-BNAbs administered to people living with HIV had similarly extended pharmacokinetics profiles as previous smaller studies of monotherapy and combination LS-bNAB studies.
4. For participants who experienced viral rebound after receiving LS-bNAbs, dynamics of viral rebound appeared to fit a maximal direct inhibitory PKPD model, with five to seven times higher bNAB concentrations estimated at viral rebound >50copies/mL compared to a previously proposed 10µg/mL threshold.
5. Rectal tissue LS-bNAB exposures were 8-9 fold lower compared to blood and the presence of the LS-mutations did not appear to increase tissue exposures compared to other IgG antibodies

6. The amount of intact HIV reservoir in the rectal tissue was too small to quantify with existing assays in most participants diagnosed early in HIV, and attempts to develop a scalable, high throughput, qualitative reservoir assay remains a challenge.
7. Compared to three participants in Arm B (placebo arm) who showed classical HIV-associated mucosal immune changes and increased tissue-associated HIV DNA reservoir in their rectal biopsies even 12 weeks after restarting ART, three Arm A participants who received bNAbs and remained suppressed showed no substantial mucosal immune cell populations or their tissue-associated HIV DNA reservoir.

I have shown that bNAbs are safe and a promising long-acting intervention associated with prolonged viral control. The aim of an eradication cure for HIV aims to target the HIV reservoir, reducing or eliminating the reservoir size such that viral replication off ART is halted. This study has demonstrated that a subgroup of people receiving bNAbs are able to achieve extended periods of viral control, beyond the duration which bNAbs are present in blood or tissue. Participants demonstrating this 'post-bNAb' effect may be exhibiting a post-intervention viral control effect, or 'vaccinal effect', driven by enhancement of cellular immune responses, similar to persistent enhancement of HIV-specific CD8 responses a year after bNAb dosing, as seen in the eCLEAR study (245).

The data on bNAbs in tissue addresses all the aims presented in my introduction. In chapter 4 I have demonstrated the safety profile of bNAbs. Reassuringly the use of bNAbs do not appear to increase the time to viral resuppression after an ATI compared to undertaking an ATI alone with frequent monitoring and the use of potent integrase inhibitor containing ART regimens on restarting ART. In my systematic review of ATI studies since 2015 (322), and also from the RIO study, most participants in the studies included resuppress within 12 weeks, comparable to treatment naïve people diagnosed with primary HIV who initiate ART. There were no serious adverse events associated with bNAbs in the RIO study, consistent with previous studies of intravenous bNAbs where no serious adverse events related to the study drug have been reported. Serious

adverse events associated with bNAbs were only reported in one phase one study of subcutaneous 10E8VLS injections, where seven of eight participants experienced erythema and induration at the injection site (248). The RIO study is the first double-blinded randomised placebo-controlled study powered to demonstrate the clinical effectiveness of bNAbs in maintaining viral suppression, and as described in chapter 5, at twenty weeks after a single dose of 10-1074LS and 3BNC117LS, participants who receive bNAbs had a 91% greater likelihood of remaining off ART compared to placebo. Six participants remained virally suppressed beyond 48 weeks after the last dose, the average time which bNAbs levels were expected to fall below 10µg/mL, compared to two participants in the placebo arm. This prolonged 'post bNAb' viral suppression is consistent with the proposed bNAbs-induced 'vaccinal effect' hypothesis. Although the investigation of HIV-specific immune responses after bNAbs are beyond the scope of this thesis, enhanced HIV-specific T cell responses have been observed a year after non-LS bNAb dosing in several studies (178,245). The finding that in people who experienced longer time to rebound, the slower doubling times may be consistent with enhanced immune control of viral replication slowing the rates of viral replication, even as bNAbs are no longer present. Understanding the pharmacokinetic-pharmacodynamic interactions of bNAbs have provided valuable insight into the thresholds and mechanism of action of bNAbs in Chapter 6. These findings will be informative for upcoming studies involving bNAbs, for example the ACACIA and PAUSE studies in Africa (378,379), where the same two LS-bNAbs would be given to people with HIV who have not started receiving treatment (ACACIA), or those who are virally suppressed on ART (PAUSE). In the UK, the AbVax study will investigate the use of a combination vaccination and LS-bNAb approach to induce a protective T cell vaccinal effect (380). Previous bNAb studies have not had adequate sample sizes or duration of ATIs to determine a threshold which bNAbs are effective. Using serum bNAb measurements and viral rebound data from participants in the intervention arm of the RIO study, I demonstrated that for participants who eventually experienced viral rebound fit a direct inhibitory model of bNAbs effect on plasma viral loads. The model not only allowed estimation of the overall drug parameters such as a maximal inhibition effect and bNAb concentrations

associated with 80% of the maximum inhibition (IC80), but also an exploration of how bNAbs may interact when given in combination. Different models as described in the introduction to chapter 6 were tested and the maximal inhibitory model appeared to best fit the rebound outcomes from the RIO study. This contrasts with a previous study by Mayer et al. (357) suggesting the multiplicative BH model better explained the interactions of bNAbs used in combination, on the overall neutralisation IC80 values observed in vitro. One key difference is the use of in vitro neutralisation IC80 as the outcome in the context of HIV-prevention studies, the biomarker which is highly relevant for the prevention of target cell infection. However, in a therapeutic study, where the outcome is prevention of viral rebound, while neutralisation likely remains a key mechanism of bNAb action, other non-neutralising effects of bNAbs may be an important component of the effect of bNAbs to suppress viral load (381), not reflected in the neutralisation IC80 values. Thus, the BH interaction proposed for models to predict neutralisation IC80 values, may not be as applicable for the modelling of bNAb therapeutic effects and it appears that viral rebound is driven by a predominant antibody under the maximal interaction model. The IC80 values estimated from the inhibitory model may not directly correlate with empirical neutralisation IC80 values but reflect the dose-response curve of the effect of bNAbs on plasma viral load.

In addition, the IC80 values appear to evolve over time, with viral rebound observed at high levels of bNAbs after a second dose despite being suppressed at lower bNAb concentrations after the first dose. Whether this was due to de novo mutations, or selection of previously missed minority resistant strains, the overall effect is an increase in resistance over time, consistent with the rebound *env* sequence-predicted bNAb mutations. Cell-to-cell transmission may be another mechanism for immune escape from bNAb-mediated neutralisation, with one study finding decreased neutralising activity of bNAbs in cell-to-cell assays of 16 bNAbs (382). The implication that there is likely ongoing viral replication and selection, despite the apparent undetectable status of many participants after bNAb dosing should be taken into consideration should bNAbs be considered for future treatment strategies to maintain viral suppression. Other antiretroviral

options to arrest viral replication and prevent the development of resistance should be considered in addition to these LS-bNAbs.

The evolution of bNAb IC80 over time may also explain the higher bNAb concentrations observed at the time of viral rebound, and it may be challenging to predict a one-size fit all threshold where bNAbs remain therapeutic, compared to classical ART drugs such as efavirenz, rilpivirine or dolutegravir (383,384). Individual participants may have different therapeutic thresholds depending on viral factors such as the baseline provirus bNAb sensitivity, and the duration on bNAb therapy in the absence of ART, as well as host factors including the development of HIV-specific T cell responses (178,245), the presence of protective HLA alleles (385,386).

In chapter 7, I compared bNAb exposure between serum and tissue, and report that tissue bNAb levels were eight to nine-fold lower than in serum, despite the presence of the LS modification to the Fc portion. Even as a proportion of total IgG that crosses over into tissue, the amount of bNAbs was two to four-fold lower than in serum. When compared to the protein-normalised serum rebound concentrations, tissue bNAb concentrations were predicted to be lower than the rebound thresholds even at peak concentrations after dosing. If bNAb concentrations are sub-optimal in preventing viral replication in tissue, leading to tissue resistance compartmentalisation and selection for resistant strains as the source of viral recrudescence, it may also explain viral rebound observed in the presence of higher serum bNAb concentrations. Strategies to address this may be to increase the dose and frequency of dosing to maintain trough bNAb concentrations above the modelled bNAb concentration at which viral rebound occurs. The favourable safety profile of bNAbs appear to favour this as a strategy, without any dose-dependent adverse events related bNAbs reported to date. Apart from modification of the Fc portion of antibodies such as engineering LS-bNAb variants, other strategies for improving tissue penetration have been reported, including the use of antibody-drug conjugates where the conjugation of antibodies to cytotoxic agents have been shown to improve targeting of tumour tissue, and are currently being developed for oncology treatments (387). Reassuringly,

for people who remain suppressed after receiving bNAbs, there did not appear to be an increase in total or intact viral reservoir measures in the three participants who donated biopsies, suggesting that even if viral replication is present, it is at negligible levels at 12 weeks, however, the numbers in this study were small, larger sample sizes and longer-term reservoir measurements would be required to draw definitive conclusions about the reservoir changes. These findings are also complicated by the fact that HIV DNA may be more difficult to measure from gut samples, due to the low frequencies of infected CD4 T cells in the samples (388) Even more challenging, people who started ART during acute and early HIV results in more rapid declines in both the total and intact HIV reservoirs, and in one study, only intact HIV DNA was detectable in PBMCs using the IPDA from fewer than half of the people treated during acute HIV (389). Studies using other assays to measure the tissue HIV reservoir have described higher frequencies of total or integrated DNA in gut tissue (70), but this may not accurately reflect the replication competent reservoir in tissue. The yield of intact HIV DNA quantification may be increased by enrichment of homogenised tissue samples with CD45 cell sorting using FACS (390). However, it is promising that people with extremely low tissue HIV reservoir measures appeared to have improved clinical outcomes in the RIO study. I also described phenotypic changes within the rectal mucosal immunity after bNAb administration and demonstrate that while people who remain virally suppressed after bNAb administration did not demonstrate substantial changes in the T cell populations, particularly those that may be target cells for viral infection. Unlike participants in arm B who experienced viral rebound and demonstrated increases in the amount of total HIV DNA measured early within 12 weeks after restarting ART with bNAbs, with corresponding changes in the mucosal immunity reflective of HIV viraemia on CD4 T cells and T regulatory cells, along with increased T cell activation, the lack of similar reservoir or mucosal changes in the Arm A participants demonstrate the protective effects of bNAbs in preventing HIV-associated mucosal immune damage in this small sub-study of RIO participants.

There were limitations within this study, which may limit the generalisability of these findings. The RIO study's rigorous inclusion criteria and study design, rightfully prioritised participant safety by ensuring only people diagnosed with early or primary HIV infection were enrolled in the RIO study along with the advantages of early HIV treatment with attenuated HIV reservoir diversity and improved immune recovery (30) . However, the findings may not be generalisable to other people living with chronic HIV infections. Despite being able to recruit more than a third of the RIO participants to contribute rectal tissue biopsies in the Gut Sub-study, due to the small nature of the biopsies and need for pooling of biopsies from individual timepoints to achieve adequate cell numbers, only twenty one participants had adequate samples for analyses. Thus the overall numbers for individual components of this study of rectal biopsies were low, and for the exploratory measures of tissue reservoir sizes and mucosal immunity, the study was underpowered to determine the statistical significance of these results. However, there were trends in the results which are the first of their kind, and generates important hypotheses to guide future research into the mechanistic effects of bNAbs on the tissue reservoir. Primary outcomes of the RIO study will be reported shortly as the study unblinding has recently been completed at the time of writing, however the study remains ongoing and further analyses of secondary outcomes, such as differences between the 1st and 2nd ATI for Arm B participants were not able to be undertaken due to incomplete outcomes as some participants had not begun their 2nd ATI at the time of this thesis submission. Due to the long duration of action of these bNAbs, I was unable to measure reservoir changes due to insufficient biopsies at later timepoints, particularly for Arm B participants. Other methodological limitations include the lack of paired serum protein or IgG results, which were overcome using reference values available from the clinical biochemistry laboratory. Similarly, comparison of baseline tissue and blood proviral sequences, to rebound viral sequences to investigate the evolution of viral rebound sequences and relationships with plasma and gut proviral sequences were not complete, as data from the rebounds for these participants were not readily available at the time of analysis.

8.2 Future research and conclusions

In this thesis, I have demonstrated that the administration of bNAbs and ATIs are safe and do not impact the time to viral resuppression in a closely monitored trial setting along with the use of potent integrase inhibitors containing ART regimen. I report outcomes from the RIO study, which demonstrates LS-bNAbs are able to maintain prolonged viral suppression off ART compared to placebo, with a sub-group of participants who received bNAbs are able to achieve viral suppression beyond the duration of bNAbs presence in blood. The study is ongoing and further analyses will be undertaken on completion of the second stage of the study. Comparing the time to viral rebound outcomes of Arm B participants who have completed their second ATI compared to their first would answer the research question if the administration of bNAbs in the presence of viraemia enhances the vaccinal effect of bNAbs, due to the formation of immune complexes after administration, to prime longer-lasting HIV-specific immune responses that persist as bNAb concentrations are washed out. The long-lasting pharmacokinetics profiles of LS-bNAbs were comparable to previous studies of LS-bNAbs, however this was the first time they have been reported in a large trial of people diagnosed early in HIV with an extended ATI protocol. For participants who experienced viral rebound after receiving LS-bNAbs, viral rebound occurred at high serum bNAb concentrations than previously reported, and may represent ongoing viral replication and development of bNAb resistance over time, even as participants remain virologically suppressed. Rectal tissue bNAb levels were eight to nine-fold lower compared to serum, and may not be able to achieve high enough concentrations compared to the serum bNAb rebound thresholds. However, at 12 weeks after dosing, participants who remained virally suppressed after receiving bNAbs did not show any noticeable changes in their viral reservoir measures, or mucosal immune cell populations. Future planned pharmacokinetic analyses would include additional variables, such as reservoir sizes, laboratory markers of HIV-specific T cell responses, predicted bNAb neutralisation affinities, to develop more complex mechanistic PKPD models to explain and predict the effects of bNAbs, particularly for people who demonstrate longer-term post-bNAb viral suppression.

Assessment of immune cell function and the HIV reservoir remains challenging, due to limited cell yields, and the rare reservoir cell populations containing replication provirus in an already rare population of reservoir cells. This is akin to searching for a needle in a needle haystack in a haystack. Future research directions would include the being able to accurately measure intact or replication reservoir in tissue from people who receive bNAbs and other interventional drugs. Once suitable high throughput assays such as SIP-Seq are available and validated for use, I would be able to quantify and qualify the replication competent viral reservoir in these low-yield but vital tissue samples. I would also be able to measure reservoir changes from samples taken at later timepoints, to determine if there are further reservoir changes in virally suppressed individuals after bNAbs administration in Arm A, and for those participants in Arm B who demonstrated higher tissue total HIV measurements after their ATI, if these changes were transient and would return to baseline after being virally suppressed on ART for a year or longer, as they do in PBMCs (320,369,370). I would also be able to use both viral and proviral sequences from tissue and blood to investigate the relationship between baseline and rebound viral sequences, particularly with regards to the development and tissue compartmentalisation of bNAb resistance. To investigate changes in mucosal immunity, I would use single cell spatial transcriptomic platforms to characterise tissue sections in situ. Spatial transcriptomics assays such as the Nanostring CosMx platform have already been used to characterise tissue SHIV reservoir in macaque models (391). They allow the interrogation of these rare reservoir cells at a single cell level and associated changes in cellular gene expression, and would be able to detect changes in mucosal immunity not present in the bulk phenotype results at these early timepoints. This thesis generates some important hypothesised evolution of the bNAb therapeutic thresholds, and the lower tissue penetration of bNAbs compared to blood may be a key consideration in the development of future interventions or dosing strategies to target the tissue reservoir.

Developments within the field of research towards a HIV cure has rapidly progressed over the recent decades since the announcement of the first person cured of HIV. bNAbs represent one of the latest developments

to demonstrate promising results in conferring long-lasting remission for people living with HIV. With a subgroup of people who received two-doses of LS-bNAbs demonstrating sustained viral control beyond 72 weeks, we are approaching one of the criteria of an ideal target product profile of a HIV cure or remission agent, the ability to confer long term viral remission for at least two years (139). However, a combination approach with other immunotherapeutics or vaccinations may still be required to improve the efficacy of LS-bNAbs (392). Due to pace of research developments within the field, the use of platform studies would allow for rapid inclusion of novel therapeutics and/or gene therapies to be evaluated and compared in clinical trials, which may revolutionise the field and accelerate achieving the goal of a HIV cure. One such example of a platform study, the RECOVERY trial for investigating treatments for COVID-19 and other pneumonia (393), has made rapid progress identifying both effective therapies such as dexamethasone, tocilizumab and Baricitinib, and ineffective therapies for COVID-19. However, as low and middle-income countries are disproportionately affected by new HIV transmissions and a lack of access and funding for the HIV response, future studies should be inclusive of people from these settings to ensure any cure or remission strategy is effective, scaleable and accessible to these populations.

In conclusion, this thesis confirms LS-bNAbs are safe and effective and maintaining longer-term viral suppression for people living the HIV. They are able to penetrate the rectal tissue, albeit at lower levels compared to blood in people living with HIV. Despite the lower tissue exposures, LS-bNAbs appeared to be protective against early increases in the tissue HIV reservoir and HIV-associated mucosal immune injury if they remain virally suppressed after dosing. These findings will inform the development of improved immunotherapeutic strategies to better target the tissue-associated HIV reservoir.

List of communications

Data contained in or relevant to this thesis published during my studies are listed below:

Publications

1. Tipoe T, Ogbe A, **Lee MJ**, et al. Impact of Antiretroviral Therapy during Primary HIV Infection on T cell Immunity after Treatment Interruption. *Eur J Immunol*. 2024; 54(11):e2451200 [doi: 10.1002/eji.202451200]
2. **Lee MJ**, Eason M, Castagna A, et al. The impact of analytical treatment interruptions and trial interventions on time to viral re-suppression in people living with HIV restarting ART in cure-related clinical studies: A systematic review and meta-analysis. *J Int AIDS Soc*. J Int AIDS Soc. 2024; 27(8):e26349 [doi: 10.1002/jia2.26349]
3. Zacharopoulou P, **Lee MJ**, Oliveira TYK, et al. Prevalence of resistance-associated viral variants to the HIV-specific broadly neutralising antibody 10-1074 in a UK bNAb-naïve population. *Front Immunol*. 2024; 15:1352123. [doi: 10.3389/fimmu.2024.1352123]
4. **Lee MJ**, Godakandaarachchi P, Collins S, et al. Understanding participant perspectives around HIV cure-related studies involving antiretroviral analytical treatment interruption in the United Kingdom. *J Virus Erad*. 2023; 9:100360 [doi: 10.1016/j.jve.2023.100360]
5. **Lee MJ**, Collins S, Babalis D, et al. The RIO Trial: Rationale, design, and the role of community involvement in a randomized placebo-controlled trial of antiretroviral therapy plus dual long-acting HIV-specific broadly neutralising antibodies (bNAbs) in participants diagnosed with recent HIV infection – Study protocol for a two-stage randomized phase II trial. *Trials*. 2022; 23:263[doi: 10.1186/s13063-022-06151-w]
6. **Lee MJ**, Fidler S, Frater J. Immunotherapeutic approaches to HIV cure and remission. *Curr Opin Infect Dis*. 2022; 35(1):31-41 [doi: 10.1097/QCO.0000000000000803.]

Presentations

- 1. The use of long-acting broadly neutralising antibodies to confer HIV viral control. Results from the RIO study and RIO Gut sub-study**
Keystone Symposium: HIV cure and ART-free remission 2025, South Africa. [Oral presentation]
- 2. Clinical Trials using bNAbs.**
HIV Cure & Immunotherapy Forum, International AIDS Society Pre-Conference Workshop 2023, Australia. [Oral Presentation]
- 3. Impact of 10-1074LS and 3BNC117LS on viral rebound dynamics following treatment interruption six months after dosing: early results from the open label arm of the RIO trial.**
HIV Persistence during Therapy Reservoirs & Eradication strategies workshop 2022, USA. [Oral Presentation]
- 4. Comparison of HIV-specific broadly neutralising antibodies resistance prediction using plasma RNA, blood, and gut tissue HIV proviral sequences**
International Union of Sexually Transmitted Infections conference 2024, Australia [Poster presentation]
- 5. The Impact of HIV Cure-Related Study Drugs and Treatment Interruption on Viral Resuppression Rates**
Conference of Retrovirus and Opportunistic Infections 2024, USA. [Poster Presentation]
- 6. Attitudes towards participation in HIV cure trials which include a treatment interruption amongst participants in an observational study.**
European AIDS Conference 2023, Poland [Poster Presentation]
- 7. Comparing Viral Re-suppression Rates Following Antiretroviral Therapy Interruption in Cure-related Clinical Studies: A Systematic Review and Meta-Analysis of Intervention vs. Non-Intervention Trials.**
European AIDS Conference 2023, Poland [Poster Presentation]

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Appendix

Appendix Table 1. List of primers and Probes, their sequences, and corresponding positions against the HIV reference sequence (HXB2)

Target region	Primer/Probe name		Sequence	Position (HXB ref)	
5' Long terminal repeat	LTR OS		GRAACCCACTGCTTAASSCTC AA	506 – 528	
	LTR AS		TGTTCGGGCGCCACTGCTAGAGA	626 – 648	
	LTR Probe FAM		FAM – AGT RGT GTG TGC CCG TCT GTT G – BHQ-1	555 - 573	
Packaging site	IPDA PS Forward		CAGGACTCGGCTTGCTGAAG	692 – 711	
	IPDA PS Reverse		GCACCCATCTCTCTCCTTCTAGC	771 – 793	
	IPDA PS Probe FAM		56FAM/TTTTGGCGT/ZEN/ACTCACCAGT/3IABkFQ	740 - 758	
	IPDA PS Forward ExonucleaseResistant		CAGGACTCGGCTTGCTGA*A*G	692 – 711	
	IPDA PS Reverse ExonucleaseResistant		GCACCCATCTCTCTCCTTCTA*G*C	771 – 793	
env	IPDA PS Probe FAM ExonucResistant		56FAM/TTTTGGCGT/ZEN/ACTCACCAG*T*/3IABkFQ	740 - 758	
	IPDA Env Forward		AGTGGTGCAGAGAGAAAAAGAGC	7736 – 7759	
	IPDA Env Reverse		GTCTGGCCTGTACCGTCAGC	7832 – 7851	
pol	IPDA Env Probe HEX		5HEX/CCTTGGGTT/ZEN/CTTGGGA/3IABkFQ	7781 - 7796	
	iSCA Forward		TTTGAAAGGACCAGCAAA	4930 – 4948	
	iSCA Reverse		CCTGCCATCTGTTTTCCA	5042 - 5059	
gag	iSCA_Probe_FAM		56FAM-AAAGGTGAA/Zen/GGGGCAGTAGTAATACA-3IABkFQ	4959 - 4984	
	Nested Gag 5Outer		CTCTAGCAGTGGCGCCCGAA	627 – 646	
	Nested Gag 3Outer		TCCTTTCCACATTTCCAACAGCC	2023 - 2045	
env	Nested Gag 5Inner		ACTCGGCTTGCTGAAGTGC	696 – 714	
	Nested Gag 3Inner		CAATTTCTGGCTATGTGCCC	1984 – 2003	
	Nested Env 5Outer		TAGAGCCCTGGAAGCATCCAGGAAG	5853 – 5877	
env	Nested Env 3Outer		TTGCTACTTGTGATTGCTCCATGT	8913 – 8936	
	Nested Env 5Inner		CACCTTAGGCATCTCCTATGGCAGGAAGAAG	5953 – 5983	
	Nested Env 3Inner		GTCTCGAGATACTCGTCCCACCC	8882 – 8904	
RPP30 (Human)	RPP30_1 Forward		GATTTGGACCTGCGAGC	-	
	RPP30_1 Reverse		GCGGCTGTCTCCACAAG	-	
	RPP30_1 HEX-Prb		/5HEX/CT GAC CTG A/ZEN/A GGC TCT /3IABkFQ/	-	
	RPP30_2 Forward		GACACAATGTTTGGTACATGGTTAA	-	
	RPP30_2 Reverse		CTTTGCTTTGTATGTTGGCAGAAA	-	
RPP30_2 FAM_Prbs		/56-FAM/CC ATC TCA C/ZEN/C AAT CAT TCT CCT TCC TTC /3IABkFQ/		-	
Novel HIV-specific MultiDisplacement Amplification Exonuclease resistant Primers					-
	Primer name	Sense or Anti-Sense	Sequence	HXB starting position	
	MDA-1	Sense	CTAATTCAC*T*C	8	
	MDA-5	Sense	CTATTAGAT*A*C	2341	
	MDA-7	Sense	GCAGTAGTA*A*T	5001	
	MDA-9	Sense	CAATTATTG*T*C	7893	
	MDA-11	Sense	AATCTCTAG*C*A	9751	
	MDA-A	Anti-Sense	GATATCTTG*T*C	27	
	MDA-C	Anti-Sense	TATGTCATT*G*A	3309	
	MDA-D	Anti-Sense	TGAAACATA*C*A	5125	
	MDA-F	Anti-Sense	TAATTTCTC*T*G	8119	
	MDA-G	Anti-Sense	ATCTCTAGT*T*A	9669	
	HXB 14R MDA	Anti-Sense	ATCTGGTCT*A*A	465	
	HXB 2F MDA	Sense	GACTTGAAA*G*C	641	
	HXB 4F MDA	Sense	CATAAAGCAA*G*A	1858	
	HXB 3R MDA	Anti-Sense	CCATAAAGC*A*A	2030	
	HXB 11F MDA	Sense	AGGATATAAT*C*A	6536	
	HXB 11R MDA	Anti-Sense	GCATACATT*G*C	7521	
	HXB 14F MDA	Sense	ACTTACTTT*G*A	8535	
	HXB 13R MDA	Anti-Sense	TCAGCTACT*G*C	8685	

Appendix table 2. Search strategies for systematic review on the Medline database till 22 Apr 2024

#	Query	Results from Medline
1	exp HIV/ or exp HIV-1/	109142
2	exp Antiretroviral Therapy, Highly Active/ or exp Anti-Retroviral Agents/ or exp Anti-HIV Agents/	101243
3	Pause*.mp.	12724
4	Stop*.mp.	165587
5	Discontinuu*.mp.	159640
6	Interrupt*.mp	90263
7	exp Therapeutics/	5327024
8	Drug*.mp.	6811851
9	ART.mp.	175030
10	2 or 7 or 8 or 9	10535853
11	3 or 4 or 5 or 6	414160
12	1 and 10 and 11	2988
13	limit 12 to (english language and humans and yr="2015 -Current" and "all adult (18 plus years)" and english and last 9 years)	430

Appendix table 3. Search strategies for systematic review on the Embase database till 22 Apr 2024

#	Query	Results from Embase
1	aids virus.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	1,088
2	aids viruses.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	93
3	acquired immune deficiency syndrome virus.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	18
4	acquired immunodeficiency syndrome virus.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	32
5	hiv.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	481070

6	htlv-iii.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	1869
7	human immunodeficiency virus.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	524380
8	human immunodeficiency viruses.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	893
9	human t cell leukemia virus type iii.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	16
10	human t cell lymphotropic virus type iii.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	177
11	human t lymphotropic virus type iii.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	229
12	immunodeficiency virus, human.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	107
13	immunodeficiency viruses, human.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	4
14	lav-htlv-iii.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	240
15	lymphadenopathy associated virus.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	299
16	lymphadenopathy-associated viruses.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	0
17	virus, aids.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	231
18	virus, human immunodeficiency.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	390
19	virus, lymphadenopathy-associated.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	11
20	viruses, aids.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	6

21	viruses, human immunodeficiency.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	115
22	viruses, lymphadenopathy-associated.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	1
23	txid12721.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	0
24	hiv-1.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	106883
25	hiv-i.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	1100
26	human immunodeficiency virus type 1.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	22676
27	human immunodeficiency virus 1.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	101666
28	immunodeficiency virus type 1, human.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	13
29	txid11676.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	0
30	24 or 25 or 26 or 27 or 28 or 29	132663
31	1 or 2 or 3 or 4 or 5 or 6 or 7 or 8 or 9 or 10 or 11 or 12 or 13 or 14 or 15 or 16 or 17 or 18 or 19 or 20 or 21 or 22 or 23	591860
32	antiretroviral therapies, combination.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	0
33	antiretroviral therapy, combination.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	16
34	antiretroviral therapy, highly active.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	101
35	combination antiretroviral therapies.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	137

36	combination antiretroviral therapy.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	5353
37	haart.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	19122
38	therapies, combination antiretroviral.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	0
39	therapy, combination antiretroviral.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	4
40	32 or 33 or 34 or 35 or 36 or 37 or 38 or 39	24474
41	agent, antiretroviral.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	9
42	agents, anti-retroviral.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	2
43	anti retroviral agents.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	230
44	antiretroviral agent.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	453
45	41 or 42 or 43 or 44	691
46	aids drug.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	682
47	aids drugs.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	455
48	agent, anti-hiv.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	2
49	agents, anti-aids.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	1
50	agents, anti-hiv.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	13
51	anti aids agents.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	107

52	anti aids drug.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	124
53	anti aids drugs.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	127
54	anti hiv agent.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	450
55	anti hiv agents.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	1,326
56	anti hiv drug.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	786
57	anti hiv drugs.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	1,338
58	drug, aids.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	13
59	drug, anti-aids.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	1
60	drug, anti-hiv.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	9
61	drugs, aids.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	42
62	drugs, anti-aids.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	0
63	drugs, anti-hiv.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	8
64	46 or 47 or 48 or 49 or 50 or 51 or 52 or 53 or 54 or 55 or 56 or 57 or 58 or 59 or 60 or 61 or 62 or 63	4840
65	pause*.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	19131
66	Stop*.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	276296

67	Discontin*.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	283679
68	Interrupt*.mp.	137726
69	65 or 66 or 67 or 68	684111
70	(Treatment or Treatments or therapeutic or therapeutics or therapy or therapies).mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	15379826
71	ART.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	265677
72	drug*.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	14359545
73	40 or 45 or 64 or 70 or 71 or 72	21493210
74	30 and 31 and 69 and 73	4608
75	limit 74 to (human and english language and embase and english and yr="2015 - Current" and adult <18 to 99 years> and last 9 years)	578

Appendix table 4. Search strategies for systematic review on the Web of Science database till 22 Apr 2024

#	Query	Results from Wos
1	HIV (All Fields) or HIV-1 (All Fields)	466970
2	TS=("Antiretroviral Therapy, Highly Active" OR "Antiretroviral Agents" OR "Anti-HIV agents" OR "Therapeutics" OR "Drug" OR "ART")	2738328
3	TS=("Pause" OR "Stop" OR "Discontinue" OR "Interrupt")	171595
4	#1 AND #2 AND #3	786
5	#4 AND English (Languages) AND 2015 TO 2024 (Publication Year)	407

Appendix table 5. List of revisions

No.	Revision	Examiner's comment	Page(s)
1	Table 1.3.3.: Added two further cases of cure recently reported at CROI 2025 (The Chicago and Oslo patients) to	N/A	33
2	Chapter 3 Section 3.3.8: New section, describing modification of assay to measure intact proviral DNA using Env and PS probes. Chapter 3 Discussion: Added paragraph 'I was unable to optimise a modified IPDA by using two probes to ψ and <i>env</i>. This may be have been due to the autofluorescent properties of the double-emulsion droplets containing the two probes, or compensation errors when analysing droplets containing both probes. However, even if successful, the assay remains more time consuming and provides few advantages over the existing commercially available IPDA.'	We accept the candidate's explanation that in the flow-based assay he tried to set up to measure the reservoir, amplification is preferable because it will allow for more detailed genetic characterization of the reservoir. However, an alternative would be to perform IPDA which still allows him to distinguish between intact versus non-intact genomes. Why wasn't this attempted and if it was, potential explanations of why it didn't work.	124, 126
3	Figure 5.3.2.1: Updated figure to 72 weeks. Data censored at 72 weeks as small numbers beyond 72 weeks resulted in wide confidence intervals.	N/A	170
4	Figure 5.3.2.2: Updated figure to 72 weeks cutoff for same reason as above	N/A	171
5	Figure 5.3.2.3: Updated figure to 72 weeks cutoff for same reason as above	N/A	172
6	Figure 5.3.2.4: Updated figure to 72 weeks cutoff for same reason as above	N/A	173
7	Chapter 5 Discussion: Added recent relevant studies – the FRESH study of 2 LS-bNAbs in S. Africa, and a meta-analysis of rates of PTC in people with HIV.	N/A	176
8	Chapter 6 Discussion: Added 'More in-depth mechanistic models may consider factors such as antibody-antigen binding and dissociation rates, target saturation, rates of viral replication and reactivation of HIV-containing latent reservoir cells. Different models of clearance of virus-antibody immune complexes have been described using data from a study of VRC01 monotherapy in people living with HIV (365), and these models may be further investigated with the two LS-bNAbs used in RIO. '	In the PK discussion, please very briefly mention other potential PK or PD measurement/type of investigation which could further characterise the PK/PD relationship, such as saturation of all receptors, half life of the binding of the antibody to the target.	212

9	Chapter 8: Expanded discussion on reasons for viral rebound at higher bNAb concentrations 1) Increased resistance over time, 2) Cell-to-cell transmission	We would like the candidate to provide potential explanations in the discussion on why there was viral rebound in the presence of antibodies in some cases.	244
10	Chapter 8: Added paragraph ‘Developments within the field of research towards a HIV cure has rapidly progressed over the recent decades since the announcement of the first person cured of HIV. BNABs represent one of the latest developments to demonstrate promising results in conferring long-lasting remission for people living with HIV. With a sub-group of people who received two-doses of LS-bNABs demonstrating sustained viral control beyond 72 weeks, we are approaching one of the criteria of an ideal target product profile of a HIV cure or remission agent, the ability to confer long term viral remission for at least two years (139). However, a combination approach with other immunotherapeutics or vaccinations may still be required to improve the efficacy of LS-bNABs (392). Due to pace of research developments within the field, the use of platform studies would allow for rapid inclusion of novel therapeutics and/or gene therapies to be evaluated and compared in clinical trials, which may revolutionise the field and accelerate achieving the goal of a HIV cure. One such example of a platform study, the RECOVERY trial for investigating treatments for COVID-19 and other pneumonia (393), has made rapid progress identifying both effective therapies such as dexamethasone, tocilizumab and Baricitinib, and ineffective therapies for COVID-19. However, as low and middle-income countries are disproportionately affected by new HIV transmissions and a lack of access and funding for the HIV response, future studies should be inclusive of people from these settings to ensure any cure or remission strategy is effective, scaleable and accessible to these populations.’	In the discussion/future directions, include some discussion on the future of immunotherapy and gene therapy for HIV cure the potential of these kinds of interventions for public health impact in low- and middle-income countries, i.e., a little bit of a crystal ball into the future of the field.	249
11	Appendix: List of revisions added	NA	291