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**Evaluation of neutralization sensitivity of HIV-1 subtype C
between plasma, peripheral blood mononuclear cells and lymph
node-derived viruses**

BY

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Science in Virology, College of Health Sciences, University of KwaZulu-Natal, HIV
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Preface

The experimental procedures presented in this thesis were performed at the Hasso Plattner Research Laboratory, Doris Duke Medical Research Institute, Nelson R. Mandela School of Medicine, University of KwaZulu Natal, Durban, South Africa. This dissertation represents the original work of the author and has not been submitted for any degree or examination at any other university. Where the work of others has been used, the authors have been duly acknowledged.

This study was performed under the supervision of Dr Bongiwe Goodness Ndlovu and the co-supervision of Dr Kamini Gounder.

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As the candidate's co-supervisor I have/have not approved this thesis for submission.

Signed:  Name: Kamini Gounder Date: 28 January 2026

Plagiarism declaration

I, Keyura Velan declare that:

(i) The research reported in this thesis is my original research and it has not been submitted for any degree or examination at any other university.

(ii) My contribution to the project was as follows: Writing a research proposal, obtaining ethics approval, conducting laboratory experiments, data collection, data analysis and manuscript preparation.

(iii) This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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a) Where their exact words have been used, then their writing has been placed in italics and inside quotation marks, and referenced.

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Date: 29 January 2026

Ethics approval

Ethics approval for this study was obtained from the Biomedical Research Committee of the Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa (BREC/00006367/2023) (Appendix). The study participants provided written informed consent prior to participating in the study.

Contents

Plagiarism declaration	iii
Ethics approval	iv
List of presentations	7
Dedication	8
Acknowledgements	9
List of tables and figures	10
List of abbreviations	xii
List of Amino acids	x
Abstract	xi
Chapter 1: General introduction	1
Study Aim and objectives	3
Chapter 2: Literature review	4
2.1. Background	4
2.2. Origin, classification and diversity of HIV-1	5
2.3 HIV structure and genome organization	6
2.4 HIV Envelope trimer and viral entry	8
2.5 HIV life cycle	10
2.6 Innate immune responses	12
2.7 Adaptive immune responses	13
2.7.1 Cell mediated immune responses	13
2.7.2 Humoral immune responses	14
2.7.2.1 B cell activation and production of the initial antibody responses	14
2.7.2.2 Fc-mediated effector functions	15
2.8 Neutralizing antibodies and factors associated with their development	16
2.9 Different classes of broadly neutralizing antibodies	17
2.10 Antibody-based vaccines	20
2.11 Latent HIV-1 reservoirs and viral compartmentalization	23
2.12 Study Rationale	26
2.13 Review of Methodology	28
Chapter 3: Methods and Materials	30
3.1 Study participants	30
3.2 Clinical sample collection	30

3.3 Sequence analysis and SGA selection.....	31
3.4 Amplification of HIV-1 <i>env</i>	32
3.5 PCR purification and gel extraction.....	32
3.6 TOPO cloning of full-length HIV-1 <i>env</i>	33
3.7 Sanger sequencing of HIV-1 <i>env</i> clones	33
3.8 Maintenance of 293T and TZM-bl cells.....	34
3.9 Production of HIV-1 pseudoviruses	34
3.10 TCID assay	35
3.11 TZM-bl micro-neutralisation assay.....	35
3.12 Statistical analysis	36
Chapter 4: Results.....	38
4.1 Clinical Characteristics	38
4.2 Partial diversity of HIV-1 subtype C <i>env</i> sequences.....	39
4.3 Compartmental assessment of HIV-1 <i>env</i> variable domain lengths and HIV-1 <i>env</i> variable domain potential N-linked glycosylation sites	42
4.4 Amino acid variations in bnAb epitopes	45
4.5 Neutralisation Sensitivity between lymph nodes, PBMC and plasma compartment.....	53
4.6. Intra-participant differences in neutralisation sensitivity and amino acid signatures	57
Chapter 5: Discussion	66
Conclusion and implications for HIV cure strategies and bnAb trials	71
Chapter 6: References:	72
Chapter 7: Supplementary information	88
Appendix.....	101

List of presentations

- **Keyura Velan**, Nombali Gumede, Ntokozo Ntshangase, Silondolozé Mtanjana, Nelisiwe Zikhali, Thumbi Ndung'u, Kamini Gounder, Bongiwe Ndlovu (2024). *Evaluation of neutralization sensitivity of HIV-1 Subtype C between lymph nodes, peripheral blood mononuclear cells and plasma derived viruses*. Oral presentation at the College of Health Science (CHS) Annual Research Symposium, Durban, South Africa
- **Keyura Velan**, Nombali Gumede, Ntokozo Ntshangase, Silondolozé Mtanjana, Nelisiwe Zikhali, Thumbi Ndung'u, Kamini Gounder, Bongiwe Ndlovu (2025). *Evaluation of neutralization sensitivity of HIV-1 Subtype C between lymph nodes, peripheral blood mononuclear cells and plasma derived viruses*. Poster presentation at the 10th Annual South African Immunology Society (SAIS) 2025- Immunology at the Centre of One Health Congress, Durban, South Africa

Dedication

*To my loving family,
My heart will always be a flower at your feet*

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- To my brother, Shiven; my parents, Ravi and Rohini Velan; my aunt and uncle Cookie and Booyesen Velan, who are nothing less than parents to me and to my family; for your endless love and sacrifices.

List of tables and figures

Figure 1.1: A schematic diagram showing the genomic organization of the HIV-1 virion

Figure 1.2: A schematic diagram showing the organization of the HIV-1 genome

Figure 1.3: Schematic diagram of the *env* construct featuring the gp120 and gp41 subunits

Figure 1.4: HIV Replication cycle

Figure 1.5: Surface representation of the epitopes of HIV-1 antibodies on an *env* glycoprotein trimer showing seven major epitopes such as the membrane proximal external region (MPER) (peach), the gp120/gp41 interface (green), CD4 binding site (red), V1V2 glycan (blue), V3 glycan (purple), silent face (orange) and the fusion peptide (pink)

Table 4.1: Characteristics of study participants

Figure 4.1: Phylogenetic analysis of full-length HIV-1 *env* sequences derived from the plasma, PBMC and lymph nodes derived viruses in four participants from the HPP and lymph node cohorts.

Figure 4.2: Comparison of the V1-V4 loop lengths between *env* sequences generated from lymph node, PBMC and plasma compartments.

Figure 4.3: Comparison of the potential N-linked glycosylations (PNGs) in the V1-V4 loops of the *env* sequences generated from plasma, PBMC and lymph nodes compartments.

Figure 4.4: Amino acid differences detected in the various bnAb epitopes (A-U)

Table 4.2: Amino acid frequencies in the various bnAb epitopes

Figure 4.5: Neutralisation sensitivity data in lymph nodes, PBMCs and plasma-derived clones.

Figure 4.6: Neutralisation data presented as IC50 titers (mg/mL) in which each virus is represented by an individual circle.

Table 4.3: HIV-1 *env* sequence alignment representing the CD4 binding site, including the D loop, CD4 binding region and the V5 loop.

Table 4.4: HIV-1 *env* sequence segments representing the V2 loop.

Table 4.5: Sequence segments representing the V3 glycan.

Figure 4.7: Comparison of the V1 loop lengths between PGT121-sensitive and resistant HIV-1 *env* clones to the V3 glycan from PL, PBMC and LN compartments

Supplementary Table 1: HIV *env* primer sequences used in the first and second round of amplification of the *env*

Supplementary Table 2: HIV-1 *env* primer sequences used in the Sanger sequencing of the TOPO clones generated after second round amplification

Supplementary figures 7.1 A-D: Phylogenetic trees generated per PID

Supplementary figure 7.2 A-D: Highlighter plots generated for each PID showing silent and non-silent mutations

Supplementary tables 7.3A-D: Logo plot frequencies

Appendix: BREC approval

Appendix: Turnitin score

List of abbreviations

AIDS: Acquired immune deficiency syndrome

ADCP: Antibody dependent cellular phagocytosis

ADCDC: Antibody dependent complement dependent cytotoxicity

ADCC: Antibody-dependent cellular cytotoxicity

ART: Antiretroviral treatment

ARV: Antiretroviral

CDRH3: Complementary heavy chain determining region

DCs: Dendritic cells

bnAbs: Broadly neutralizing antibodies

CCR5: C-C Chemokine receptor 5

CXCR4: C-X-C chemokine receptor type 4

CRFs: circulating recombinant form

Env: Envelope glycoprotein

HIV-1: Human immunodeficiency virus type 1

HIV-2: Human immunodeficiency virus type 2

LN: lymph node

mAb: Monoclonal antibody

mRNA: Messenger Ribonucleic Acid

NK cells: Natural killer cells

nNAbs: non-neutralizing antibodies

PBMC: Peripheral blood mononuclear cells

PEP: Post-exposure prophylaxis

PL: Plasma

PrEP: Pre-exposure prophylaxis

RNA: Ribonucleic acid

RT: Reverse Transcriptase

SGA: single genome amplicon

SIV: Simian immunodeficiency virus

ssRNA: Single stranded ribonuclease acid

T/F: Transmitted Founder

μL: Microlitre

w.p.i.: Weeks post infection

List of Amino acids

Amino acid	3-letter abbreviation	1-letter abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Abstract

Background: Genetic variations have been observed in the HIV-1 *env* single genome amplicons (SGA) derived from plasma, peripheral blood mononuclear cells (PBMCs) and lymph node samples. However, the impact of these amino acid variations on the sensitivity of broadly neutralizing antibodies is not known. Therefore, the aim of this study was to evaluate HIV-1 *env* sequence differences within bnAb epitopes and assess their neutralization sensitivity in HIV-1 subtype C between plasma (PL), PBMC and lymph node (LN)-derived clones.

Methods: Full-length HIV-1 *env* gene were amplified from four HIV-1 subtype C-infected ART-naïve individuals selected from the FRESH cohort based in KwaZulu-Natal, South Africa. Single genome amplicons (SGAs) were produced from plasma, PBMC and LN samples, sequenced by Sanger sequencing and cloned into a TOPO 2.1 vector. The *env* pseudoviruses were prepared through co-transfection of an envelope plasmid with the pSG3Δ Env backbone into 293T cells using the TransGENE 9 reagent. The neutralization sensitivity was assessed using the TZM-bl neutralization assay against a panel of broadly neutralizing antibodies (bnAbs) targeting the CD4 binding site, V1V2 loop, V3 glycan, gp120/gp41 interface, MPER, fusion peptide and silent face epitopes. HIV-1 *env* sequences were analyzed within bnAb epitopes and the neutralisation sensitivity was compared between LN, PBMCs and plasma samples.

Results: Phylogenetic analysis of HIV-1 *env* sequences across participants revealed consistent and strong participant-specific clustering, confirming that viral populations evolved independently within each individual. Plasma sequences tended to have a slightly longer V1 loop lengths and more potential N-linked glycosylation sites (PNGS) in the V4 loop, compared to PBMC and LN clones, suggesting a marginally denser glycan shield. A high frequency of discordant amino acid mutations was observed in all three compartments in the V2 apex and the V3 loops while few discordant amino acid mutations were observed in the conserved regions including the CD4 binding site, MPER and gp120-gp41 interface. The highest frequency of resistant mutations was observed in the plasma compartment followed by PBMCs and LN. Discordant mutations were associated with differences in neutralization sensitivity between viruses derived from LN, PBMCs and plasma.

Conclusion: Our findings suggest that plasma viruses have a higher frequency of resistance mutations within bnAb epitopes, and lower neutralisation sensitivity compared to PBMC and lymph nodes. However, there was no evidence of compartmentalisation, and minor differences in variable loop length and potential N-linked glycosylation sites. These findings provide a preliminary insight on the ability of bnAbs to reduce the size of viral reservoirs in PL and LN compartments. These findings may also influence the selection of bnAbs in immunotherapy studies.

Chapter 1: General introduction

Human immunodeficiency virus (HIV) infection is one of the leading pandemics globally; approximately 1.3 million new infections and 630,000 AIDS-related deaths occurred in 2023, despite the availability of effective HIV treatments (UNAIDS, 2024). Current biomedical prevention strategies include pre-exposure prophylaxis (PrEP), post-exposure prophylaxis (PEP), medical male circumcision, condom usage and access to sterile needles and syringes (Mody et al., 2024, Swendeman et al., 2024). However, these prevention strategies are inadequate due to various challenges such as lack of access to healthcare, HIV-related stigma and non-adherence; therefore, an effective vaccine is needed (Haberer et al., 2023, Obeagu and Obeagu, 2024b). However, the main challenges in the HIV-1 vaccine development include genetic diversity, and the design of immunogens that are capable of eliciting broadly neutralizing antibodies (bnAbs) that have the ability to neutralize diverse HIV-1 strains (Mahomed, 2024, Boomgarden and Upadhyay, 2025).

Previous studies have shown that bnAbs are capable of preventing Simian-Human Immunodeficiency Virus (SHIV) infection or suppress viremia in non-human primates and humanized mice (Nishimura and Martin, 2017, Parsons et al., 2018, Sun et al., 2021). In addition, bnAbs also reduced plasma viremia and delayed viral rebound in participants living with HIV during antiretroviral treatment interruption (ATI) (Bar-On et al., 2018, Thavarajah et al., 2024). Other studies have shown that bnAbs enhance the clearance of HIV-infected cells through enhancement of effector functions such as antibody-dependent cellular cytotoxicity (ADCC) and expansion of CD4⁺ and CD8⁺ T cell responses (Briel et al., 2017, Davies et al., 2019, Powell et al., 2020). Recently, the antibody mediated prevention (AMP) clinical trial found that VRC01 (CD4-binding site antibody) did not prevent the overall HIV-1 acquisition more effectively than placebo in participants at high risk of HIV acquisition. However, analyses of VRC01-sensitive HIV-1 isolates provided proof-of-concept that bnAb prophylaxis could be effective in humans (Corey et al., 2021). While the use of bnAbs is promising, optimal combinations, dosing regimens, and their relevance in other body compartments remain to be fully established. Furthermore, there is an emergence of antibody-resistant strains, therefore a combination of bnAbs that target different epitopes may be required to protect against globally diverse strains (Landais et al., 2016, Dufloo et al., 2022, Mkhize et al., 2023, Ndlovu et al., 2025).

Neutralization susceptibility varies geographically, with contemporary Indian subtype C viruses showing greater sensitivity to V3- and CD4bs-directed bnAbs, while South African subtype C viruses are more sensitive to CD4bs and V2 apex bnAbs. These findings highlight inter-subtype differences across geographical regions and underscore the importance of continuous viral surveillance to ensure that interventions remain relevant to currently circulating viruses (Mullick et al., 2021, Sutar et al., 2025).

Other studies compared viral populations circulating in peripheral blood and viral reservoir sites; they found differences in viral populations, particularly in HIV-1 *env* and *gag* sequences between blood and lymph nodes (Mandizvo et al., 2022, Jeewanraj et al., 2023). In addition, discordant drug resistance mutations and coreceptor usage were also reported between cerebrospinal fluid (CSF) and plasma viral populations (Pillai et al., 2006, Stefic et al., 2017, Sojane et al., 2018, Kelentse et al., 2020). Furthermore, HIV-1 *env* variants derived from the CSF were more resistant to bnAbs compared to the paired plasma variants (Stefic et al., 2019). However, there is limited information on bnAb sensitivity between plasma and LN-derived clones, which could be due to the difficulty of collecting lymph node samples in people living with HIV. This technique is quite invasive and it is associated with various risks, hence there are few studies that characterize lymph nodes. This highlights the need for neutralisation sensitivity testing to determine the differences in sensitivity profiles between LN, PBMC and plasma compartments.

Study Aim and objectives

The aim of this study was to analyze the HIV-1 *env* sequence variations and evaluate the neutralisation sensitivity between plasma, PBMC and lymph node-derived viruses.

Objectives

- 1) Analysis of HIV-1 *env* variable loop lengths, potential N-linked glycosylation sites, and amino acid signatures within bnAb epitopes between plasma, PBMC and lymph node compartments.
- 2) Evaluation of neutralisation sensitivity between variants derived from plasma, PBMC and lymph nodes.

Chapter 2: Literature review

2.1. Background

Approximately 61% of new HIV infections occurred in sub-Saharan Africa, with nearly 50% in adolescent girls and young women between the ages of 15-24 years (Chen-Charles et al., 2025). The high rate of HIV transmission in young women is attributed to low socio-economic status and relationships with older men, which reinforces unequal gender power dynamics (Mabaso et al., 2021). Various HIV prevention interventions are available worldwide; these include social, behavioral and biomedical tools. Social interventions include HIV counseling and testing, sexual education, changing social norms and policies to promote prevention (Haberer et al., 2023, Obeagu and Obeagu, 2024b).

Behavioral interventions include abstinence, condom use and practicing safe sex. While, biomedical tools include the use of sterile needles and syringes, treatment of sexually transmitted infections (STIs), medical male circumcision, the use of pre-exposure prophylaxis (PrEP) and post-exposure prophylaxis (PEP) (Larki et al., 2025). These prevention tools are highly recommended for high-risk individuals including adolescent girls and young women, men who have sex with men (MSM), transgender and female sex workers (Jiang et al., 2016). However, current prevention tools are sub-optimal due to various challenges including lack of awareness, inadequate access to healthcare, side effects associated with PrEP, fear of HIV-related stigma and self-perceptions of not being a suitable candidate for PrEP (Bogart et al., 2025). These challenges highlight the need for an effective prevention and treatment strategy.

2.2. Origin, classification and diversity of HIV-1

HIV belongs to the family of *Retroviridae*, sub-family of *orthoretrovirinae* and *lentivirus* genus (Luciw et al., 1984, Weis, 1994, Himathongkham and Luciw, 1996, Douek et al., 2009, Bogart et al., 2025). HIV-1 and HIV-2 are two distinct types of the human immunodeficiency virus (Luciw et al., 1984). HIV-1 has high virulence and faster disease progression, while HIV-2 has less virulence and slower disease progression (Bayón-Gil et al., 2025). HIV-1 origin is traced back to Simian Immunodeficiency Virus (SIV) isolated from chimpanzees known as *Pan troglodytes* (SIVcpz) (Hirsch et al., 1989, Huet et al., 1990, Sharp et al., 2000). In contrast, HIV-2 origin is traced back to Simian Immunodeficiency Virus isolated from sooty mangabeys (SIVsmm), monkeys and apes (Gao et al., 1999, Sharp and Hahn, 2010).

HIV-1 was first isolated in 1983 and was identified as the causative agent of acquired immunodeficiency syndrome (AIDS) (Barré-Sinoussi et al., 1983, Gallo and Montagnier, 1988). HIV-1 is the most prevalent, and is responsible for almost 95% of all HIV infections worldwide (Locatelli et al., 2008). HIV-1 is subdivided into various groups such as the major (M), outlier (O), group N and group P (Gustchina and Weber, 1991, De Cock et al., 1993). HIV-1 group M is responsible for the pandemic which likely began in the region of Kinshasa, Democratic Republic of Congo in the early 1980s (Sharp and Hahn, 2010). Group M is further classified into nine distinct subtypes, denoted with letters namely: A, B, C, D, F, G, H, J and K (Gao et al., 1998). Subtype A remains the most prevalent subtype in East Africa, Russia and former Soviet Union countries (Hemelaar et al., 2006, Kerina et al., 2013, Hemelaar et al., 2020). Subtype B is the most prevalent in Europe, Americas and Oceania; while subtype C is the most prevalent in Southern Africa, Ethiopia and India; CRF01_AE is prevalent in Asia, subtype G and CRF02_AG are prevalent in West Africa (Tully and Wood, 2010, Kerina et al., 2013).

While HIV-1 is distributed worldwide, HIV-2 has been reported in West Africa, Europe, South America, Asia and the United States (McCutchan, 2006). HIV-2 is classified into five subtypes (subtype A- E), with subtypes A and B being the most prevalent (Gao et al., 1999). Studies have shown that HIV-2 infected participants elicit higher levels of humoral immune responses that appear to be more protective against disease progression (Nyamweya et al., 2013, Balasubramaniam et al., 2019). This high genetic diversity has made the development of effective monitoring tests, vaccine development and cure strategies a significant challenge in the field (Kerina et al., 2013, Salimi et al., 2020).

2.3 HIV structure and genome organization

HIV-1 virion is spherical in shape and the outer layer is made up of the lipid bilayer membrane and *env* spike glycoproteins (Fig 2.1) (Gelderblom et al., 1987, Salimi et al., 2020). Each HIV-1 envelope spike is made up of three molecules of gp120 and gp41 subunits joined together by disulphide bonds (Li et al., 2000). Beneath the envelope, there is a matrix (MA) protein, which is involved in viral assembly and plays an important role in viral replication (Frankel and Young, 1998). Beneath the matrix, there is a viral capsid which forms the core of the virus particle that surrounds single stranded RNA, nucleocapsid (NC) and viral enzymes (Frankel and Young, 1998). The nucleocapsid (NC) proteins, also known as p7, protects RNA inside the virion core from digestion by nucleases and they are involved in virion assembly (Clever and Parslow, 1997, Laughrea et al., 1997). Viral enzymes are involved in replication and maturation of the viruses.

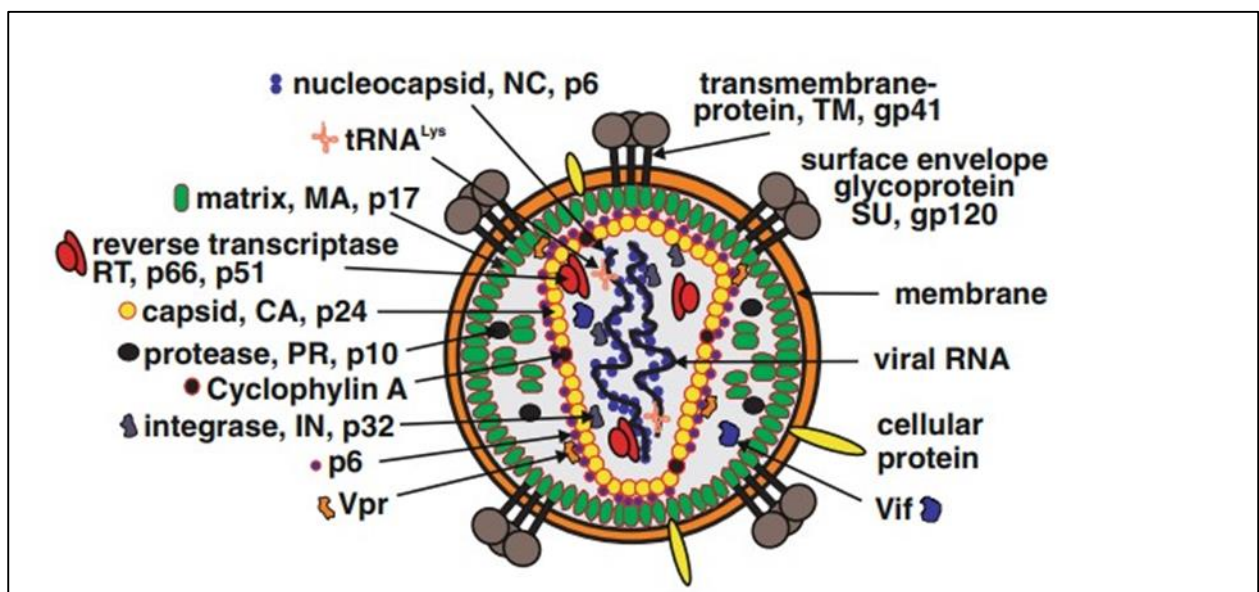


Figure 2.1: A schematic diagram showing the genomic organization of the HIV-1 virion (Kirchhoff, 2013).

The HIV genome is made up of single stranded RNA, which is ~9 kb long and it is the largest component of the virion (Guntaka, 1993). HIV genome is flanked by two long terminal repeats (LTRs), at the 5' and 3' ends; the 5' LTR acts as a promoter for viral gene transcription, while the 3' LTR is involved in gene transcription termination (Guntaka, 1993, Klaver and Berkhout,

1994). The genome is divided into structural, regulatory and accessory genes; structural genes include *gag*, *env* and *pol* and these are located after the 5' LTR (Guntaka, 1993, Himathongkham and Luciw, 1996, Madlala et al., 2023). The *gag* gene encodes structural proteins that are essential for viral assembly and maturation. It encodes a Gag p55 polyprotein precursor that is cleaved by the viral protease into matrix (MA), capsid (CA), and nucleocapsid (NC) (Fig 2.1) (Fun et al., 2012, Zhang et al., 2025).

The *env* gene codes for gp160 glycoprotein, which is cleaved by furin-protease into gp120 surface protein and gp41 transmembrane glycoprotein, and they play an important role in viral entry into host cells (Fig 2.2) (Anokhin and Spearman, 2022). In contrast, *pol* gene codes for protease (PR), reverse transcriptase (RT), integrase (IN) and RNase H enzymes (Avidor and Giladi, 2004). Reverse transcriptase is responsible for converting the viral RNA into double-stranded DNA (Del Amo et al., 2020). Integrase facilitates the integration of the viral DNA into the host genome (Hu and Hughes, 2012), while protease is responsible for cleaving the Gag and Gag-Pol polyproteins into functional proteins necessary for viral assembly and maturation (Hu and Hughes, 2012). RNase H is crucial for viral replication as it degrades the RNA strand which allows for the synthesis of viral DNA and removal of primers needed to facilitate this process; resulting in making it a target for anti-HIV targets (Hu and Hughes, 2012).

The HIV genome also contains two regulatory genes such as *tat* and *rev*; *tat* codes for the Tat protein, an important regulator of viral transcription and recruits cellular transcription factors to the HIV-1 promoter (Gatignol, 2007). The *rev* gene codes for Rev protein that regulates post-transcriptional processing of viral mRNAs and exports unspliced and partially spliced viral RNAs from the nucleus into the cytoplasm (Suhasini and Reddy, 2009).

Finally, HIV-1 also contains four accessory genes such as *nef*, *vif*, *vpr* and *vpu*, the *nef* gene codes for the Nef protein that downregulates CD4 receptors on CD4⁺ T helper cells (Seelamgari et al., 2004, Virgilio, 2024). Nef also downregulates HLA-class I molecules which are expressed by all nucleated cells (Garcia and Miller, 1991, Mann et al., 2013). In addition, *vif* gene codes for Vif protein and it is involved in the inhibition of cytoplasmic defenses. While *vpr* codes for the Vpr protein which induces cell-cycle arrest (Andersen et al., 2008). The *vpu* codes Vpu protein which promotes degradation of CD4 molecule in the endoplasmic reticulum, it also enhances viral assembly and release from the cells (Cohen et al., 1996, Stephens et al.,

1997). Together, these proteins enhance HIV-1 replication, spread into naïve cells and allow HIV to evade host immune responses.

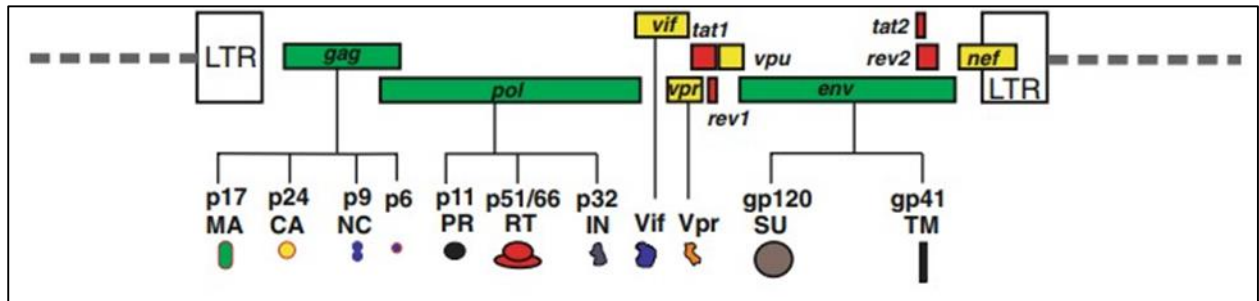


Figure 2.2: A schematic diagram showing the organization of the HIV-1 genome (Kirchhoff, 2013).

2.4 HIV Envelope trimer and viral entry

HIV-1 envelope (Env) trimer is a complex, membrane-bound protein structure encoded by the Gp160 precursor, the trimer plays a crucial role in viral entry into host cells (Bour et al., 1995, Salzwedel, 1997, Elalouf et al., 2024). It is composed of three subunits, each consisting of a gp120 exterior subunit and a gp41 transmembrane subunit (Fig 2.3) (Chen, 2019, Andeweg, 1995). The Env trimer serves as the primary target for neutralizing antibodies (Jenness et al., 2017). Gp120 subunit is located on the outer surface of the viral membrane, it is made up of five conserved regions (C1-C5) interspersed with five variable regions (V1-V5) (Fig 2.3) (Kleim et al., 1996, Cashin et al., 2014).

Gp120 has a very high genetic diversity, it evolves faster (changing 1–2% per year) than any other region of the HIV genome and this ability to mutate plays an important role in immune evasion (Crandall, 1999). It is heavily glycosylated with approximately 20 potential N-linked glycans that mask the surface of the trimer and prevent interactions with neutralizing antibodies (Yu et al., 2015). Gp120 contains the CD4-binding site, formed by several conserved regions including the D-loop, CD4 binding region, and the V5 loops across the gp120, the CD4bs interacts with the CD4 receptors on the cell surface and initiates viral attachment and entry of the virus into the host cell (Kwong et al., 1998, Sullivan et al., 2003). Furthermore, the V3 loop

binds to CCR5 or CXCR4 coreceptors during viral entry and it is an important epitope for neutralizing antibodies (Arrildt et al., 2012).

In contrast, gp41subunit is a conserved region, it is divided into three major domains: the ectodomain, transmembrane domain (TM) and the cytoplasmic domain (CT) (Fig 2.3) (Blumenthal et al., 2014). The ectodomain, is further subdivided into the fusion peptide (FP), N-terminal heptad repeat (NHR), C-terminal heptad repeat (CHR) with heptad-repeat regions (HR1 and HR2) and the membrane-proximal external region (MPER) (Daste et al., 2018, Cano Muñoz, 2022). The trimeric heptad repeat domains HR1 and HR2 play a key role in HIV-1 entry during membrane fusion (Mzoughi et al., 2019). While, the fusion peptide facilitates fusion of the host and viral membranes (Garg et al., 2011, Haqqani and Tilton, 2013, Blumenthal et al., 2014). The MPER of gp41 interacts with the fusion peptide to promote membrane perturbations that ensure fusion pore opening (Liu et al., 2000). Finally, the cytoplasmic tail plays an important role in mediating envelope intracellular trafficking and incorporation into assembling virions (Sigfrid et al., 2017). Gp41 is highly conserved, with only four N-linked glycosylation sites within a region flanked by two highly conserved cysteine amino acids and a hydrophobic membrane domain (Brodsky et al., 1990).

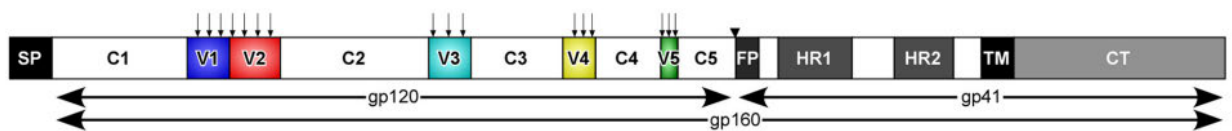


Figure 2.3: Schematic diagram of the *env* construct featuring the gp120 and gp41 subunits (Nakane et al., 2015).

2.5 HIV life cycle

HIV infects the host cells such as dendritic cells, monocytes and macrophages that express CD4 receptors and chemokine receptors on their surface. During HIV entry (Fig 2.4) (step 1), gp120 binds to the CD4 receptor on the surface of the host cell, this is followed by binding of chemokine receptors (CCR5 or CXCR4) to the V3 loop of gp120 (Moore and Chaisson, 1999, Rossi et al., 2021). This interaction induces additional conformational changes that allow exposure of the gp41 transmembrane protein, the fusion peptide is inserted into the cell membrane (Dore et al., 2004, Cai et al., 2012). The trimeric gp41 complex then forms a helical bundle structure that joins the cellular and viral membranes together resulting in virion entry into the host cell (Wilén et al., 2012, Kirchhoff, 2013).

In the cell cytoplasm, single stranded viral RNA is converted into double-stranded DNA by reverse transcriptase enzyme and this process is known as reverse transcription (step 2) (Payne and Kohler, 2017). This is followed by uncoating (step 3) which refers to the disassembly of the viral capsid into the cytoplasm (Arhel and Kirchhoff, 2010, Ambrose and Aiken, 2014). After uncoating, the viral capsid migrates into the nucleus through the nuclear pore complexes, and this process is known as the nuclear import (step 4). Reverse transcription continues in the nucleus, which is followed by the integration (step 5) of cDNA into the host genome and this process is facilitated by the integrase enzyme. The integrated cDNA is known as the proviral DNA (Colin and Van Lint, 2009). Thereafter, the integrated HIV proviral DNA serves as a template for viral transcription (step 6) where the integrated viral DNA is copied into viral mRNA (messenger RNA) and genomic RNA to make new virus particles. After transcription, mRNA is transported out (step 7) of the nucleus into the ribosomes, where mRNA is translated (step 8) into proteins (Kirchhoff, 2013). Some proteins are produced as precursors such as Gag, Gag-Pol, while other proteins are produced as functional proteins including Tat, Rev, Nef, Vpr and Vif (Williams et al., 2006, Goff, 1990). Translation is followed by viral assembly (step 9); the viral genome and proteins are assembled together to form a new viral particle (Kirchhoff, 2013).

This is followed by the budding (step 10) process; the immature virion buds off from the plasma membrane of the host cell. Thereafter the virion is released from the cell. As the virion buds from the cell, it acquires a lipid envelope from the host cell membrane (Kirchhoff, 2013, Welch et al., 2007). The final step is maturation (step 11) where Gag, Gag-Pol and Env proteins are

then cleaved by HIV protease into mature viral proteins that can perform various functions (Barrie et al., 1996).

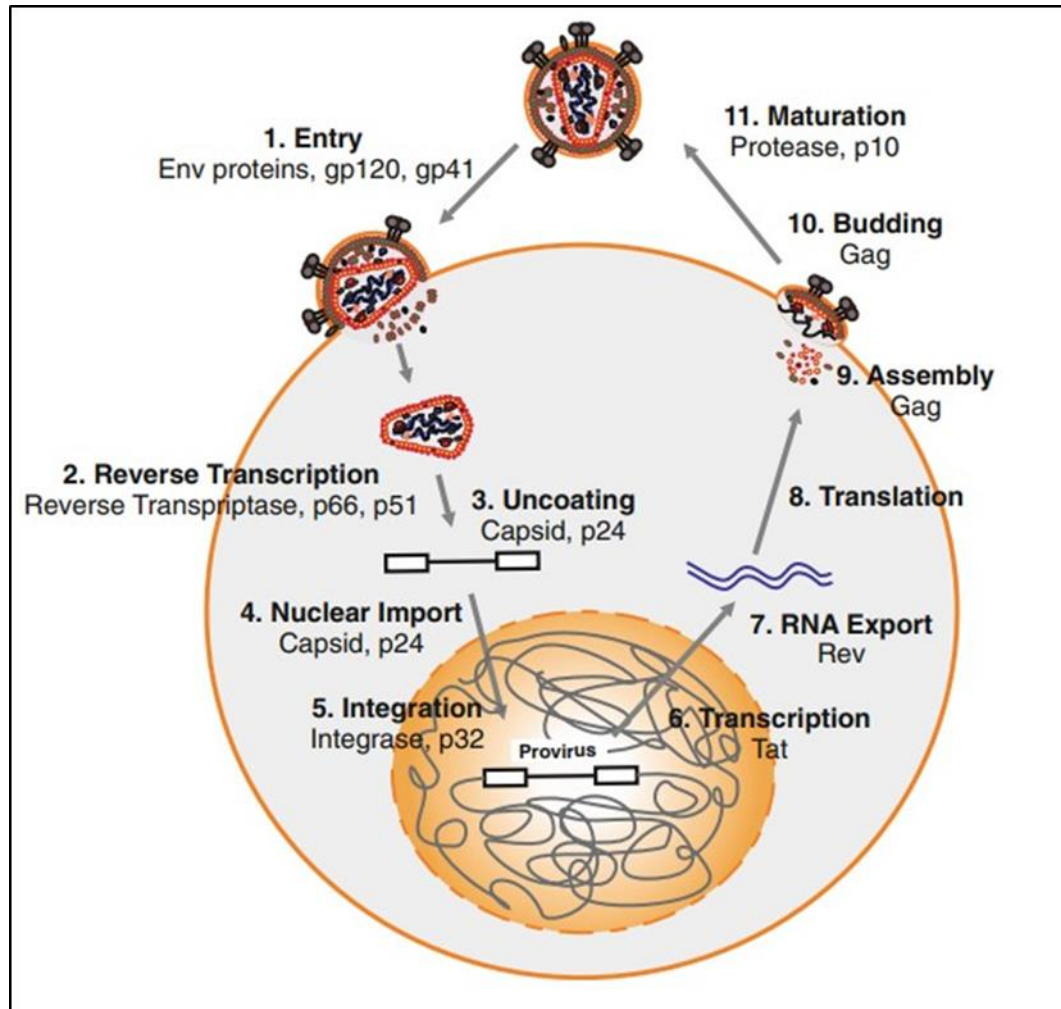


Figure 2.4: HIV Replication cycle (Kirchhoff, 2013).

2.6 Innate immune responses

During acute HIV infection, there is an intense pro-inflammatory response known as the cytokine storm, which is characterized by the secretion of high levels of cytokines and chemokines (Fiebig et al., 2003, Kramer et al., 2010). Pro-inflammatory cytokines such as IL-1 α , IL-1 β , IL-6, TNF- α , IL-8, IP-10 are secreted first, followed by anti-inflammatory cytokines IL-4, IL-10, IL-11 and IL-13 and T cell homeostatic cytokines (IL-2, IL-7) (Kramer et al., 2010, Roberts et al., 2010). These cytokines play an important role in the activation of adaptive immune responses, regulation of immune responses and tissue repair. However, the cytokine storm may also contribute to harmful immune activation that leads to excessive inflammation and allergic responses and loss of CD4⁺ T cells (Obeagu, 2025).

The cytokine storm is then followed by the activation of innate immune cells including neutrophils, monocytes/macrophages and dendritic cells. Macrophages and dendritic cells (DCs) are crucial mediators of innate immunity, and they promote the development of adaptive immune responses (Hespel and Moser, 2012). Both DCs and macrophages initiate CD4⁺ and CD8⁺ T cell responses by presenting antigens to naïve and memory CD4⁺ T cells via MHC-class I and class II molecules (Obeagu and Obeagu, 2024a). They also promote the activation, proliferation, and differentiation of T lymphocytes, and this is critical for initiating adaptive immune responses (Martín-Moreno and Muñoz-Fernández, 2019). Furthermore, natural killer cells contribute to the early containment of viral replication, while activated natural killer (NK) cells secrete perforins and granzymes that directly kill viral infected cells (Alter et al., 2011). They also produce additional cytokines such as IFN- γ , TNF- α and IL-10, which modulate adaptive immunity (Shibata et al., 1998). Thus, NK cells are crucial for the early control of HIV infection and they also modulate the maturation of DCs through the NK-DC cross-talk (Altfeld and Gale Jr, 2015).

2.7 Adaptive immune responses

Adaptive immune responses include both cell-mediated and humoral immune responses which are induced by T and B lymphocytes and develop only after 4-6 weeks after primary infection (Alter et al., 2011, Cumming and Peters, 2024). These responses are antigen-specific resulting in a delay between exposure and the development of immune responses. Adaptive immunity has the capacity for immunological memory which allows the host to generate a more rapid and efficient immune response against the second exposure to an antigen (Cumming and Peters, 2024).

2.7.1 Cell mediated immune responses

Cell-mediated immune responses are made up of CD4⁺ T helper cells that provides help to the other arms of adaptive immune system, and CD8⁺ T cells which are involved in cellular cytotoxicity (Porichis and Kaufmann, 2012). CD4⁺ T cell activation occurs when the T cell receptor (TCR) binds to a specific antigen on the surface of the antigen-presenting cell through the interaction with MHC class II molecules (Zhu, 2025). There are different subsets of CD4⁺ T cells, these are T helper 1 (Th1), T helper 2 (Th2), T helper 17 (Th17) and regulatory T cells (Tregs) (Steinman and Germain, 1998). Activated T helper 1 CD4⁺ T cell subsets secrete IL-21 and IL-4 cytokines that activate CD8⁺ T cell responses and macrophages and enhance the destruction of viral infected cells (Porichis and Kaufmann, 2012).

In contrast, activated TH2 CD4⁺ T cells secrete IL-2, IL-5, IL-6, IL-10, IL-13 that activate B cells to differentiate into antibody producing plasma cells (de Bree et al., 2017, Baiyegunhi et al., 2018). TH2 cytokines are also involved in class switching where mature B-cells change the constant region of an antibody without altering the antigen-binding variable region creating antibodies of different classes (isotypes). Different antibody isotypes are important for production of a stronger immune response against pathogens such as HIV (Alberts et al., 2002). Th17 cells promote inflammation in mucosal tissues, while Tregs regulate the immune responses (Yan et al., 2020).

As plasma viremia approaches its peak (first two weeks), there is a reduction in CD4⁺ T cells and an increase in CD8⁺ T cell responses (Borrow et al., 1994, Radebe et al., 2011). Early cytotoxic T lymphocytes (CTL) responses determine the course of disease progression and are associated with lower viral set point and slower disease progression (Nazziwa et al., 2024). Individuals with poorly controlled viremia are able to maintain viral suppression in the absence of ART (elite controllers) (ECs) (Goonetilleke et al., 2009, Salgado et al., 2024). A post-peak decline of viremia then appears as a result of the emergence of HIV-specific cytotoxic CD8⁺ T cells (Ndhlovu et al., 2015). However, HIV-1 introduces mutations to escape from CD8⁺ T cell responses, which leads to the loss of viral recognition and killing by the CD8⁺ T cell functions (Obeagu and Obeagu, 2024a, Vanegas-Torres and Schindler, 2024).

2.7.2 Humoral immune responses

2.7.2.1 B cell activation and production of the initial antibody responses

Humoral immune responses to HIV-1 infection develops within two weeks following infection, however they are only effective during the later stages of infection. After initial HIV-1 infection, B lymphocytes are activated when the virus or viral proteins bind to B cell receptors on the cell surface (Mitchell et al., 2025). Activated B lymphocytes divide and differentiate into plasma cells that produce anti-gp41 IgM non-neutralizing antibodies (Tomaras et al., 2008, Moshgabadi et al., 2015). These antibodies develop and reach detectable levels and form virion-antibody immune complexes at approximately 5 days after infection (Tomaras et al., 2008, De Biasi et al., 2020). Anti-gp41 IgM antibodies are followed by class switching into gp41-IgG and IgA non-neutralizing antibodies (Tomaras et al., 2008, Moshgabadi et al., 2015). Whereas anti-gp120-IgG antibodies are delayed and only develop approximately 28 days after infection (Bonsignori et al., 2009). Anti-Gag p24, p17 and Tat antibodies are critical components of the humoral immune responses, anti-p24 IgG antibodies develop at approximately 14 weeks post infection, while anti-p17 and anti-Tat IgG antibodies are generated as the immune response matures (Pelaez-Prestel et al., 2024, Nandy and Kumar, 2025). They are associated with lower viremia and serve as early prognostic markers for slower disease progression and better immune control (Wu et al., 2021).

Furthermore, anti-Tat antibodies have been linked to immune restoration in participants on suppressive combination antiretroviral therapy (cART), including increases in CD4⁺ T-cell counts and reductions in proviral DNA (Cafaro et al., 2019). These findings suggest that early non-neutralizing antibody responses in HIV are important as markers of infection, however, they have very little impact in disease progression. However, antibodies that develop few months following infection are involved in viral control, immune restoration and they have a potential in the development of effective vaccines and therapies.

2.7.2.2 Fc-mediated effector functions

Antibody-dependent effector functions are important components of the immune response that provide mechanisms for clearance of infected host cells, immune complexes, or opsonized pathogens (Igietseme et al., 2014). These interactions provide a direct link between the innate and adaptive immune systems by harnessing the functions of the innate immune system. They also overcome the limitations of innate immunity such as pattern recognition capacity by utilizing the diversity and specificity of the adaptive immune response (Pincetic et al., 2014). Fc-mediated effector functions play a critical role in the immune response against HIV-1 by extending the activity of antibodies beyond neutralisation. These functions are mediated through the Fc (fragment crystallizable) region of antibodies, which engages Fc gamma receptors (FcγRs) on immune effector cells and activates pathways such as antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and complement-dependent cytotoxicity (CDC) (Alter et al., 2018, Richardson et al., 2018).

In HIV infection, Fc-mediated mechanisms contribute to viral control by facilitating the clearance of infected cells, enhancing antigen presentation, and modulating inflammatory responses (Chung et al., 2018). For example, VRC01-class antibodies have demonstrated ADCC activity that correlates with decreased viral replication, while non-neutralizing antibodies elicited in the RV144 clinical trial were associated with partial protection through Fc-dependent mechanisms (Haynes et al., 2012). The glycosylation pattern of the Fc domain strongly influences binding affinity to FcγRs and determines the potency of these effector functions (Ackerman et al., 2013a, Ackerman et al., 2013b). Therefore, optimizing both Fab-mediated neutralisation and Fc-mediated effector activity is essential for designing effective HIV vaccines and antibody-based therapeutics.

2.8 Neutralizing antibodies and factors associated with their development

Approximately, 3-12 months after infection, activated B lymphocytes or plasma cells produce strain specific neutralizing antibodies that target the variable regions of the HIV-1 Env trimer including the V1-V2 and V3 loops (Archary et al., 2016). These antibodies bind to cell-free viruses and prevent further infection of the host cells. They have the potential to suppress viral replication by exerting immune pressure on the virus, however the virus mutates the HIV Env trimer and escape from strain specific neutralizing antibodies (von Overbeck and Zinkernagel, 2006).

Previous studies have shown that broadly neutralizing antibodies (bnAbs) only develop in 15-30% of HIV-infected individuals at approximately 2-3 years of infection (Euler et al., 2010, Gray et al., 2011, Moore et al., 2015, Landais et al., 2016, Landais and Moore, 2018, Ndlovu et al., 2020b). They target seven epitopes on the HIV-1 Env trimer, namely: the CD4 binding site, V2 loop at the trimer apex, V3 glycan supersite, gp120-gp41 bridging region, the silent face, the fusion peptide and the membrane-proximal external region (MPER) (Fig 2.5) (McCoy, 2018, Wion and Miller, 2021). Virological factors that are associated with the development of bnAbs are superinfection, high viral load, infection with HIV subtype C and viral diversity (Moore et al., 2015, Landais et al., 2016). While immunological features include high levels of T follicular helper cells, high *env* IgG titers and high levels of Fc effector functions were associated with the development of bnAbs and clinical factors such as dysfunctional natural killer cells (CD56-, CD16+) (Landais et al., 2016, Moody et al., 2016, Richardson et al., 2018, Bradley et al., 2020). Further studies are required to provide more insight on the factors associated with the development of neutralisation breadth, which may provide important information for vaccine design.

2.9 Different classes of broadly neutralizing antibodies

CD4 binding site bnAbs are subdivided into three sub-epitopes, namely: VRC01-class, VH1-46 class and CHR3 binders. VRC01-class utilize the VH1-2 alleles in their heavy chains (mimics CD4 receptors), while VH1-46 class utilize the VH1-46 alleles in their heavy chains and complementary heavy chain determining region (CDRH3) binders utilize the CDRH3 region to bind to the *env* trimer (Kim et al., 2016, Liao and Zhang, 2018). VRC01 class bnAbs develop after approximately five years following infection, however they have the highest neutralisation breadth (>90%) (Liao and Zhang, 2018). They also have high levels of somatic hypermutation (20-30%) in their heavy and light chains, examples of the most broad and potent VRC01-class bnAbs are VRC01, NIH45-46, N6, 3BNC117, and VRC07 (Wu et al., 2011, Zhou et al., 2010). They interact with the N-linked glycans closely bordering the CD4 binding sites; such as positions N197, N234, N276, asparagine at positions D279 and D280 and arginine at position R456 and glycine at position G458 (Julien et al., 2013, Lyumkis et al., 2013, Zhou et al., 2015).

The V2 apex bnAbs are derived from rare precursors with long anionic CDRH3 loops that are often deleted in the B cell repertoire (Doria-Rose et al., 2014, Moore et al., 2015, Doria-Rose et al., 2016, Morgand et al., 2016). This unique characteristic allows them to penetrate the glycan shield (McCoy, 2018). There are two classes of V2 apex bnAbs; PG9-like and CAP256-like bnAbs (Doria-Rose et al., 2014). V2 apex-directed bnAbs (e.g., CAP256-VRC26.25 and PG9/PG16) typically show exceptionally high potency and broad activity across diverse clades due to their ability to engage a conserved quaternary glycan-peptide epitope at the trimer apex. However, they are sensitive to changes in V2 loop length and glycan occupancy (notably N160), which can limit breadth in certain subtypes. PG9-like bnAbs recognize a quaternary epitope which is primarily composed of the N-linked glycans at positions N156 and N160 and positively charged residues such as lysine (K) at position 169 (Hoffenberg et al., 2013). In contrast, CAP256-like bnAbs have the longest CDRH3 loop (Doria-Rose et al., 2014, Doria-Rose et al., 2016). They interact with various amino acids in the V2 apex, such as the aspartic acid (D) at position 164, arginine (R) at position 166, arginine (R) or lysine (K) at position 169, as well as the N-linked glycans at position N156 and N160 respectively (Walker et al., 2009, Sok et al., 2014, Doria-Rose et al., 2014, Doria-Rose et al., 2016). However, they do not depend on the N156 and N160 glycans. The maturation pathways of these V2 apex bnAbs differ in that PG9-like bnAbs have intermediate to high levels of somatic hypermutation

(SHM), while CAP256- like bnAbs have very high levels of SHM with early signs of bnAb potential (Andrabi et al., 2015). Antibodies that target the V2 apex are important as they frequently develop especially in subtype C settings, and account for up to a third of bnAb responses (Gray et al., 2011, Yates et al., 2011, Landais et al., 2016).

The V3 glycan supersite is a glycan-rich region that is targeted by bnAbs; there are three classes of V3 glycan supersite bnAbs such as the PGT121, PGT128/10-1074 and BG18 families (Garces et al., 2014). The PGT121 family targets the N332 glycan which is a primary anchor for binding, however they also target other N-linked glycans including the N301, N137 and N156 (Krumm et al., 2016). The PGT128/10-1074 family heavily depends on position N332 which enhances their potency, and the GDIR motif at positions 324-327 of the HXB2 (Wibmer et al., 2015). V3 glycan bnAbs (e.g., PGT121, 10-1074 and BG18) show consistently broad neutralisation across most circulating strains and high potency, driven by recognition of a conserved glycan patch centered on the N332 glycan. Shifts in glycan positioning (such as N332 and N334) can reduce activity in a subset of viruses. The BG18 family has the highest neutralization potency, they target the N332 glycans and other additional glycans at positions N392 and N386 located near the V3 loop base as well as the GDIR motif (Sok et al., 2014, Barnes et al., 2018).

Gp120-gp41 interface is a heavily glycosylated region located between the gp120 and gp41 molecules. Interface-targeting bnAbs (e.g., PGT151 and 8ANC195) recognize quaternary sites bridging gp120–gp41 and although often potent, their breadth can be narrower or more strain-dependent due to reliance on intact glycan shields. The most broad and potent interface bnAbs are PGT151 and 8ANC195 (Scharf et al., 2014). PGT151 family has an unusual feature of long HCDR3 loop which is shared by several HIV-1 bnAbs targeting glycan-dependent epitopes (Liao et al., 2013). However, the CDRH3 of these bnAbs folds back towards the light chain forming part of an antigen binding site that makes significant contact with the gp41 interacting region of gp120 (Scharf et al., 2014, Wibmer et al., 2015).

The MPER epitope is highly conserved, it is divided into two overlapping functional regions namely; the proximal MPER (closer to the ectodomain/fusion peptide) and the distal MPER (closer to the transmembrane domain) (Binley et al., 2004). The proximal MPER region is targeted by 2F5 and Z13e1 bnAbs. Key amino acids within the proximal MPER are residues at positions 662-667 and aspartic acid/ lysine (D/K) at position 659 (Bricault et al., 2019). Resistant mutations including serine (S), asparagine (N), glutamic acid (E) at positions 664 and

665 are associated with 2F5 resistance and the alanine (A) at position 667 is associated with sensitivity to 2F5 antibody (Ofek et al., 2004, Williams et al., 2017, Bricault et al., 2019). The distal MPER spans residues 671-683 which are contact positions for 10E8, 4E10 and VRC42.01 (Salzwedel et al., 1999, Bricault et al., 2019). BnAbs targeting the MPER interact with the viral membrane through long CDRH3 loops that may promote high levels of autoreactivity (Verkoczy et al., 2014). MPER- bnAbs such as 10E8 and 4E10 demonstrate high neutralisation breadth, among the highest of any bnAb class, due to the MPER being highly conserved; however, their potency is often moderate due to the epitope's membrane-proximal location, restricted accessibility, and the need to accommodate the Env dynamic movement near the viral membrane.

The fusion peptide (FP), located at the N-terminus of gp41 at positions 512-527, plays a crucial role in mediating viral and host membrane fusion, making it a conserved and vulnerable target for broadly neutralizing antibodies (bnAbs) (Liang et al., 2018, Monteiro et al., 2021). FP targeting bnAbs such as VRC34 antibody lineage, and ACS202 recognize the hydrophobic residues including L520, G521, F522, L523 and G524, which are essential for membrane insertion and sensitivity to neutralisation (Kong et al., 2016, Van Gils et al., 2016). Amino acid substitutions at these positions, such as A525G or S528T can disrupt antibody binding and confer resistance by altering the peptide conformation or glycan positioning (Xu et al., 2018). FP-targeting bnAbs are generally characterized by their recognition of the gp120-gp41 interface and dependence on the N88 glycan for stable binding (Kong et al., 2016). The conformational flexibility of this region, together with glycan shielding around N88 and N611, modulates antibody access and determines the breadth of neutralisation across clades (Kong et al., 2016). Fusion peptide bnAbs (e.g., VRC34.01 and ACS202) show intermediate breadth and potency due to variations in glycans shielding this epitope.

The silent face bnAbs include VRC-PG05, SF10, SF12 ACS114 and ACS117 target the highly conserved N-linked glycans such as the N262, N295, and N448, and this is a sterically shielded epitope (Zhou et al., 2018, Schoofs et al., 2019). Silent face antibodies are typically characterized by high levels of somatic hypermutation and glycan accommodation. Historically this epitope was considered difficult to target, but new bnAbs show surprisingly high neutralisation breadth and potency. For example, VRC-PG05 had a modest breadth and potency, while SF12 has a much higher breadth and stronger potency (Schoofs et al., 2019). The ACS silent-face antibodies have relatively limited breadth and moderate potency compared to SF12. Overall, both the fusion peptide and silent face epitopes represent structurally

conserved, glycan-influenced regions targeted by potent bnAbs with distinct recognition modes and neutralisation mechanisms.

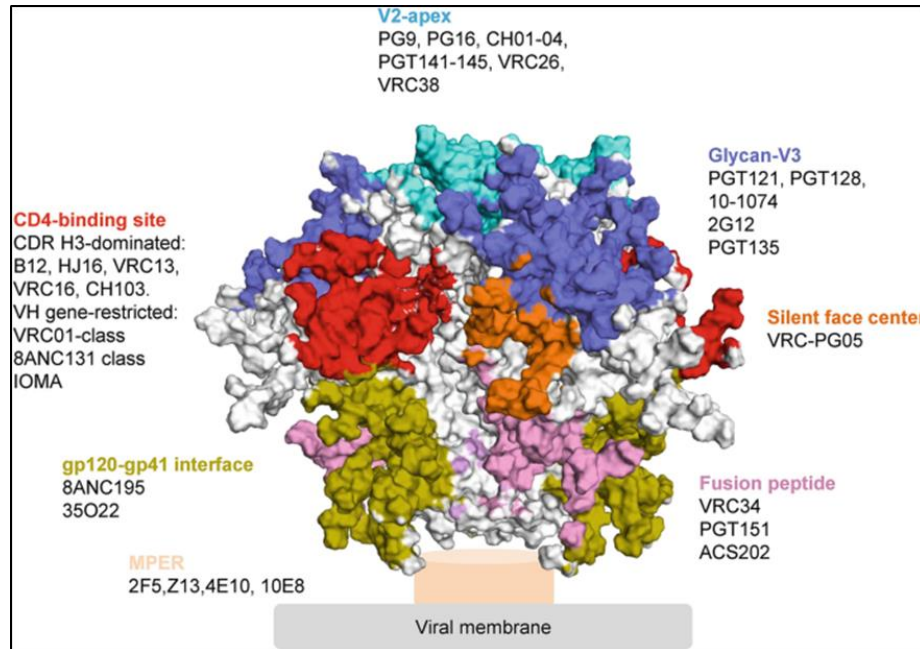


Figure 2.5: Surface representation of the epitopes of HIV-1 antibodies on an *env* glycoprotein trimer showing seven major epitopes such as the membrane proximal external region (MPER) (peach), the gp120/gp41 interface (green), CD4 binding site (red), V1V2 glycan (blue), V3 glycan (purple), silent face (orange) and the fusion peptide (pink) (Zhou et al., 2015).

2.10 Antibody-based vaccines

Previous studies have shown that an effective HIV vaccine may require inducing the development of bnAbs (Walker et al., 2009, Walker et al., 2011, Haynes et al., 2019). Despite extensive efforts, most vaccine regimens to date have failed to elicit bnAbs in humans. Previous studies used recombinant monomeric gp120 HIV-1 Env proteins as immunogens, however they failed to elicit bnAbs. The VAX003 and VAX004 Phase III HIV vaccine trials evaluated bivalent recombinant gp120 vaccines (AIDSVAX B/E in Thailand and AIDSVAX B/B in North America and the Netherlands, respectively) (Pitisuttithum et al., 2013, Pitisuttithum et al., 2020). Both trials induced strong gp120-binding and CD4-blocking antibody responses but failed to provide protection against infection.

The RV144 efficacy trial conducted in Thailand, vaccinated participants with (ALVAC canarypox vector and AIDSVAX gp120 protein booster (bivalent CRF_01AE/B gp120 in alum), this vaccine provided 31.2% vaccine efficacy against HIV acquisition (Group, 2005, Gilbert et al., 2005, Pitisuttithum et al., 2006). The correlates of protection in this vaccine were high levels of V1V2-IgG antibodies that mediate ADCC activity (Haynes et al., 2012, E. Ackerman and Alter, 2013, Chung et al., 2014). The RV144 was followed by the HVTN 702 clinical trial that was conducted in South Africa, where healthy uninfected participants were given ALVAC-HIV gp120 (bivalent gp120s in MF59), but this vaccine was ineffective in preventing HIV acquisition (Zolla-Pazner and Gilbert, 2019). However, HVTN 702 vaccinated participants had lower V1V2 IgG binding antibody responses compared to the RV144 (Gray et al., 2021). These studies suggest that recombinant monomeric gp120 immunogens failed to elicit broadly neutralizing antibodies in humans, and did not provide protection against HIV acquisition.

The use of the HIV Env trimer in vaccination is a promising approach aimed at inducing broadly neutralizing antibodies (bnAbs) against HIV (Bontempo et al., 2020, Turner et al., 2021). BG505 SOSIP.664 trimer is a soluble HIV-1 Env trimer derived from a clade A transmitted/founder virus (BG505) (Sanders et al., 2013, Julien et al., 2015). It includes stabilizing a disulfide linkage between gp120 and gp41, introduction of an I559P mutation in gp41 to favour the prefusion closed conformation, deletion of the membrane proximal external region (MPER) for better solubility, a truncation at residue 664 in gp41, and addition of a glycan at position 332 (T332N) to recreate a broadly neutralizing epitope (Sanders et al., 2013). In animal studies (rabbits, macaques, guinea pigs), BG505 SOSIP.664 induced autologous Tier-2 neutralizing antibodies, often against “holes” in the glycan shield and C3, V1/V2/V3 (Moore, 2014, Klasse et al., 2016, Klasse et al., 2018).

HIV-1 Env trimers such as the BG505 SOSIP trimer mimic the native viral envelope spikes and contains all seven bnAb epitopes (Turner et al., 2021). In humans, early Phase I trials (e.g. IAVI W001 and more recently HVTN 137A) have shown that BG505 SOSIP.664 trimer is safe and immunogenic (Hahn et al., 2024). In HVTN 137A, when BG505 SOSIP.664 gp140 was given with a TLR7/8 adjuvant (3M-052-AF/Alum), autologous Tier-2 neutralizing antibodies developed after 2-3 doses with responses mapped to multiple *env* regions (Hahn et al., 2024). In addition, weak heterologous neutralisation was seen in only the best responders (Hahn et al., 2024). However, there are remaining challenges such as the need for further stabilization and optimization to enhance their immunogenicity and breadth of protection (Bontempo et al.,

2020, Turner et al., 2021). Recent innovations like the triple tandem trimer design aim to improve trimer-specific responses by genetically linking Env protomers to ensure consistent trimer formation (del Moral-Sánchez et al., 2024).

Recently, the antibody-mediated prevention (AMP) trial was conducted in Sub-Saharan Africa, North and South America (Corey et al., 2021). The participants received intravenous VRC01 monoclonal antibody every eight weeks over a period of 72 weeks. VRC01 prevention efficacy was 75% in the most sensitive viruses, although no prevention efficacy was observed in resistant and intermediate viruses (Corey et al., 2021). The analysis of VRC01-sensitive HIV-1 isolates provided proof-of-concept that bnAb prophylaxis could be effective (Corey et al., 2021). However, it was clear that high levels of bnAbs are required to achieve HIV-1 prevention in humans. The study also highlighted that the majority of circulating viruses are resistant to VRC01 monoclonal antibody. Other studies also reported that resistant strains may pre-exist in the latent reservoirs before the initiation of ART, although they could evade detection due to their low frequencies (Curtis et al., 2022).

The germline targeting strategy is an HIV vaccine design approach that aims to activate the rare naïve B cells that have the genetic potential (germline precursors) to develop into broadly neutralizing antibody (bnAb) lineages. Recently, germline targeting vaccines were also explored in the HIV vaccine field. This strategy uses a class-specific priming immunogen, this is followed by the series of boosting immunogens to train B cells to develop bnAbs of specific classes (Steichen et al., 2024, Caniels et al., 2024). Recently, a randomized placebo-controlled phase I clinical trial (IAVI G001) was conducted to evaluate the ability of eOD-GT8 60mer immunogen to activate germline B lymphocytes (Venkatesan, 2022). HIV negative participants received two administrations of low-dose or high-dose vaccine (eOD-GT8 60mer) at 8-weeks intervals, all vaccinated recipients produced VRC01-class IgG antibodies, however, no neutralizing activity was found in these responses (Venkatesan, 2022, Cohen et al., 2023).

In addition, the IAVI G003 companion trial evaluated the eOD-GT8 60mer immunogen delivered via the mRNA platform to activate bnAb precursors in the African population (Willis et al., 2025). This vaccine also elicited CD4bs-specific IgG memory B cells in peripheral blood and germinal center B cells in lymph nodes. Analysis of antibody sequences showed that >90% of vaccinated recipients produced VRC01-class IgG B cell responses (Willis et al., 2025). Furthermore, the IAVI G002 trial also evaluated the ability of an engineered immunogen to guide responses primed by eOD-GT8 60mer for the development of bnAb. This immunogen

was shown to successfully engage the target germline B cells (Cohen et al., 2022, Cohen et al., 2023).

However, there are remaining challenges in vaccine development, such as an increase in resistance in circulating viruses. The high frequency of VRC01-resistant mutations in circulating strains is a challenge for therapeutic strategies (Saha and Dixit, 2020). Most bnAbs require years of somatic mutation and selection during chronic infection, their unmutated precursors often do not recognize native *env* efficiently. Although, germline targeting vaccine strategy may overcome this challenge. Future vaccine clinical trials may need to use a combination of monoclonal antibodies that target different epitopes, as well as computational protein designs and artificial intelligence to address the development of escape mutations.

2.11 Latent HIV-1 reservoirs and viral compartmentalization

Latent HIV reservoirs are a collection of cellular and anatomical sites in the body that harbor transcriptionally silent proviruses (Pace et al., 2012). These reservoirs are located throughout the body in tissues such as the liver, gut, lungs, kidneys, genital tract, breasts and brain and can be activated again and produce infectious viruses (Seipone et al., 2018, Sojane et al., 2018). There are different cellular reservoirs such as macrophages, lymphoid cells, monocytic cells and follicular dendritic cells (Murzin et al., 2024, Chen et al., 2022). Latency is maintained through mechanisms like transcriptional interference, epigenetic regulation, and the interaction of transcription factors. These mechanisms suppress viral gene transcription, allowing the virus to evade the immune system (Castro-Gonzalez et al., 2018, Chen et al., 2022). A significant portion of proviral reservoirs consists of defective proviruses, which do not produce replication-competent virus but can contribute to chronic immune activation. Despite ART, proviral reservoirs persist and can reactivate if treatment is interrupted or ineffective causing a major barrier to curing HIV (Wang et al., 2021). Since latently infected cells are not actively producing viral proteins, these cells have the ability to escape the viral cytopathic effect and remain resistant to clearance by the immune system (Schiralli Lester and Henderson, 2012).

Lymphoid tissues act as major sites of viral persistence. The T follicular helper cells (Tfh) and other CD4⁺ subsets here harbor integrated provirus, protected within germinal centers where immune surveillance and drug penetration are limited (Li et al., 2025). Peripheral blood

mononuclear cells (especially resting memory CD4⁺ T cells) contain latent, replication-competent proviruses. This reservoir is the most accessible for study and reflects systemic infection dynamics (Ortiz et al., 2022). Plasma mainly contains cell-free viral RNA, reflecting active replication or viral release from tissue reservoirs (Pasternak et al., 2013). Together, these reservoirs demonstrate how HIV persists in both cellular and anatomical niches, maintaining infection despite effective therapy.

Compartmentalization in viral reservoirs refers to the phenomenon where genetically or functionally distinct populations of a virus persist and evolve independently within different anatomical or cellular sites of the body; for example, between lymph nodes, blood (PBMCs/plasma), gut, or the central nervous system (Kearney et al., 2015, Salemi and Rife, 2016). Compartmentalization reflects the complex dynamics of viral evolution and adaptation in distinct tissue reservoirs as compartment-specific immune pressures, such as broadly neutralizing antibody (bnAb) escape mutations, contribute to *env* compartmentalization in some individuals (Salemi and Rife, 2016). This occurs because limited viral trafficking, local immune pressures, or tissue-specific environments restrict gene flow between compartments (Vanderford et al., 2011). As a result, each site can harbor unique viral variants, often showing differences in sequence, tropism, replication capacity, or drug sensitivity (Vanderford et al., 2011, Bobe et al., 2021).

Studies have revealed significant compartmentalization of HIV-1 *env* between plasma, PBMC and lymph nodes, indicating limited viral gene flow across these anatomical sites (van Marle et al., 2007). HIV-1 *env* sequences from lymph nodes often display increased N-linked glycosylation and immune escape mutations, reflecting local adaptation to strong humoral pressures in germinal centers (Zhang et al., 2021). In contrast, plasma-derived *env* variants are typically less glycosylated and represent actively replicating, transmission-competent virus populations (Frost et al., 2001). PBMC-associated *env* sequences frequently showed evidence of latent virus, often differing significantly from plasma/lymph node variants (Lorenzo-Redondo et al., 2016, Jeewanraj et al., 2023). Other studies showed differences in neutralisation sensitivity and the presence of discordant drug-resistance mutations in CSF and plasma as well as cervicovaginal lavage (CVL) and plasma viral populations (Wong et al., 1997, Cunningham et al., 2000, Venturi et al., 2000, Cinque et al., 2003, Strain et al., 2005, Kelentse et al., 2020). These studies suggest that compartmentalization may have implications for vaccine development and cure strategies. During ART, plasma viremia is usually undetectable, but transient “blips” can indicate ongoing low-level replication or release from latent

compartments (Jones and Perelson, 2007, Gupta, 2016). Combination ART only targets active HIV-1 replication, therefore, it is ineffective against latent proviruses and does not cure HIV infection (Chavez et al., 2015). The latent HIV reservoir is a major barrier to developing an HIV cure. Thus, further research is required to reduce or eliminate reservoirs to achieve a functional cure for HIV.

2.12 Study Rationale

HIV-1 *env* diversity evolves differently across anatomical and cellular compartments, yet the extent to which these compartment-specific patterns influence neutralisation sensitivity and epitope exposure is not completely understood. Previous studies have shown that features such as glycan occupancy, fusion-peptide variability, and silent-face accessibility can differ across plasma, lymph nodes, and mucosal reservoirs, but most analyses have relied on bulk sequencing or limited subsets of viral quasiespecies. As a result, in depth intra-participant and inter-participant diversity has often been under-represented, specifically when trying to link phylogenetic clustering with neutralisation sensitivity.

SGA derived *env* sequences provide a high-resolution view of viral evolution, yet there is still a gap in the field regarding how best to select SGA clones that accurately capture the breadth of diversity found across compartments. Many earlier data sets have not systematically used phylogenetic structure, highlighter mutational patterns and alignment-based mutational profiling to ensure that selected sequences fully represent compartment-specific diversification, particularly in participants with heterogeneous viral reservoirs. This limits the ability to draw conclusions on how intra-participant variation impacts neutralisation profiles.

In parallel, although bnAbs across the seven epitopes have been well characterized, the relationship between their breadth/potency and naturally occurring *env* patterns across compartments remains insufficiently mapped (Mandizvo et al., 2022). Prior work has shown notable variability in HIV-1 *env* particularly in bnAb epitopes, and resistance patterns between plasma and CNS compartments. However, the neutralisation sensitivity was not determined in participants with plasma, PBMC and lymph node samples. Recently, discordant amino acid mutations were observed in the CD4 binding site, V1V2 region, the V3 glycan, the gp120/gp41 interface and the MPER regions, these variations could alter the bnAb sensitivity between the compartments (Jeewanraj et al., 2023). The variability of the neutralisation sensitivity in the different compartments may be an important aspect in the development of effective therapeutic vaccines. However, the neutralisation sensitivity was not assessed in-vitro, and new bnAb epitopes such as the fusion peptide and silent face were not investigated in this study. Thus, the aim of this study was to evaluate HIV-1 *env* sequence differences within broadly neutralizing antibody (bnAb) epitopes and to assess their neutralisation sensitivity in HIV-1 subtype C across plasma, PBMC and lymph nodes-derived clones. HIV-1 *env* single-genome amplicons

(SGAs) previously generated from four participants living with HIV, they were analysed within variable loops, and bnAb epitopes. Few SGAs were selected in each participant for in-vitro neutralisation sensitivity testing. Thereafter, the bnAb escape patterns were evaluated and compared between plasma, PBMC and lymph nodes. This work provides a more comprehensive framework for interpreting how anatomical compartmentalisation shapes *env* evolution and impacts susceptibility to antibodies targeting diverse epitopes.

2.13 Review of Methodology

Sanger sequencing is a critical tool in molecular biology due to high accuracy, simplicity and allows for longer read lengths and uses low-throughput applications. Sanger sequencing is widely used for confirming genome edits and screening clones in genome editing workflows. It provides unambiguous identification of sequence changes and can detect mixed single nucleotide polymorphisms (SNPs). This sequencing method is cost-effective, has a simple workflow, and produces data that is straightforward to analyze (Crossley et al., 2020). In contrast, next generation sequencing (NGS) provides a high-throughput discovery and cost-effective screening of larger genomic regions, however disadvantages include short read lengths produced, the technology is sensitive to sample quality; degradation, contamination, or impurities in the sample can compromise the accuracy and reliability of the results and the NGS workflow involves many complex steps requiring high experimental skill and knowledge of bioinformatics (Baudhuin et al., 2015).

Furthermore, TOPO cloning is a rapid cloning method that uses the enzyme topoisomerase I to ligate PCR-amplified DNA fragments into a vector (Celie et al., 2016). This method is especially useful for cloning PCR products from heterogeneous samples, such as those from genome editing experiments, allowing isolation of individual alleles or variants for detailed sequence analysis (Hartley, 2006).

The pseudovirus neutralisation assay is a critical tool in HIV vaccine clinical trials because it provides a safe, standardized, and quantitative way to assess the neutralizing antibody responses induced by vaccination. Using replication-deficient viral particles that express the HIV Env glycoprotein, the assay measures how effectively vaccine-induced antibodies can prevent viral entry into target cells. Importantly, pseudovirus assays enable comparison of immune responses between participants and across studies, identification of correlates of protection, and guidance for vaccine optimization by highlighting which *env* regions or antibody specificities contribute to neutralisation. As such, they are a cornerstone of HIV vaccine evaluation, bridging laboratory immunogenicity findings with potential clinical efficacy. The principle of the pseudovirus neutralisation assay is based on measuring how well antibodies can block HIV entry into target cells using a safe, non-replicating viral system. The TZM-bl cell line are used in the tissue culture assays due to their ability to express the CCR5 and CXCR4 coreceptors and CD4 receptors (Montefiori, 2004, Montefiori, 2009).

The TZM-bl assay is a cell-based reporter neutralisation assay. TZM-bl cells are HeLa-derived cells engineered to constitutively express CD4, the CCR5 and CXCR4 coreceptors, to carry integrated reporter genes (firefly luciferase and β -galactosidase) under HIV-1 long terminal repeat (LTR) control (Tat-inducible) (Montefiori, 2004). In the neutralisation assay, serial dilutions of the antibody are incubated with *env*-pseudotyped HIV-1. The mixture is added to TZM-bl cells with DEAE-dextran to enhance infection. The advantages of this assay include high sensitivity, reproducibility, straightforward readout (luminescence). While the limitations include the cell line expressing high levels of CD4/CCR5 and CXCR4, which may lead to over estimation of neutralizing potency compared to primary target cells. Since the assay measures only a single round of infection, it does not capture later replication or viral spread factors (Montefiori, 2009).

Chapter 3: Methods and Materials

3.1 Study participants

Four participants living with HIV were selected from the HIV Pathogenesis Programme (HPP), Females Rising through Education, Support and Health (FRESH) acute infection and the Lymph Node cohorts in Durban, South Africa (Ndung'u et al., 2018, Dong et al., 2018, Ndhlovu et al., 2019, Jeewanraj et al., 2023). “This study employed an intensive intra-participant sampling strategy, analyzing all successfully amplified single-genome amplicons (SGAs) from plasma (PL, n=8), PBMC (n=12) and lymph node (LN, n=13) compartments from four treatment naïve participants. Three females and one male participant between the ages of 20-29 years were characterized through HIV-1 *env* sequence analysis and evaluation of neutralisation sensitivity. All participants were ART-naïve. All participants provided written informed consent and the study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BREC/00006367/2023).

3.2 Clinical sample collection

Lymph node samples were collected after participants had undergone physical assessments and lab tests to determine whether they meet the safety threshold. Lymph nodes were identified under ultrasound guidance and excised through a 4cm incision made by a qualified, board-certified surgeon. Lymph node samples were fixed in 10% formal-saline (Sigma Aldrich, St Louis, Missouri, USA) and tissue blocks were processed using a microtome (Leica Microsystems, Wetzlar, Germany), thereafter, they were stored on ice until staining was performed. The remaining tissue was processed to release lymph node mononuclear cells (LMCs) as previously described (Lorenzo-Redondo et al., 2016). Cryopreserved lymph node mononuclear cells (LNMCs) (10 million cells) were lysed, and viral RNA was quantified using the Amplicor ver 1.5 assay (Roche Molecular Systems, Branchburg, NJ, USA) at an accredited clinical laboratory using standardized protocols.

In addition, blood samples were collected and centrifuged to separate peripheral blood mononuclear cells (PBMC) and plasma (PL). Sample storage was managed under the Laboratory Information Management Systems (LIMS) where each sample was given a unique number that describes sample type and storage conditions. Plasma samples were stored at -80°C, while PBMC samples were stored at -196°C in liquid nitrogen tanks. HIV viral loads were measured using the Amplicor ver 1.5 assay (Roche Molecular Systems, Branchburg, NJ, USA) and CD4+ T cell counts were measured using Tru-count technology (Ndung'u et al., 2018). Thereafter, they were further analysed using flow cytometry according to manufacturer's instructions (Becton Dickinson, BD Biosciences, San Jose, CA, USA) (Ndung'u et al., 2018).

3.3 Sequence analysis and SGA selection

Analysis of *env* sequences was performed using the Geneious Prime software programme v10.1.3, FigTree software and the Los Alamos database (Biomatters Ltd, Auckland, New Zealand). Maximum likelihood phylogenetic trees were constructed using the Geneious Prime software programme v10.1.3, FigTree software for each participant to assess phylogenetic relatedness, to identify major and minor clusters and visualize compartment-specific clustering (Supplementary figures 7.1A-D). (Biomatters Ltd, Auckland, New Zealand). SGAs were selected from each major phylogenetic cluster to ensure representation of dominant lineages, low frequency variants, long branch-length sequences and any compartment-restricted populations. Highlighter plots and Logo plots were generated using the AnalyzeAlign tool in the Los Alamos database website; to identify silent and non-silent mutations and to analyze various positions previously reported to affect neutralisation sensitivity on each of the seven epitopes and a consensus sequence was chosen to represent each unique mutation. Highlighter plots generated using the HIV Los Alamos database were used to evaluate mutational patterns and screen for hypermutated sequences, and confirm the presence of both synonymous and non-synonymous substitutions (Supplementary fig 7.2). SGAs passing all quality and diversity criteria were selected for downstream cloning, pseudovirus production and neutralization assays.

HIV Los Alamos database:

(<http://www.hiv.lanl.gov/content/sequence/GLYCOSITE/glycosite.html>).

3.4 Amplification of HIV-1 *env*

In this study, HIV-1 *env* was re-amplified from previously generated single genome amplicons derived from LN (n=13), PBMC (n=12) and plasma (n=8) samples (Jeewanraj et al., 2023). Second-round HIV-1 *env* PCR was performed using 5X Phusion Buffer, dNTPs, *env*1A forward primer (5' - CAC CGG CTT AGG CAT CTC CTA TGG CAG GAA GAA-3'), and *env*1M reverse primer (5' - TAG CCC TTC CAG TCC CCC CTT TTC TTT TA -3'), together with Phusion HF *Taq* Polymerase (Invitrogen, Thermo Fisher Scientific, Massachusetts, United States). Amplification was performed at 94°C for 4 min, 35 cycles of 94°C for 15 sec, 55°C for 30 sec, 68°C for 4 min, and final extension of 68°C for 20 min followed by the hold at 4°C. Second-round PCR products were resolved on a 1% agarose gel and successful amplification of *env* was confirmed by the presence of a single band at approximately 3,000 bp position relative to a 1 kb DNA ladder (Invitrogen, Thermo Fisher Scientific, Massachusetts, United States).

3.5 PCR purification and gel extraction

Second round HIV-1 *env* was purified using the QIAquick PCR Purification kit as per manufacturer's instructions (Qiagen Inc., Valencia, CA, USA). Purified amplicons were quantified using the NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts). Purified DNA was then incubated with Platinum *Taq* DNA polymerase at 72°C for 8 minutes (Invitrogen, Thermo Fisher Scientific) to generate 3'-overhangs required for TOPO cloning and the DNA was immediately placed on ice until further processing.

3.6 TOPO cloning of full-length HIV-1 *env*

Purified HIV-1 *env* PCR products were cloned using the pcDNA 2.1/V5-His-TOPO TA cloning kit (Invitrogen, Thermo Fisher Scientific, Massachusetts, USA) following the manufacturer's instructions. PCR products were diluted 1:2 or 1:4 in nuclease-free water prior to ligation. The TOPO cloning reaction consisted of diluted PCR product, salt solution, DEPC-treated water and the TOPO vector was incubated on ice for 30 minutes. Following ligation, the reaction mixture was transformed into NEB 5-alpha chemically competent *E.coli* cells (New England Biolabs). Cells were incubated on ice for 30 minutes and heat shocked at 42°C in water bath for 30 seconds and immediately returned to ice for 5 minutes. Pre-warmed SOC outgrowth media was added to the cells and the cultures were incubated at 37°C for 1 hour in a shaking incubator. Bacterial culture was inoculated on pre-warmed Luria-Bertani (LB) ampicillin plates containing *X-gal* and incubated at 37°C for 18 hours. The presence of HIV-1 *env* insert was confirmed using colony PCR using a mastermix containing Supertherm *Taq* polymerase enzyme, HIV-1 *env* primers, dNTPs, High-Fidelity PCR Buffer, MgSO₄ and DEPC-treated water (Separation Scientific, South Africa). Plasmid DNA was isolated using the GeneJET Plasmid MiniPrep or Maxiprep (Thermo Fisher Scientific, USA) kit as per manufacturer's instructions.

3.7 Sanger sequencing of HIV-1 *env* clones

Full-length HIV-1 *env* inserts were sequenced directly with the ABI 3500xl Genetic Analyzer and the BigDye™ Terminator v3.1 Sequencing Kit (Thermo Fisher Scientific, Massachusetts, United States) as previously described (Jeewanraj et al., 2023). The sequencing master mix was prepared with 5X Big Dye sequencing buffer, 2pmol of overlapping sequencing primers that span the entire *env* gene, Big Dye v3.1 Ready Reaction Mix enzyme, PCR product and DEPC treated water. Sequencing reactions were incubated in the thermocycler with the

following conditions: 1 cycle at 96 °C for 1 minute; 25 cycles of 96°C for 10 seconds, 50 °C for 5 seconds and 60°C for 4 minutes and hold at 4°C. Raw chromatograms were assembled and edited using Sequencher v3.4.1 software programme (Gene Codes, Ann Arbor, USA, Michigan).

3.8 Maintenance of 293T and TZM-bl cells

Both the TZM-bl (HeLa) and 293T (HEK) cell lines were obtained from the NIH AIDS Research Center (NIH, AIDS Reagent Program, Bethesda, MD, USA) and the American Type Culture Collection (ATCC, Manassas, CA, USA) respectively. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies, Carlsbad, CA, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco BRL Life Technologies, Carlsbad, CA, USA), 25 mM HEPES (Life Technologies, Taipei City, Taiwan) and 50 µg/ml gentamicin. The cells were maintained in T75 flasks and incubated overnight at 37°C, in a 5% CO₂ incubator. Growth media was then removed from the flasks after 2-3 days and the cells were washed with phosphate buffered saline (PBS) (Invitrogen, Grand Island, NY, USA). The cells were trypsinized for 30 seconds, and incubated in the CO₂ incubator for the cells to detach from the surface. Afterwards, the cells were re-suspended in fresh growth media, counted using an automated TC20™ cell counter (Bio-Rad Laboratories, Berkeley, California United States) following the manufacturer's instructions.

3.9 Production of HIV-1 pseudoviruses

Pseudotyped viruses were produced through co-transfection of the pSG3Δ^{Env} backbone and HIV-1 *env* plasmid DNA into the 293T (HEK) cell line as previously described (Ndlovu et al., 2020a). Briefly, *env*-pseudotyped viruses were prepared by the co-transfection of an HIV-1 *env* expressing plasmid with an *env*-deleted HIV-1 backbone plasmid (pSG3Δ^{Env}) into 293T cells in 6 well plates culture flasks using a TransGENE 9 transfection reagent (Roche Diagnostics, Mannheim, Germany). Culture plates were incubated at 37°C for 48 hours in a 5% CO₂ incubator. After incubation, the resulting supernatants were filtered and the FBS concentration was adjusted to 20%. Viral stocks were titrated by doing serial dilutions starting with a 1:4 dilution factor in a 96-well plate. TZM-bl cells containing dextran-hydrochloride (DEAE) were

added to the virus, and TZM-bl cells not treated with the virus were used as a negative control. The plate was incubated at 37°C, 95% relative humidity, and 5% CO₂ for 48 hours. After incubation, culture media was removed from each well and replaced with Bright Glo Luciferase reagent (Promega, Madison, Wisconsin, United States) and read using the Victor Nivo plate reader (PerkinElmer, Massachusetts, USA).

3.10 TCID assay

To determine the infectivity of pseudoviruses, the infectivity of viral stocks was assessed in TZM-bl cells as previously described (Ndlovu et al., 2020a). Briefly, viral stocks were serially diluted in growth media in four-fold dilutions in a 96 well plate (Sigma-Aldrich, St Louis, MO, USA). TZM-bl cells (10,000 cells/well) were added into the wells, untreated TZM-bl cells were used as a negative control, and the plates were incubated for 48 hours at 37°C in a 5 % CO₂ incubator. After incubation, culture media was removed from each well and replaced with Bright Glo Luciferase reagent (Promega Madison, Wisconsin, United States) and incubated in the dark for 2 minutes. After incubation, the mixture was transferred into a 96-well black plate (Sigma Aldrich, St Louis, MO, USA) and read using the Victor Nivo plate reader (PerkinElmer, Massachusetts, USA). A cut-off value of 10 times higher than the cell control in relative light units (RLU) was used to identify a positive infection.

3.11 TZM-bl micro-neutralisation assay

To assess the neutralisation sensitivity of pseudoviruses, the TZM-bl neutralisation assay was performed, as previously described (Ndlovu et al., 2020a). Monoclonal antibodies that target the CD4 binding site (VRC01, VRC07-523LS, N6), V1V2 loop (PGDM1400, CAP256-VRC26.25, PG9), V3 glycan (PGT121), gp120/gp41 interface (PGT151), the MPER (10E8), fusion peptide (N123.VRC34) and the silent face region (SF12) were serially diluted in 96-well plates, with the starting concentration of 20µg/ml. Afterwards, viral suspensions were prepared and added into the wells and the plates were incubated for 1 hour at 37°C in a 5% CO₂ incubator. After incubation, freshly trypsinized TZM-bl cells (1×10^4 cells/well), containing dextran were added into each well and the plates were incubated for 48 hours at

37°C, 5% CO₂ incubator. After 48-hour incubation, culture media (150µL) was removed and Bright Glo Luciferase reagent was added (Promega Madison, Wisconsin, United States). The plates were incubated for 2 minutes at room temperature and the contents were transferred into 96-well black plates (Sigma-Aldrich, St Louis, MO, USA). The plates were read on a Victor Nivo Multimode luminometer (Perkin Elmer, Massachusetts, USA) and the results were presented in relative light units (RLUs). A cell control containing only TZM-bl cells, and a virus control containing both the pseudotyped virus and TZM-bl cells were included as negative and positive controls respectively. All neutralisation experiments were performed in at least two independent replicates, and average neutralisation IC₅₀ titers were recorded.

3.12 Statistical analysis

Statistical analysis was performed using GraphPad Prism 9 software for Windows (GraphPad Software, San Diego, California USA). Non-parametric Mann–Whitney test was performed to compare the V1, V2, V3, and V4 loop lengths, and the number of PNGs across plasma, PBMC and lymph nodes within each of the four variable regions. For each region, three pairwise comparisons were conducted, and significance was assessed after correction for multiple testing. For the neutralisation assay, a sigmoidal standard curve was drawn using the RLUs to calculate the inhibitory concentration (IC₅₀) for each antibody. The difference in neutralisation sensitivity between LN, PBMC and PL viruses were determined by comparing the IC₅₀ titers using the Mann Whitney t-test where a p-value <0.05 indicates significant differences between the compartments. Compartmental assessment of HIV-1 *env* variable domain potential N-linked glycosylation sites and compartmental assessment of HIV-1 *env* variable domain lengths were determined using the Kriskal-Wallis and Mann Whitney U test to evaluate the differences across the LN, PBMC and plasma compartments.

Statistical justification of sample size

This study employed an intensive intra-participant sampling strategy, analyzing all successfully amplified single-genome amplicons (SGAs) from plasma (PL, n=8), PBMC (n=12) and lymph node (LN, n=13) compartments from four treatment naïve participants. The sample size was small due to the unavailability of matching plasma, LN and PBMC samples. The collection of lymph node samples is quite invasive, and therefore it was limited to few individuals in the cohort. However, a comprehensive analysis was performed within each participant, all SGA-derived *env* sequences were analysed and selected sequences were selected for functional characterisation in each compartment. This design increases statistical power for detecting compartment-specific differences in both genetic and neutralisation sensitivity profiles, while controlling for inter-participant variability through paired and mixed-effects analyses.

Chapter 4: Results

4.1 Clinical Characteristics

Four untreated participants were selected from the Females Rising through Education, Support and Health (FRESH) cohort. The study participants had a median plasma viral load of 124,500 copies/ml (4,100- 200,000 copies/ml), three of the four participants had high viral load > 50,000 copies/ml, and one participant (PID 0053) had a low VL (4,100 copies/ml) (Table 4.1). Prior to LN excision, the median CD4+ T cell count was 530 cells/mL (165-752 cells/mL), notably one of the participants (PID 118) had very low CD4+ T cell count (165 cells/mL) indicating a weak immune system. Blood and lymph node samples were collected around the same time (921-926 days post enrollment) for PID 0053, and (1427-1464 days) for PID 0011 following enrollment into the study. Plasma and PBMC samples were collected on the same day as lymph node excision for PIDs 118 and 3011. Lymph node samples were not collected from PID 118 as this participant was immunocompromised.

Table 4.1: Characteristics of study participants

PID	Gender	Age during LN excision	CD4 count (cells/ μ L)	PL viral load (copies/ml)	PL collection (days*)	LN collection (days*)	PBMC collection (days*)	No. of LN SGAs selected	No. of PBMC SGAs selected	No. of PL SGAs selected
0011	F	26	642	59,000	1464	1464	1427	8	4	2
0053	F	26	752	4,100	921	926	921	3	3	2
118	M	29	165	200,000	0	Not done	0	-	2	2
3011	F	20	561	190,000	0	3	0	2	3	2
Median		26	530	124,500	461	923	461	-	-	-
Total no. of SGAs selected								13	12	8

days- days post enrollment*

4.2 Partial diversity of HIV-1 subtype C *env* sequences

The phylogenetic analysis was performed using the HIV Los Alamos database and showed that all four participants were infected with HIV-1 subtype C (Fig 4.1). To determine the intra-participant and inter-participant diversity, HIV-1 *env* single genome amplicons (SGA) sequences were produced from plasma (n=33), PBMC (n=49) and LN (n=33), from the four participants. PID 0011 had the greatest intraparticipant *env* diversity among the four participants. HIV-1 *env* sequences formed two clearly distinct clusters on the phylogenetic tree, the major cluster had sequences from all three compartments intermingling with each other (Fig 4.1). While, the second smaller cluster only had PBMC RNA and LN DNA sequences. This second cluster was genetically distinct from the main intra-participant lineage, indicating the coexistence of divergent viral populations across compartments. The PBMC RNA compartment in particular displayed partial clustering from PL and PBMC DNA with the remaining sequences showing equilibration, consistent with compartment-specific evolutionary pressures or replication dynamics. Overall, PID 0011 sequences did not cluster with sequences from other participants, forming a participant-specific branch, demonstrating strong inter-participant structure and limited evidence of cross-participant similarity. A total of 14 *env* sequences representing intra-participant diversity were chosen; these include two sequences from PL, four sequences from PBMC and eight sequences from LN compartments (Fig 4.1, Table 4.1).

Sequences from PID 0053 had moderate intra-participant diversity with the evidence of partial compartmentalization (Fig 4.1). PBMC and LN sequences showed partial compartmentalization, the same pattern was observed in PL and PBMC compartments. PID 0053 sequences formed a single participant-specific lineage that remained distinct from the clusters of other participants with no intermingling with other PIDs observed. A total of eight sequences representing intra-participant diversity were chosen; these include two sequences from PL, three sequences from PBMC and three sequences from LN compartments (Fig 4.1, Table 4.1).

PID 118 exhibited two distinct clusters with partial intra-participant compartmentalisation, primarily between PBMC and PL *env* sequences (Fig 4.1). PL-derived sequences clustered together and were partially compartmentalized from the PBMC sequences. The observed divergence suggests separate evolutionary changes for cell-associated versus circulating viruses in this participant. Similarly, *env* sequences from PID 118 remained phylogenetically

distinct from the other PIDs with no cross-participant intermingling occurred, confirming strong participant-specific clustering. A total of four sequences representing intra-participant diversity were chosen; these include two sequences from PL and two sequences from PBMC compartments (Fig 4.1, Table 4.1).

PID 3011 showed partial intra-participant *env* diversity, with only subtle compartment-specific patterns. Although some partial compartmentalisation was observed between compartments, especially between LN and PL compartments or PBMC and PL compartments (Fig 4.1). Majority of sequences remained equilibrated, indicating a homogenous viral population across compartments compared with the other PIDs. Despite limited intra-participant structuring, PID 3011 sequences formed a participant-specific lineage that did not overlap with sequences from the other three PIDs. A total of seven sequences representing intra-participant diversity were chosen; these include two sequences from PL, three sequences from PBMC and two sequences from LN compartments (Fig 4.1, Table 4.1).

Phylogenetic analysis of HIV-1 *env* sequences across participants revealed consistent and strong participant-specific clustering, confirming that viral populations evolved independently within each individual. In contrast, the degree of intra-participant diversity and compartmental structuring varied markedly between participants, ranging from highly structured and diverse viral populations to largely equilibrated populations across compartments. These differences highlight substantial heterogeneity within-host viral evolutionary dynamics. While HIV-1 *env* evolution is universally participant-specific, the extent of compartmentalization and viral diversity within individuals is highly variable.

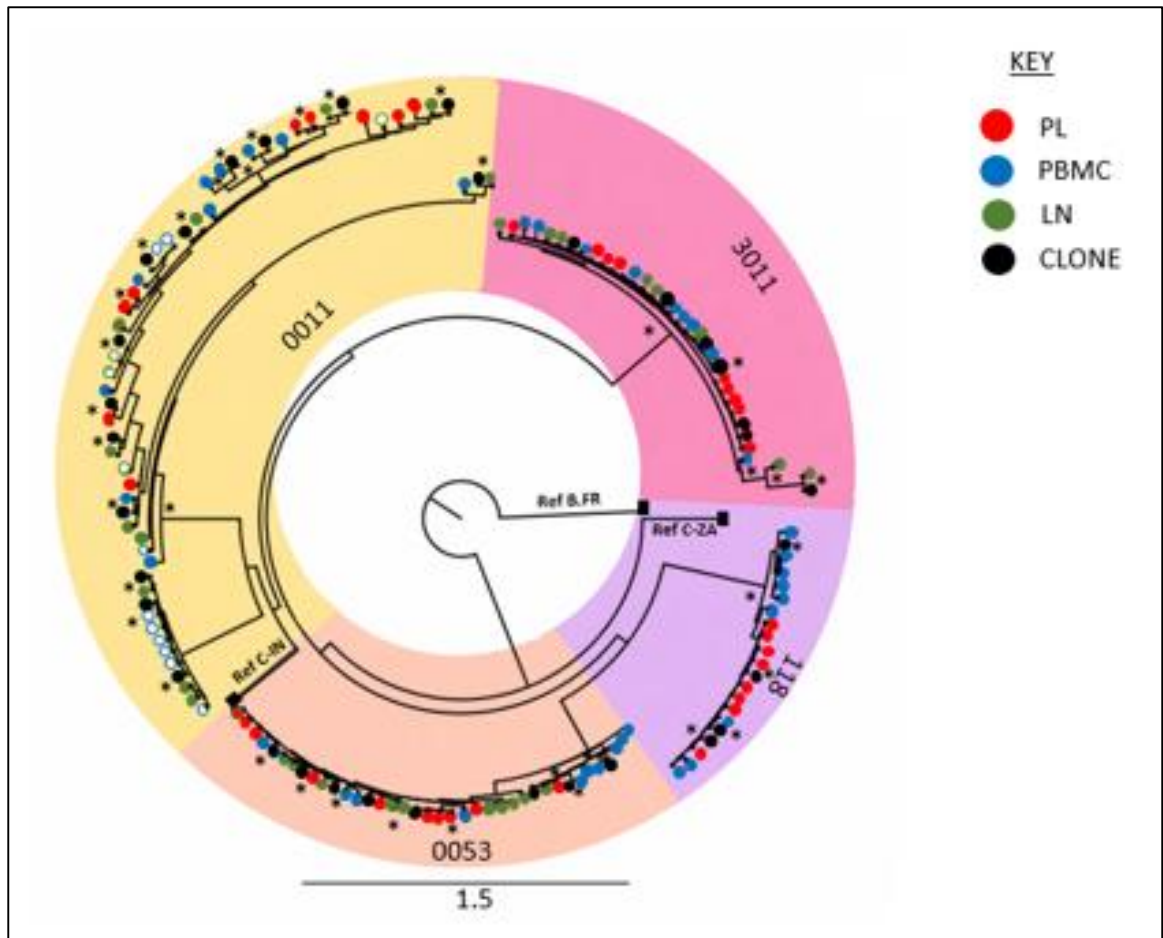


Figure 4.1: Phylogenetic analysis of full-length HIV-1 *env* sequences derived from the plasma, PBMC and lymph node derived viruses in four participants from the HPP and lymph node cohorts. Red circles represent plasma, solid blue circles represent PBMC DNA, open blue circles represent PBMC RNA, solid green circles represent LN DNA and open green circles represent LN RNA. Black symbols represent the selected SGAs that were cloned and clustered with the original SGA that was previously generated. HIV-1 reference sequences. (Ref.B.FR.83.HXB2_LAI_IIIB_BRU.K03455, Ref.C.IN.95.95IN21068.AF067155 and Ref.C.ZA.04.04ZASK146.AY772699 were obtained from the HIV Los Alamos database.

* Indicates bootstrap proportions <70.

4.3 Compartmental assessment of HIV-1 *env* variable domain lengths and HIV-1 *env* variable domain potential N-linked glycosylation sites

Variable loop lengths contribute to the formation of the glycan shield that protects conserved regions from broadly neutralizing antibodies (Doores, 2015). Longer variable loops often carry additional potential N-linked glycosylation sites (PNGS) which may reduce accessibility of neutralizing epitopes (Sagar et al., 2006). Analysis of the V1 loop had a slightly longer V1 loop in PL sequences compared to PBMC and LN (Fig 4.2). While the median V2 loop was longer in both plasma and PBMC compared to LN sequences ($p=0.02$). There was no difference in the median V3 and V4 loop lengths between the three compartments. However, the median V4 loop was slightly longer in LN compared to plasma and PBMC, although these differences were not statistically significant ($p>0.05$) (Fig 4.2A, C, D). These findings suggest minor variations in the V1, V2 and V4 loop lengths, while the V3 loop length remains the same in plasma and PBMC compared to LN compartments.

Potential N-linked glycosylation sites (PNGS) are main targets for the V2 apex, V3 glycan, gp120-gp41 interface and the silent face bnAbs, the loss of PNGS may lead to escape from bnAbs (Doores, 2015). To assess the differences in the number PNGS across the compartments, PNGS of the V1-V4 regions were computed and compared between compartments. The number of PNGS were largely similar for the V1 and V3 loops across the PL, PBMC and LN compartments ($p>0.05$) (Fig 4.3A & C). In contrast, the number of PNGS in the V2 loop was significantly higher in PBMC compared to plasma ($p = 0.03$) (Fig 4.3B). Similarly, the number of PNGS in the V4 loop was significantly higher in the plasma compared to PBMC ($p = 0.01$) (Fig 4.3D).

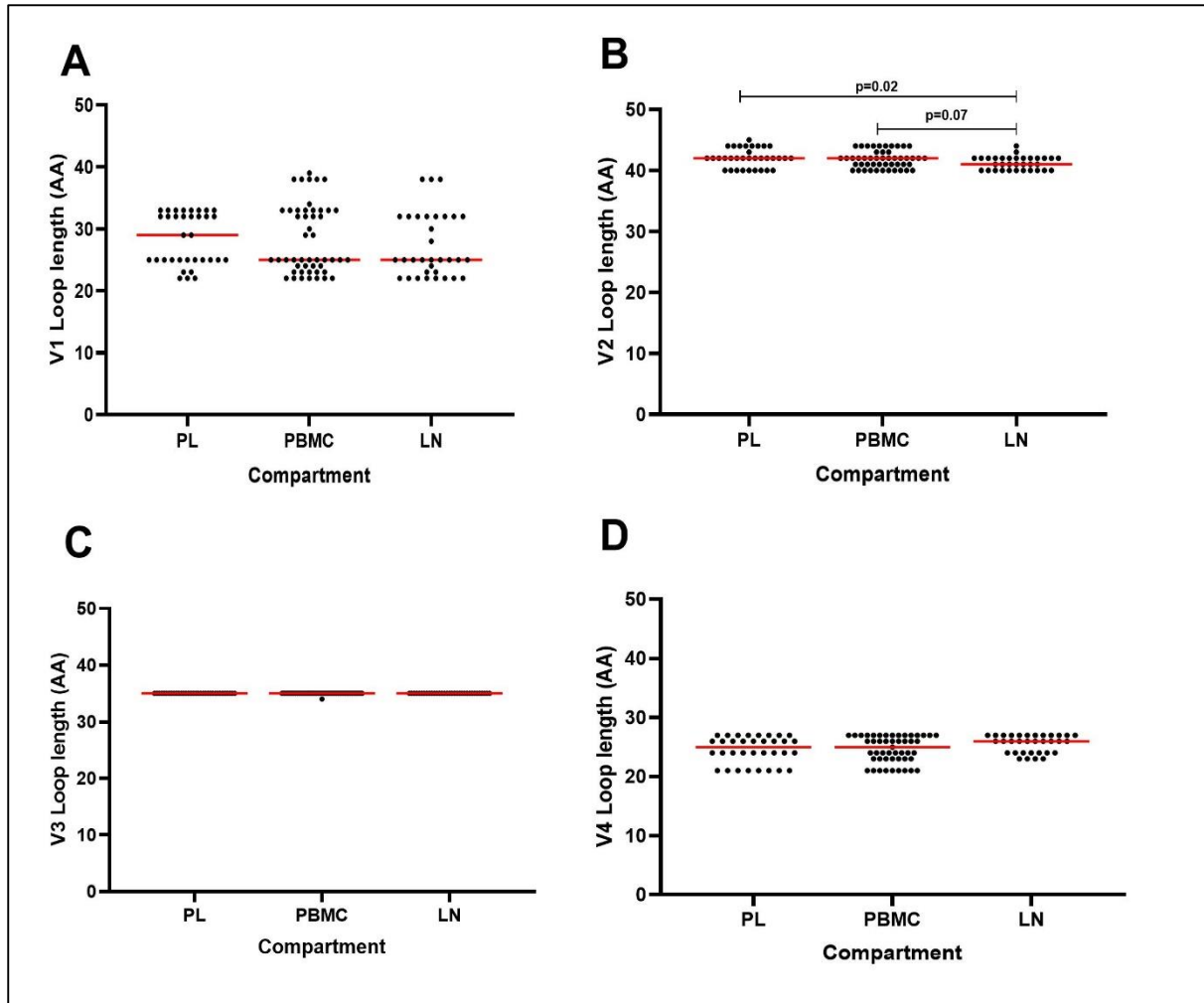


Figure 4.2: Comparison of the V1-V4 loop lengths between *env* sequences generated from lymph node, PBMC and plasma compartments. Scatterplots show *env* loop lengths for V1 (A), V2 (B), V3 (C), and V4 (D) in plasma (PL), peripheral blood mononuclear cells (PBMC) and lymph node (LN). Each dot is an individual sequence; red bars denote medians. Between-compartment differences were assessed with two-sided Mann–Whitney U tests; nominally significant p-values (and trends, where shown) are annotated on the plots. PL, plasma; PBMC, peripheral blood mononuclear cells; LN, lymph node

*Note: Medians are represented as red bars

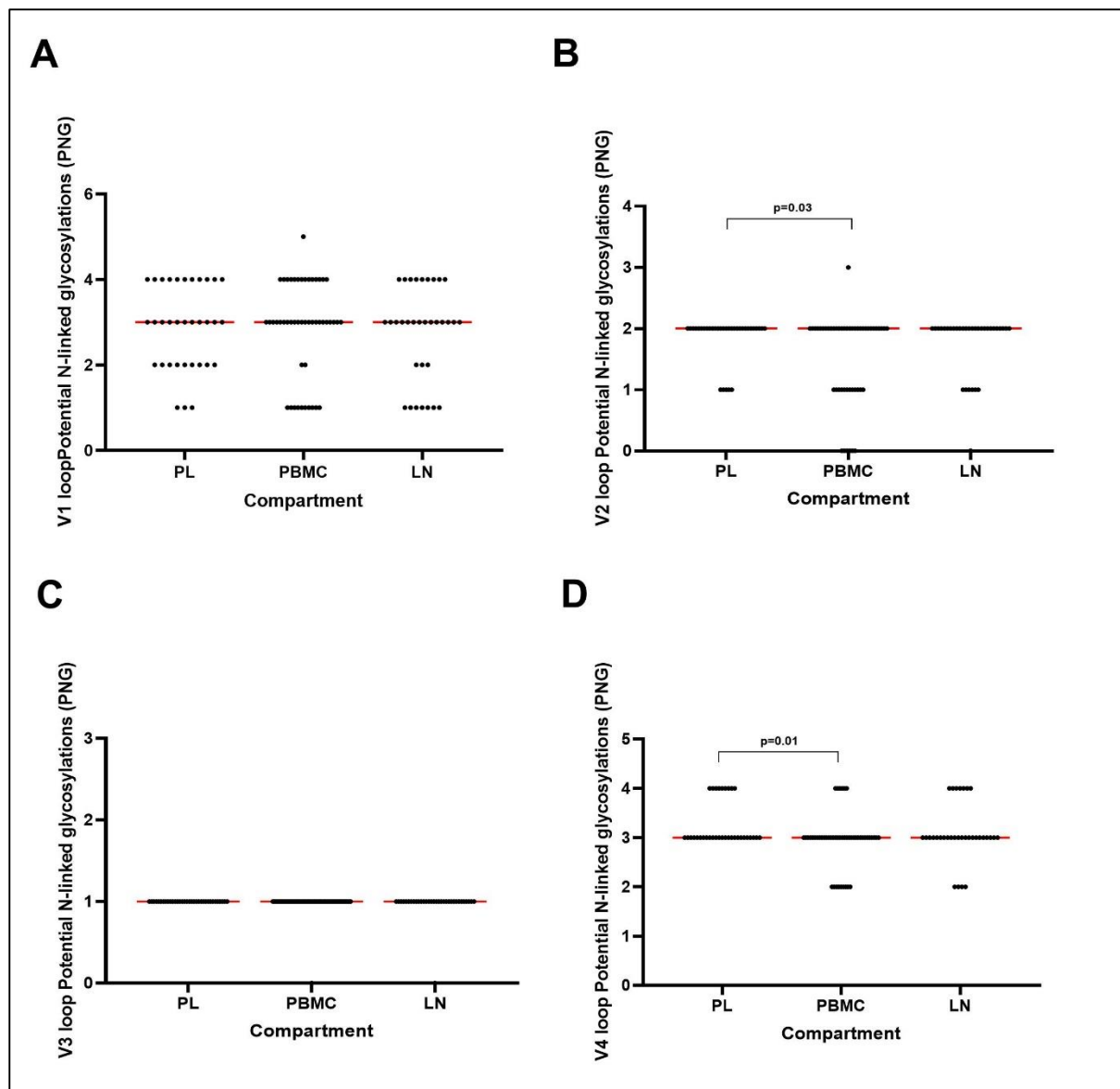


Figure 4.3: Comparison of the potential N-linked glycosylations (PNGs) in the V1-V4 loops of the *env* sequences generated from plasma, PBMC and lymph nodes compartments. Each dot represents an individual sequence and the horizontal bars indicate the median number of PNGs for each compartment. Differences between compartments were evaluated using the Mann-Whitney U test, statistically significant p-values are indicated in the plots. PL, plasma; PBMC, peripheral blood mononuclear cells; LN, lymph node

*Note: Medians are represented as red bars

4.4 Amino acid variations in bnAb epitopes

4.4.1 Minor amino acid variations in the CD4 binding site of the HIV-1 *env* sequences obtained from plasma, PBMC and lymph node compartments

Amino acid signatures within loop D, the CD4 binding site and β 20- β 25 of the V5 loop were analyzed; the N-linked glycan at position 197 was completely conserved in PL, PBMC and LN compartments (Fig 4.4 A-C). However, few amino acid differences were observed in positions 234, 276, 279 of loop D. The N276 glycan was found in all PL and LN sequences and 88% of the PBMC-derived sequences. The remaining PBMC sequences at this position featured the aspartic acid (D) and asparagine (N). A low frequency (11%) of serine residues at position 234 were found only in the lymph node compartment, the remaining LN, PL and PBMC sequences had the N-linked glycan (O), asparagine (N) and aspartic acid (D) (Fig 4.4A-C, Table 4.2A).

All three compartments had either an asparagine (N) or an aspartic acid (D) at position 279; PL and LN compartments had 50% of asparagine and 50% of aspartic acid residues (Fig 4.4 A-C, Table 4.2A, B). While 70% of *env* sequences obtained from the PBMC compartment had asparagine residues at position 279, while only 30% of sequences had aspartic acid (D) residues. Asparagine residues were previously associated with reduced sensitivity, while aspartic acid was associated with high sensitivity to the CD4 binding site bnAbs (Bricault et al., 2019). All sequences in all three compartments featured the asparagine residues at position 280. Furthermore, we analysed amino acids in position 368, aspartic acid (D) was highly conserved in all three compartments. Moreover, arginine (R) residues at position 456 were highly conserved in all three compartments. These findings suggest minor variations in the CD4 binding site of the HIV-1 *env* sequences obtained from PL, PBMC and LN compartments.

4.4.2 Substantial amino acid differences in the V2 apex of plasma, PBMC and lymph node-derived sequences

Amino acid signatures in the V2 loop were analyzed; the N-linked glycans at position 156 and 160 were highly conserved in all three compartments (Figure 4.4 D-F). However, a low frequency of threonine (T) mutations at position 160 in the PBMC and LN compartments, yet this mutation was not found in the plasma compartment. In addition, glutamic acid (E) residues at position 164 were highly conserved in all three compartments. However, PBMC and LN sequences also had a glycine (G) mutation. In addition, majority of sequences across the three compartments featured an arginine (R) or lysine (K) at position 166, and both residues are associated with high neutralisation sensitivity (Fig 4.4 D-F). Furthermore, aspartic acid residues (D) were also highly conserved at position 167, although glycine (G) mutation was found in PBMC and LN -derived sequences.

Similarly, a majority (>80%) of *env* sequences had lysine (K) or arginine (R) residues at position 169, however variations in escape mutations were observed in various compartments (Fig 4.4 D-F). Glutamine (Q), isoleucine (I) and alanine (A) mutations were found in the LN compartment, while glutamine (Q) and asparagine (N) mutations were observed in the PBMC compartment. In contrast, PL sequences had aspartic acid (D), serine (S) and isoleucine (I) mutations. Our findings suggest that the N156 and N160 glycans are highly conserved between the three compartments, although, a low frequency of mutations was observed in the PBMC and LN compartments while plasma-derived sequences were highly conserved. In addition, discordant amino acid mutations were observed at position 164, 166, 167 and 169 and these are important target sites for V2 apex bnAbs, especially CAP256-VRC26.25 monoclonal antibody. These differences highlight the dynamic viral adaptation and selective pressures within distinct anatomical reservoirs.

4.4.3 Minor amino acid differences identified in the V3 Glycan Supersite

In the V3 loop, the N332 glycan was highly conserved in all three compartments, although asparagine (N) mutation was observed in 12 % of PBMC sequences (Fig 4.4 G-I) (Table 4.2A). The N-linked glycan was highly conserved at position 301, although, there was a low frequency of the asparagine (N) in both LN and PL-derived sequences as well as glutamine (Q) and aspartic acid (D) in PBMC and LN sequences respectively. The GDIR motif (HXB2 positions 324-327), was analysed and this is an important target site for V3-glycan bnAbs (Sok et al., 2016). More than 30% of sequences in the LN, 35% of PL sequences, and 16% of PBMC sequences had an asparagine (N) at position 325 which has been associated with resistance to V3 targeting antibodies (Sok et al., 2014, Ndlovu et al., 2025). The glycan at position 386 was completely conserved in all three compartments and has been associated with high sensitivity to BG18 mAb. Minor variability was seen at position 392; the LN and PBMC had an N-linked glycan in more than 50% of their sequences and varying frequencies of asparagine (N), aspartic acid (D) and threonine (T). In contrast, 74% of PL sequences featured the glycan, while the remaining sequences had the aspartic acid residues. The aspartic acid mutation at position 392 was previously associated with resistance to V3 bnAbs (Doria-Rose et al., 2010).

4.4.4 Minor differences in amino acid signatures identified in the MPER

Analysis of the proximal MPER (positions 662-667) showed that the frequency of alanine (A) mutation at position 662 was extremely high across all compartments, this mutation is associated with resistance to 2F5 mAb (Bricault et al., 2019). Aspartic acid (D) at position 664 was completely conserved in all compartments (Fig 4.4 M-O, Table 4.2B). At position 665, lysine (K) was found in less than 40% of sequences in all three compartments, while majority of the sequences featured the serine (S) mutation, which is associated with resistance to 2F5 (Bricault et al., 2019). A high frequency of resistant mutations was also seen in position 667 including asparagine (N), lysine (K), glycine (G), and glutamine (Q) (Fig 4.4 M-O).

Analysis of the distal MPER (positions 671-683), particularly amino acids at position 674 showed variations, 74% of the LN sequences had serine (S) residues, which is associated with resistance to 10E8 antibody, while the remaining sequences had aspartic acid (D), which is associated with high sensitivity to 10E8 antibody. In contrast, 60% of the PBMC-derived sequences had serine (S) residues, while 40% of sequences had aspartic acid (D), lysine (K) residues as well as an N-linked glycan (O). While the majority of plasma-derived sequences

had serine (S), while the remaining 40% had the N-linked glycan (O) and aspartic acid (D) and these amino acids are associated with high sensitivity to 10E8. Amino acids at position 671 were highly conserved and featured either the asparagine (N) or serine (S) which are associated with sensitivity to 10E8. Positions 672 and 673 featured tryptophan (W) and phenylalanine (F) respectively, and are associated with sensitivity.

Majority of the LN, PBMC and PL sequences had threonine (T) residues at position 676, which is associated with sensitivity to 10E8 mAb, while the remaining 10-20% had serine (S) residues and this AA is associated with resistance to 10E8 mAb (Li et al., 2012, Do Kwon et al., 2021). At position 680 the tryptophan (W) was completely conserved in all compartments. Majority (>70%) of LN and PBMC-derived sequences had a high frequency of lysine (K) at position 683, which is associated with high sensitivity to 10E8. While, the remaining LN sequences (30%) had arginine (R) residues, and the PBMC sequences had arginine and glutamine (Q), and they are associated with resistance to 10E8. In contrast, only 53% of plasma-derived sequences had lysine (K), the remaining sequences featured arginine (R) and glutamine (Q) residues. In addition, the PBMC and PL compartments featured G and S residues respectively. Interestingly, glutamine mutation was only observed in PBMC and PL compartments. In summary, the distal MPER region was highly conserved and minor variations were observed in the three compartments.

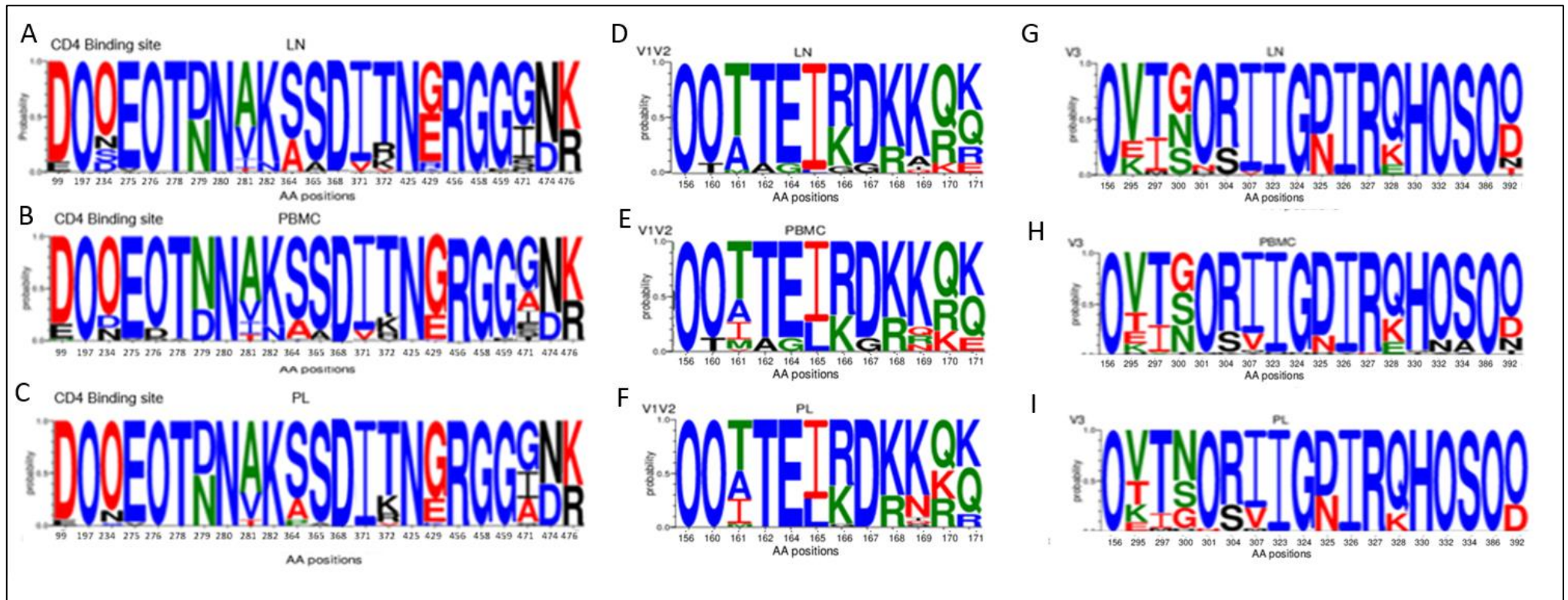
4.4.5 Minor differences in amino acid signatures in the gp120-gp41 interface, fusion peptide and the silent face

In the gp120/gp41 interface, sequences in all three compartments were highly conserved (Fig 4.4 J-L) (Table 4.2B). The N-linked glycans were conserved in both position 611 and 637. The N-linked glycan at position 637 plays a critical role in maintaining the structure of loop D, which is responsible for the interaction between gp120 and gp41 and the positioning of the viral spike for the binding to the CD4 for viral entry.

In the fusion peptide, residues 512–527 across all three compartments remained conserved. Alanine (A) at position 512 and 517 were conserved in all three compartments. While, minor differences were seen at positions 514 and 516, where both the PBMC and PL compartments had glycine (G) in 100% of their sequences, while 96% of the LN sequences had glycine, the remaining sequences had arginine (R) mutation which is associated with resistance (Fig 4.4 P-

R) (Table 4.2B). All three compartments had valine (V) and methionine (M) at position 518, and these amino acids are associated with neutralization sensitivity.

For the silent face region, the N-linked glycan was completely conserved at position N262 in all compartments. However, minor differences in the frequency of N-linked glycans was observed at positions 295 and 448 (Fig 4.4 S-U, Table 4.2B). Loss of a glycan at position N295 and N448 are associated with reduced sensitivity to the silent face bnAbs (Zhou et al., 2018). PID 0053 had a lysine (K) in the LN compartment and all other SGAs had glutamic acid (E). Valine (V) substitution at position 295 disrupts the N295 glycan site and reduces sensitivity to VRC-PG05 and ACS114, while SF12 retain partial activity (Zhou et al., 2018). At position 448, only 39% of LN, 49% of PBMC and 50% of PL compartments had an N-linked glycan, majority of the sequences had either serine (S) or threonine (T) which are both associated with resistance to silent face bnAbs (Fig 4.4 S-U). The lack of the N448 glycan results in strong resistance or complete escape from VRC-PG05 and ACS114 mAbs (Schoofs et al., 2019).



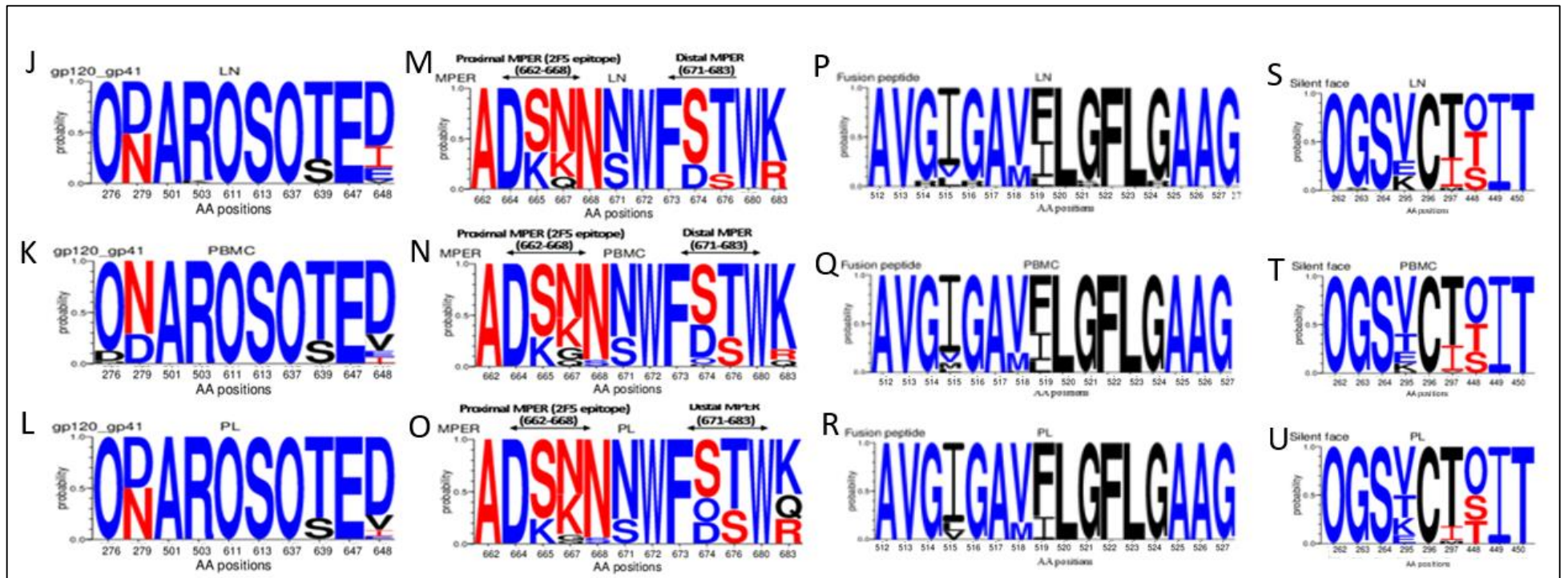


Figure 4.4: Amino acid differences detected in the various bnAb epitopes (A-U)

Sequence Logo plots from HIV-1 *env* sequences obtained from plasma, PBMC and lymph node compartments showing the frequency of important amino acid positions within epitopes for broadly neutralizing antibodies targeting the CD4-binding site (A-C), V2-glycan (D-F), V3 glycan (G-I), gp120-gp41 interface (J-L), MPER (M-O), fusion peptide (P-R) and silent face (S-U). The size of the amino acid is directly proportional to the frequency. The “O” shows the N-linked glycans. Red is associated with resistant amino acid residues; blue is associated with sensitive amino acid residues; green is associated with differences in neutralisation sensitivities in antibodies from the same class, and black is associated with uncharacterized amino acids.

A

Compartment	Epitope positions																			
	CD4 binding site					V2 loop							V3 glycan							
	234(%)	276(%)	279(%)	368(%)	456(%)	156(%)	160(%)	164(%)	166(%)	167(%)	169(%)	295(%)	301(%)	324(%)	325(%)	326(%)	327(%)	332(%)	386(%)	392(%)
PL	O-88 N-9 D-3	O-100	D-44 N-56	D-100	R-100	O-100	O-100	E-100	R-62 K-35 G-3	D-100	K-71 N-20 I-3 S-3 Q-3	V-50 T-24 K-18 E-8	O-97 N-3	G-100	D-65 N-35	R-98 K-2	R-100	O-100	O-100	O-74 D-26
PBMC	O-76 N-12 D-12	O-88 D-10 N-2	N-70 D-30	D-100	R-100	O-100	O-88 T-12	E-88 G-12	R-68 K-32	D-88 G-12	K-78 R-5 Q-5 N-12	V-52 T-24 E-16 K-8 N-2	O-98 Q-2	G-98 R-2	D-82 N-16 R-2	I-98 Y-2	I-100	O-88 N-12	O-100	O-63 D-20 N-12 T-5
LN	O-67 N-12 S-10 D-11	O-100	D-46 N-54	D-100	R-100	O-100	O-91 T-9	E-91 G-9	R-58 K-36 G-6	D-91 G-9	K-85 A-9 Q-3 I-3	V-70 E-15 K-15	O-91 N-6 D-3	G-100	D-64 N-36	I-100	R-100	O-100	O-100	O-55 D-30 N-9 T-6

B

Compartment	Epitope positions													
	gp120/gp41 interface		MPER						Fusion peptide		Silent face			
	611(%)	637(%)	662(%)	664(%)	665(%)	667(%)	674(%)	676(%)	683(%)	515(%)	518(%)	262(%)	295(%)	448(%)
PL	O-100	O-100	A-100	D-100	S-76-K-23	N-53 K-38 G-6 Q-3	S-56 O-24 D-21	T-68 S-32	K-53 Q-24 R-24	I-79 V-12 L-9	V-79 M-21	O-100	V-70 K-15 E-15	O-40 T-36 S-24
PBMC	O-100	O-100	A-100	D-100	S-71 K-29	N-53 K-29 G-12 Q-6	S-60 D-33 O-5 K-2	T-71 S-23	K-84 R-10 Q-6	I-80 V-10 M-6 L-4	V-80 M-18 L-2	O-100	V-59 T-18 E-13 K-8 N-2	O-49 T-29 S-22
LN	O-100	O-100	A-100	D-100	S-64 K-36	N-63 K-24 Q-13	S-76 D-24	T-85 S-15	K-70 R-27	I-82 V-9 L-9	V-82 M-18	O-100	V-50 T-24 K-18 E-8	O-50 S-26 T-24

Table 4.2: Amino acid frequencies in the various bnAb epitopes

Logoplot frequency table listing the proportion of sequences containing each residue at each epitope for the PL, PBMC and LN compartments

4.5 Neutralisation Sensitivity between lymph nodes, PBMC and plasma compartment

Neutralisation sensitivity of pseudoviruses was assessed against a panel of 11 bnAbs targeting the CD4 binding site (VRC01, VRC07-523LS, N6), V2 glycan (PGDM1400, CAP256-VRC26.25, PG9), V3 glycan (PGT121), MPER (10E8), gp120/41 interface (PGT151), fusion peptide (N123.VRC34), and silent face (SF12) bnAbs (Fig 4.5, Fig 4.6). Neutralisation activity was determined by assessing the neutralisation breadth and potency.

Across all participants, neutralisation sensitivity varied notably between the lymph node (LN), PBMC, and plasma compartments. Overall, majority of viruses were sensitive to VRC07-523LS, and there was no difference in the neutralisation sensitivity of PL, PBMC and LN-derived viruses (Fig 4.5, Fig 4.6). However, the highest resistance was noted against VRC01, plasma-derived viruses were more resistant to VRC01 compared to PBMC and LN viruses ($p=0.02$). The high VRC01 resistance could be due to a high frequency of N-linked glycans at position 234 compared to LN and PBMC sequences (Fig 4.5, Fig 4.6). Furthermore, N6 had a high neutralisation breadth and potency (geometric mean titers= $0.09\mu\text{g/ml}$), however neutralisation titers were similar between LN and PL, while PBMC had a higher neutralisation sensitivity to compared to LN and PL compartments ($p=0.01$).

In addition, V2 glycan bnAbs including CAP256-VRC26.25 and PGDM1400 also had exceptional neutralisation breadth and potency. All PL-derived viruses were highly sensitive to CAP256-VRC26.25, with a geometric mean of $0.03\mu\text{g/ml}$, while 67% of PBMC viruses and 77% of LN-derived viruses were sensitive to CAP256-VRC26.25 with a slightly lower potency ($0.38\mu\text{g/ml}$ and $0.14\mu\text{g/ml}$ respectively) (Fig 4.6). In addition, majority of the viruses from all three compartments had a higher neutralisation sensitivity to PGDM1400, the neutralisation breadth ranged from 88-92%, and the potency ranged from 0.03 - $0.06\mu\text{g/ml}$. These minor differences in neutralisation sensitivity could be due to substantial variations in amino acids at position 164, 166, 167 and 169 (Fig 4.4 D-F).

In contrast, V3 glycan bnAb such as PGT121 displayed variable levels of resistance against viruses from all three compartments (38-62%); plasma pseudoviruses generally exhibited the highest resistance, with only 38% neutralisation coverage, and a geometric mean of $3.41\mu\text{g/ml}$. While, PGT121 neutralised 62% of LN, and $0.54\mu\text{g/ml}$ and 50% of PBMC viruses with $1.91\mu\text{g/ml}$ geometric mean (Fig 4.6). This high resistance in PL could be due to the longer V1 loop lengths observed in PL compared to PBMC and LN, as longer loop length is associated with

resistance to V3 glycan bnAbs. Interestingly, the N301, and N332 glycans were highly conserved in the majority of the clones, except PID 0011_PL SG9, which lacked the N301 glycan, hence the resistance to PGT121.

Furthermore, MPER mAb (10E8) achieved high neutralisation coverage (85-92%), with moderate potency (1.58-4.78 ug/ml), and no differences in neutralisation sensitivity between the PBMC and LN compartments. The interface mAb (PGT151) demonstrated high neutralisation coverage across all compartments, the neutralisation coverage was >70% in both PL and LN, while PBMC neutralisation coverage was only 58%. The reduced sensitivity seen in only PBMC despite minor sequences variation could be due to compartment specific differences in *env* cleavage efficiency and trimer stability. PBMC viruses may express a higher proportion of uncleaved or conformationally flexible *env*, which disrupts the gp120–gp41 interface epitope required for PGT151 binding. In contrast, PL and LN viruses appear to maintain more native prefusion *env* conformations with glycan contexts that favor PGT151 neutralization. Fusion peptide mAb, N123.VRC34 mAb displayed the lowest neutralisation coverage (0-25%) and potency in all compartments. This may be attributed to poor epitope accessibility as many Tier 2 viruses do not present the fusion peptide in a way that VRC34 can efficiently access. Furthermore, the silent face SF12 mAb had a high neutralisation coverage against all compartments, ranging from 92-100% with a strong potency of 0.03-0.6 µg/ml (Fig 4.6). All viruses had the N262 glycan, while some viruses lacked the N448 glycan. The lack of the N448 glycan is known to only slightly reduce the neutralisation sensitivity for VRC-PG05, but it is less effective of SF12.

IC50 titers µg/ml	
	<0,1
	0,1-0,9
	1,0-3,0
	3,1-9,9
	>10

PID	SGA	Compartment	CD4 binding site			V2 Apex			V3 Glycan	Interface	MPER	Fusion Peptide	Silent Face
			VRC07-523L5	VRC01	N6	CAP256-VRC26	PGDM1400	PG9	PGT121	PGT151	10 E 8	N123_VRC34	SF12
PID 0011	PL9	Plasma	10,00	20,00	0,01	0,01	20,00	0,04	20,00	0,01	2,46	20,00	0,12
	PL13	Plasma	0,03	15,36	0,58	0,03	0,04	0,04	0,20	0,19	2,31	20,00	0,03
	PBMCDA6	PBMC	0,12	14,12	0,06	0,01	0,01	0,24	20,00	0,01	5,81	20,00	0,28
	PBMCDA11	PBMC	0,21	0,18	0,14	0,01	0,16	0,02	2,31	0,05	7,80	0,02	0,07
	PBMCDA14	PBMC	0,01	0,01	0,01	20,00	0,01	0,02	0,02	20,00	20,00	20,00	0,03
	PBMCRA1	PBMC	0,01	0,01	0,32	0,01	0,01	0,09	0,07	20,00	2,30	20,00	0,11
	LNDNA1	LN	0,02	0,01	1,87	0,01	0,01	0,42	0,06	0,01	8,00	20,00	0,03
	LNDNA2	LN	0,01	19,00	0,27	20,00	0,34	0,05	14,18	20,00	3,23	20,00	0,07
	LNDNA3	LN	0,06	12,04	20,00	0,01	0,01	0,88	0,23	0,02	6,97	20,00	0,31
	LNDNA6	LN	0,06	0,08	0,13	0,01	0,43	20,00	10,72	0,32	2,60	20,00	0,26
	LNDNA7	LN	0,01	12,26	0,42	11,25	0,03	0,02	0,06	7,97	0,19	0,22	0,18
	LNDNA10	LN	0,50	7,84	20,00	11,75	0,02	0,06	0,35	0,03	20,00	20,00	0,03
PID 0053	LNRNA5	LN	20,00	0,01	0,34	0,02	0,01	0,08	0,05	11,00	0,30	20,00	20,00
	LNRNA6	LN	0,62	10,50	0,98	0,05	0,05	0,01	10,00	0,01	2,63	20,00	0,01
	PL3	Plasma	0,24	12,25	0,50	0,02	0,19	20,00	0,27	20,00	5,11	20,00	0,02
	PL8	Plasma	0,01	20,00	0,70	0,12	0,01	0,06	17,98	0,02	3,40	20,00	0,08
	PBMCDA4	PBMC	0,01	10,55	0,79	0,26	0,01	0,11	20,00	0,02	2,74	20,00	0,02
	PBMCDA5	PBMC	0,01	0,01	0,01	0,02	0,01	0,02	20,00	20,00	0,18	0,05	15,35
	PBMCDA11	PBMC	20,00	0,01	0,10	15,00	0,02	0,04	0,12	11,95	3,29	20,00	0,15
	LNDNA3	LN	0,01	0,15	0,05	0,01	0,05	0,02	0,02	0,02	0,06	20,00	0,03
PID 118	LNDNA7	LN	0,04	0,01	1,21	2,45	0,01	0,06	0,05	0,81	18,21	20,00	0,01
	LNDNA10	LN	0,15	20,00	0,53	0,04	20,00	0,02	18,63	0,01	2,47	20,00	0,09
	PL6	Plasma	0,01	20,00	0,34	0,01	0,02	20,00	15,95	20,00	10,29	0,16	0,33
	PL9	Plasma	0,06	16,80	0,10	0,02	0,01	0,49	20,00	0,43	8,27	20,00	0,06
PID 3011	PBMCDA1	PBMC	0,19	17,60	0,01	20,00	0,04	20,00	0,28	0,04	4,85	20,00	0,01
	PBMCDA4	PBMC	0,02	20,00	0,05	0,04	20,00	0,01	19,92	0,01	8,93	20,00	0,06
	PL1	Plasma	20,00	0,45	1,30	0,01	0,01	0,04	0,16	12,04	7,54	20,00	0,03
	PL2	Plasma	0,02	10,47	0,01	0,82	0,04	0,02	18,27	0,52	4,30	0,23	0,25
	PBMCDA1	PBMC	0,01	5,57	0,08	0,68	0,01	0,03	18,51	0,01	4,82	20,00	0,01
	PBMCDA5	PBMC	0,04	0,05	0,03	0,60	0,01	20,00	18,98	0,02	0,18	0,07	0,01
	PBMCDA7	PBMC	0,06	20,00	0,80	20,00	0,02	20,00	0,38	20,00	2,06	20,00	0,03
	LNDNA8	LN	0,04	0,04	0,46	0,41	0,01	0,01	0,04	20,00	0,19	20,00	0,05
PID 3011	LNDNA14	LN	0,16	3,91	0,45	0,01	0,03	0,04	20,00	0,03	0,52	20,00	0,02

Figure 4.5: Neutralisation sensitivity data in lymph nodes, PBMCs and plasma-derived clones. The data is presented as scatterplots for each IC₅₀ titer (μg/mL) where each virus is represented by an individual shape corresponding to a specific compartment. Red squares indicate neutralisation IC₅₀ scores <0.1 (sensitivity), orange squares indicate neutralisation IC₅₀ titers between 0.1–0.9, yellow squares indicate IC₅₀ titers between 1.0–3.0 and light yellow indicates IC₅₀ titers between 3.1–10 μg/ml (partial sensitivity at varying degrees), clear squares indicate titers>10 (resistance).

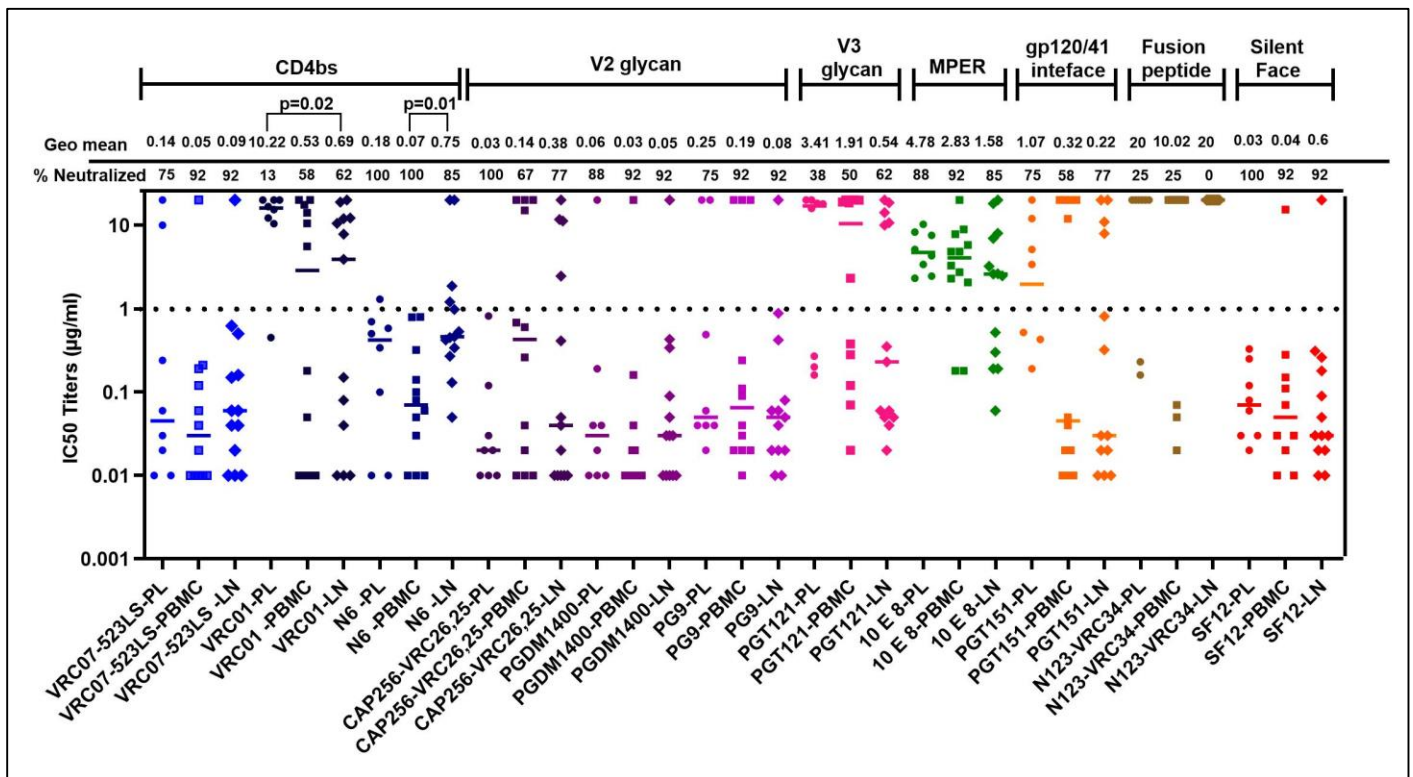


Figure 4.6: Neutralisation data presented as IC₅₀ titers (mg/mL) in which each virus is represented by an individual circle. Various bnAbs were grouped and colour coded by their epitopes, including the CD4 binding site (blue), V2 glycan (purple), V3 glycan (pink), MPER (green), gp120/41 interface (orange), fusion peptide (brown) and the silent face (red). Plasma (PL) neutralisation IC₅₀ titers are shown in circles, PBMC neutralisation titers are shown in squares,

lymph nodes (LN) titers are shown in diamond shapes. The dotted horizontal line indicates the neutralisation IC₅₀ at 1 µg/ml, which serves as a conventional threshold for potent neutralisation. The top rows show the significant p values obtained from the non-parametric Mann Whitney U tests and geometric means “Geometric mean” across isolates for each antibody and the “% Neutralized” shows the fraction of viruses neutralized by each antibody.

4.6. Intra-participant differences in neutralisation sensitivity and amino acid signatures

Analysis of the amino acid differences and the effect of these variations on neutralisation sensitivity within each participant was investigated (Tables 4.3-4.6) (Supp Tables 7.3 A-D).

PID 0011

In participant 0011, two PL, four PBMC and eight LN derived clones were evaluated against the panel of bnAbs. One of the two PL clones (PL9) was resistant, while another clone (PL13) was sensitive to VRC07-523LS. Both clones were sensitive to N6, and resistant to VRC01 (Table 4.3). In PBMC, all clones were sensitive to VRC07-523LS or N6, three of the four clones were sensitive to VRC01, however only one clone (PBMCDNA6) was resistant to VRC01. Seven of the eight LN clones were highly sensitive to CD4 binding site antibodies, while one clone (LNRNA5) was resistant to VRC07-523LS. However, no differences in the CD4 binding site between sensitive and resistant clones, all clones had asparagine (N) at position 279, and 280. An isoleucine (I) at position 281 was found in both sensitive and resistant clones (Table 4.3).

Furthermore, both PL clones were sensitive to CAP256-VRC26.25 and PG9, while one clone (PL13) was sensitive, and another clone (PL9) was resistant to PGDM1400 (Table 4.4). All PBMC clones were sensitive to PGDM1400 and PG9, while three of the four clones were sensitive to CAP256-VRC26.25 and the remaining clone (PBMCDNA14) was resistant to CAP256-VRC26.25. All LN clones were sensitive to PGDM1400, while seven of the eight clones were sensitive to PG9 with only one resistant clone (LNDNA6). Five of the eight LN clones were sensitive to CAP256-VRC26.25, and the remaining three were resistant. Two LN, and one PBMC clones lacked the N160 glycan and/or had glycine (G) mutation in position 164, but they remained

sensitive to V2 glycan bnAbs. Some of the LN (LNDNA1, LNDNA2) and PBMC (PBMC RNA1) clones lacked the N160 glycan, with glycine mutation at position 164, and they remained partially sensitive to CAP256-VRC26.25.

One PL clone (PL13) was highly sensitive, and another clone (PL9) was resistant to PGT121 (Table 4.5). Three of the four PBMC clones were sensitive to PGT121, and the remaining clone (PBMC DNA6) was resistant, while five of the eight LN clones were sensitive and three clones were resistant to PGT121. PL resistant clone (PL9) lacked the N301 glycan, and this may have led to resistance to PGT121 neutralisation. However, most of the resistant clones had a conserved N301 and N332 glycans. Although, no significant differences were found in the V1 loop length between PGT121 sensitive and resistant clones (Fig 4.7).

Both PL and six of the eight LN clones were sensitive to PGT151. While two of the four PBMC clones were sensitive, and the remaining two clones (PBMC DNA14, PBMC RNA1) were resistant. All PL and most PBMC and LN clones were sensitive to 10E8, only one PBMC clone (PBMC DNA14) was resistant to 10E8, this clone featured glutamine mutation at position 667. LN clone (LNDNA10) was also resistant to 10E8 (Supp Fig. 7.3B).

All PL, and majority of the LN and PBMC clones were resistant to N123.VRC34. Only a single PBMC (PBMC DNA11), and LN (LNDNA7) clones were sensitive which could be attributed to valine (V) residues at position 518 and isoleucine (I) at position 519 (Supp Table 7.3C). Majority of the PL, PBMC and LN clones were sensitive to SF12, only one clone (LNRNA5) was resistant to SF12 (Supp Table 7.3D). Collectively, majority of the LN, and PBMC clones were sensitive to most bnAbs, while PL viruses had a slightly higher resistance to most bnAbs. These findings suggest a greater preservation of sensitive *env* conformations in tissue reservoirs compared to circulating blood compartment.

PID 0053

In this participant, two PL, three PBMC and three LN derived clones were evaluated against the panel of bnAbs. Both PL clones were sensitive to VRC07-523LS and N6, but resistant to VRC01, while all PBMC clones were sensitive to N6 and only two clones were sensitive to both VRC07-523LS and VRC01. All LN clones were sensitive to VRC07-523LS and N6, while only two clones were sensitive to VRC01. These partially resistant clones (PL3, PL8, PBMCDNA4, PBMCDNA11, LNDNA10) lacked the N234 glycan. LNDNA10 had a serine mutation at position 234, which is associated with resistance to VRC01, while the other clones had aspartic acid (D) and were highly sensitive to VRC01 (Table 4.3). A valine (V) mutation at position 281 has been associated with reduced sensitivity. Both PL clones were sensitive to CAP256-VRC26.25 and PGDM1400, while one clone (PL3) was resistant to PG9 (Table 4.4), and all clones featured the N156 and N160 glycans. All PBMC clones were sensitive to PGDM1400 and PG9, while two of the three clones were sensitive to CAP256-VRC26.25. All LN clones were sensitive to CAP256-VRC26.25 and PG9, while two clones were sensitive to PGDM1400. Both sensitive (PL8) and resistant clones (LNDNA10) featured 166K (lysine) mutation. Most of the clones had lysine at position 169, except for LNDN13, which has an isoleucine (I) mutation.

Furthermore, one of the two PL clones was sensitive to PGT121, while the second clone was resistant. Two of the three PBMC clones were resistant, and one clone (PBMCDNA11) was sensitive to PGT121. In addition, two of the three LN clones were sensitive, while the third clone (LNDNA10) was resistant to PGT121. PGT121-resistant clones all featured the N301 and N332 glycans (Table 4.5). Similarly, one PL clone (PL3) was sensitive, while another clone (PL3) was resistant to PGT151. In contrast, one PBMC clone (PBMCDNA4) clone was sensitive, and two clones were resistant to PGT151. In contrast, all LN clones showed sensitivity to PGT151. All clones from this PID featured the N611 and N637 glycans as well as an aspartic acid (D) mutation at position 648 (Supp Table 7.3A).

All PL and PBMC clones were sensitivity to 10E8 and one LN clone (LNDNA7) was resistance. The resistance seen in this LN clone could be due to the lysine (K) at position 683, in contrast to the arginine (R) seen in the two clones that were sensitive to 10E8. All PL and LN clones were resistant to N123.VRC34, while one PBMC clone (PBMCDNA5) was sensitive and two were resistance. All PL and LN clones were sensitivity to SF12 and one PBMC clone (PBMCDNA5)

were resistance to SF12. Overall, PID 0053 also displayed the highest neutralisation breadth in LN clones, especially for CD4bs and V1V2 loop epitopes, indicating that circulating infected cells may retain more neutralisation-sensitive viral species than those persisting in lymphoid tissue or plasma. Together, these intra-participant patterns indicate localized evolution and immune compartmentalization, where viral quasispecies adapt differently depending on tissue environment and antibody exposure.

PID 118

In this participant, two PL and two PBMC derived clones were evaluated against the panel of bnAbs. Both PL and PBMC clones were sensitive to VRC07-523LS, and N6, but all clones were resistant to VRC01. Both PL clones were sensitive to CAP256-VRC26.25 and PGDM1400, while only one clone (PL6) was resistant to PG9, and the second clone (PL9) were moderately sensitive. One of the four VRC01-resistant clones had aspartic acid (D) mutation at position 276, which was previously associated with resistance to VRC01. While the other three clones had no resistance mutations. One of the two PBMC clones (PBMCDNA1) was resistant to both CAP256-VRC26.25 and PG9, while remained sensitive to PGDM1400 and there were no resistance mutations. While the second clone (PBMCDNA4) was sensitive to both CAP256- VRC26.25 and PG9, but resistant to PGDM1400 and this clone had asparagine at position 169.

Both PL clones were resistant to PGT121, while one PBMC clone (PBMCDNA1) was sensitive and the other clone (PBMCDNA4) was resistant. One of the three PGT121 resistant clones (PBMCDNA1) lacked the N332 glycan, while the other two resistant clones had both the N301 and N332 glycans. One of the two PL clones was sensitive to PGT151, while both PBMC clones were sensitive to PGT151 (Supp. Table 7.3A). Most PL and PBMC clones were resistant to 10E8, although one of the PBMC clones was moderately sensitive. One of the two PL clones was sensitive to N123.VRC34, while both PBMC clones were resistant. Both PL and PBMC clones were sensitive to SF12.

PID 3011

In this participant, two PL, three PBMC and two LN derived clones were evaluated against the panel of bnAbs. Both PL clones showed sensitivity to N6; while only one clone (PL2) was sensitive, and another clone (PL1) was resistant to either VRC07-523LS or VRC01 (Table 4.3). All PBMC clones were highly sensitive to VRC07-523LS and N6, while two clones (PBMCDNA1, PBMCDNA7) were resistant to VRC01. In contrast, both LN clones were highly sensitive to VRC07-523LS, and N6, although one of the clones (LNDNA14) had a higher resistance to VRC01. VRC07-523LS-resistant clone (PL1) had a threonine at position 281 VRC01, while the other resistant clones had glutamic acid (E) mutation at position 459 and 471 (Table 4.3). Mutations at position 281 and 459 were associated with increased resistance to VRC01 bnAbs. All PL and LN clones were sensitive to CAP256-VRC26.25, PGDM1400 and PG9 (Bricault et al., 2019). In contrast, PBMC clones were partially resistant to V2 apex bnAbs; all PBMC clones were sensitive to PGDM1400, while two of the three clones were sensitive to CAP256- VRC26.25, and two of the three clones were resistant to PG9. However, these clones had intact N156, and N160 glycans and lysine (K) at position 169 (Table 4.4).

Only one clone in each compartment was sensitive to PGT121, majority of the clones were resistant to this antibody. However, all clones had intact N301, and N332 glycans. In addition, PL, PBMC clones were moderately sensitive to 10E8, while both LN clones were more sensitive to 10E8. Only one of the two PL and PBMC clones was sensitive to N123.VRC34, while all LN clones were resistant to N123.VRC34. All PL, PBMC and LN clones were sensitive to SF12. Collectively, majority of LN and PBMC clones were sensitive to CD4bs, V2-glycan, and MPER epitopes, while PL clones had a slightly higher resistance to these bnAbs.

PID	HXB2 numbering		99	197	198	199	234	235	236	275	276	277	278	279	280	281	282	368	369	371	372	425	429	456	458	459	462	471	474	476	CD4 binding site			
			D	N	T	S	N	G	T	V	N	F	T	D	N	A	K	D	P	I	V	N	K	R	G	G	N	G	D	R	VRC07-5231S	VRC01	N6	
PID0011	PL9	Plasma	.	.	S	.	.	.	.	E	.	.	.	N	.	.	.	.	L	.	T	.	E	.	.	.	D	.	.	.	10,00	20,00	0,01	
	PL13	Plasma	.	.	.	.	.	.	.	E	.	.	.	N	.	.	.	.	L	.	T	.	E	.	.	.	D	.	N	.	0,03	15,36	0,58	
	PBMCDNA6	PBMC	.	.	.	.	.	.	.	E	.	.	.	N	.	.	.	.	L	.	T	.	E	.	.	.	D	.	N	.	0,12	14,12	0,06	
	PBMCDNA11	PBMC	.	.	.	.	.	.	.	E	.	.	.	N	.	.	.	.	L	.	T	.	E	.	.	.	D	.	N	.	0,21	0,18	0,14	
	PBMCDNA14	PBMC	.	.	S	.	.	.	.	E	.	.	.	N	.	.	.	.	L	.	T	.	E	.	.	.	D	.	N	.	0,01	0,01	0,01	
	PBMCRNA1	PBMC	.	.	.	.	D	.	I	E	.	.	.	N	.	I	N	.	.	V	T	.	E	.	.	.	T	.	N	K	0,01	0,01	0,32	
	LNDNA1	LN	.	.	.	.	D	.	.	E	.	.	.	N	.	I	N	.	.	V	T	.	E	.	.	.	T	.	N	K	0,02	0,01	1,87	
	LNDNA2	LN	.	.	.	.	D	.	.	E	.	.	.	N	.	I	N	.	.	V	T	.	E	.	.	.	T	.	N	K	0,01	19,00	0,27	
	LNDNA3	LN	.	.	S	.	.	.	.	E	.	.	.	N	.	.	.	.	L	.	T	.	E	.	.	.	D	.	N	.	0,06	12,04	20,00	
	LNDNA6	LN	.	.	S	.	.	.	S	E	.	.	.	N	.	.	.	.	L	.	T	.	E	.	.	.	D	.	N	.	0,06	0,08	0,13	
	LNDNA7	LN	.	.	S	.	.	.	.	E	.	.	.	N	.	.	.	.	L	.	T	.	E	.	.	.	D	.	N	.	0,01	12,26	0,42	
	LNDNA10	LN	.	.	.	.	.	.	.	E	.	.	.	N	.	.	.	.	L	.	T	.	E	.	.	.	D	.	N	.	0,50	7,84	20,00	
LNRNA5	LN	.	.	S	.	.	.	.	E	.	.	.	N	.	.	.	.	L	.	T	.	E	.	.	.	D	.	N	.	20,00	0,01	0,34		
LNRNA6	LN	E	.	S	.	.	.	.	E	.	.	.	N	.	.	.	.	L	.	T	.	E	.	.	.	D	.	N	.	0,62	10,50	0,98		
PID0053	PL3	Plasma	.	.	.	.	.	.	A	E	.	.	.	.	.	V	.	.	L	.	T	.	G	.	.	.	S	I	N	K	0,24	12,25	0,50	
	PL8	Plasma	.	.	.	.	.	.	.	E	.	.	.	.	.	V	.	.	L	.	T	.	G	.	.	.	S	I	N	K	0,01	20,00	0,70	
	PBMCDNA4	PBMC	.	.	.	.	.	.	A	E	.	.	.	.	.	V	.	.	L	.	T	.	G	.	.	.	S	I	N	K	0,01	10,55	0,79	
	PBMCDNA5	PBMC	.	.	.	.	.	.	.	E	.	.	.	N	.	V	.	.	L	.	T	.	G	.	.	.	R	I	N	K	0,01	0,01	0,01	
	PBMCDNA11	PBMC	.	.	.	.	.	.	A	E	.	.	.	N	.	V	.	.	L	.	T	.	G	.	.	.	G	I	N	K	20,00	0,01	0,10	
	LNDNA3	LN	.	.	.	.	.	.	.	E	E	.	.	.	.	.	V	.	.	L	.	T	.	G	.	.	.	S	I	N	K	0,01	0,15	0,05
	LNDNA7	LN	.	.	.	.	.	.	.	E	E	.	.	N	.	V	.	.	L	.	T	.	G	.	.	.	R	I	N	K	0,04	0,01	1,21	
LNDNA10	LN	.	.	.	.	E	.	E	E	.	.	.	.	.	.	V	.	.	L	.	T	.	G	.	.	.	S	I	N	K	0,15	20,00	0,53	
PID118	PL6	Plasma	.	.	.	.	.	.	.	E	.	.	.	N	.	.	.	.	L	.	T	.	G	.	.	.	D	A	.	.	0,01	20,00	0,34	
	PL9	Plasma	.	.	.	.	.	.	.	E	.	.	.	N	.	.	.	.	L	.	T	.	G	.	.	.	E	A	.	.	0,06	16,80	0,10	
	PBMCDNA1	PBMC	.	.	.	.	.	.	.	E	D	.	.	N	.	.	.	.	L	.	T	.	G	.	.	.	E	A	.	.	0,19	17,60	0,01	
	PBMCDNA4	PBMC	.	.	.	.	.	.	.	E	.	.	.	N	.	.	.	.	L	.	T	.	G	.	.	.	E	A	.	.	0,02	20,00	0,05	
PID3011	PL1	Plasma	.	.	.	.	.	.	.	E	.	.	.	.	.	T	.	.	L	.	R	.	G	.	.	.	.	.	N	K	20,00	0,45	1,30	
	PL2	Plasma	.	.	.	.	.	.	.	E	.	.	.	.	.	.	.	.	L	.	K	.	G	.	.	.	.	.	N	K	0,02	10,47	0,01	
	PBMCDNA1	PBMC	.	.	.	.	.	.	.	E	.	.	.	.	.	.	.	.	L	.	K	.	G	.	.	.	.	.	N	K	0,01	5,57	0,08	
	PBMCDNA5	PBMC	.	.	.	.	.	.	.	E	.	.	.	.	.	.	.	.	L	.	K	.	G	.	.	.	.	.	N	K	0,04	0,05	0,03	
	PBMCDNA7	PBMC	.	.	.	.	.	.	.	E	.	.	.	.	.	.	.	.	L	.	K	.	G	.	.	.	E	N	K	0,06	20,00	0,80		
	LNDNA8	LN	.	.	.	.	.	.	.	E	.	.	.	.	.	.	.	.	L	.	R	.	G	.	.	.	.	.	N	K	0,04	0,04	0,46	
LNDNA14	LN	.	.	.	.	.	R	.	E	.	.	.	.	.	.	.	.	L	.	K	.	R	.	.	E	.	R	N	K	0,16	3,91	0,45		

Table 4.3: HIV-1 *env* sequence alignment representing the CD4 binding site, including the D loop, CD4 binding region and the V5 loop. This table lists the important amino acid stretches extracted from the full-length alignment and highlights the amino acid differences observed for each defined epitope. N-linked glycans are shaded in grey, and the corresponding neutralisation IC₅₀ titers are shown alongside each table. Plasma clones are shown in red, PBMC shown in blue and lymph node clones are shown in green.

PID	HXB2 numbering		130	131	132	156	157	158	160	161	162	164	165	166	167	168	169	170	171	172	184	197	V2 Apex		
			K	C	T	N	C	S	N	I	S	S	I	R	G	K	V	Q	K	E	I	N	CAP256-VRC26	PGDM1400	PG9
PID0011	PL9	Plasma	.	.	K	.	.	.	.	T	T	E	.	G	D	.	K	.	Q	A	L	.	0,01	20,00	0,04
	PL13	Plasma	H	.	.	.	.	.	.	M	T	E	L	.	D	.	Q	K	.	E	L	.	0,03	0,04	0,04
	PBMCDNA6	PBMC	N	.	.	.	.	.	.	M	T	E	L	.	D	.	Q	K	.	.	L	.	0,01	0,00	0,24
	PBMCDNA11	PBMC	.	.	.	.	.	.	.	M	T	E	L	.	D	.	Q	K	.	.	L	.	0,01	0,16	0,02
	PBMCDNA14	PBMC	.	.	K	.	.	.	.	T	T	E	.	.	D	.	K	.	Q	A	L	.	20,00	0,01	0,02
	PBMCRNA1	PBMC	N	.	.	.	.	.	T	T	A	G	.	K	.	.	K	.	E	A	L	.	0,01	0,01	0,09
	LNDNA1	LN	N	.	.	.	.	.	T	T	A	G	.	K	.	.	K	K	E	A	L	.	0,01	0,01	0,42
	LNDNA2	LN	N	.	.	.	.	.	T	T	A	G	.	K	.	.	K	K	E	A	L	.	20,00	0,34	0,05
	LNDNA3	LN	N	.	.	.	.	.	.	T	T	E	.	.	D	.	K	.	Q	A	L	.	0,01	0,01	0,88
	LNDNA6	LN	N	.	.	.	.	.	.	T	T	E	.	.	D	.	K	.	Q	A	L	.	0,01	0,43	20,00
	LNDNA7	LN	.	.	K	.	.	.	.	T	T	E	.	G	D	.	K	.	Q	A	L	.	11,25	0,03	0,02
	LNDNA10	LN	H	.	.	.	.	.	.	M	T	E	L	.	D	.	Q	K	K	.	L	.	11,75	0,03	0,06
PID0053	LNRNA5	LN	N	.	.	.	.	.	.	T	T	E	.	.	D	.	K	.	Q	A	L	.	0,02	0,01	0,08
	LNRNA6	LN	N	.	.	.	.	.	.	T	T	E	.	.	D	.	K	.	Q	A	L	.	0,05	0,09	0,01
	PL3	Plasma	E	.	R	.	.	.	.	A	T	E	.	.	D	.	K	R	R	.	L	.	0,02	0,19	20,00
	PL8	Plasma	E	.	S	.	.	.	.	A	T	E	.	K	D	.	K	R	R	.	L	.	0,12	0,01	0,06
	PBMCDNA4	PBMC	E	.	S	.	.	.	.	A	T	E	.	.	D	.	K	R	.	.	L	.	0,26	0,01	0,11
	PBMCDNA5	PBMC	E	.	S	.	.	.	.	A	T	E	.	.	D	R	K	R	.	.	L	.	0,02	0,01	0,02
	PBMCDNA11	PBMC	E	.	N	.	.	.	.	A	T	E	.	.	D	R	K	R	.	.	L	.	15,00	0,02	0,04
PID118	LNDNA3	LN	E	.	R	.	.	.	.	A	T	E	.	.	D	.	I	R	.	.	L	.	0,01	0,05	0,02
	LNDNA7	LN	E	.	R	.	.	.	.	A	T	E	.	.	D	R	K	R	.	.	L	.	2,45	0,01	0,06
	LNDNA10	LN	E	.	R	.	.	.	.	A	T	E	.	K	D	.	K	R	R	.	L	.	0,04	20,00	0,02
	PL6	Plasma	T	.	N	.	.	.	.	.	T	E	L	.	D	.	N	K	Q	.	.	.	0,01	0,02	20,00
PID3011	PL9	Plasma	T	.	N	.	.	.	.	.	T	E	L	.	D	.	N	K	Q	.	.	.	0,02	0,01	0,49
	PBMCDNA1	PBMC	T	.	N	.	.	.	.	.	T	E	L	.	D	.	R	R	Q	.	.	.	20,00	0,04	20,00
	PBMCDNA4	PBMC	T	.	N	.	.	.	.	.	T	E	L	.	D	.	N	K	Q	.	.	.	0,04	20,00	0,01
PID3011	PL1	Plasma	N	.	.	.	.	.	.	T	T	E	.	K	D	R	K	.	.	W	L	.	0,01	0,01	0,04
	PL2	Plasma	N	.	.	.	.	.	.	T	T	E	.	K	D	R	K	.	.	W	L	.	0,82	0,04	0,02
	PBMCDNA1	PBMC	N	.	.	.	.	.	.	T	T	E	.	K	D	R	K	.	.	W	L	.	0,68	0,01	0,03
	PBMCDNA5	PBMC	N	.	.	.	.	.	.	T	T	E	.	K	D	R	K	.	.	W	L	.	0,60	0,01	20,00
	PBMCDNA7	PBMC	N	.	.	.	.	.	.	T	T	E	.	K	D	R	K	.	.	W	L	.	20,00	0,02	20,00
	LNDNA8	LN	N	.	.	.	.	.	.	T	T	E	.	K	D	R	K	.	.	W	L	.	0,41	0,01	0,01
PID3011	LNDNA14	LN	N	.	.	.	.	.	.	T	T	E	.	K	D	.	K	.	.	W	L	.	0,01	0,03	0,04

Table 4.4: HIV-1 *env* sequence segments representing the V2 loop. This table lists the important amino acid stretches extracted from the full-length alignment and highlights the amino acid differences observed for each defined epitope. The N-linked glycans are shaded in grey and the corresponding neutralisation IC₅₀ titers are shown alongside each table. Plasma clones are shown in red, PBMC shown in blue and lymph node clones are shown in green.

PID	HXB2 numbering	295	296	297	300	301	302	303	304	323	324	325	326	327	328	330	332	333	334	368	386	387	388	392	393	394	417	V3 Glycan
		N	C	T	N	N	N	T	R	I	G	N	M	R	Q	H	N	I	S	D	N	S	T	N	S	T	P	PGT121
PID0011	PL9	Plasma	V	.	I	G	.	.	I	.	.	.	I	.	.	.	.	.	.	.	.	T	S	.	A	.	.	20,00
	PL13	Plasma	V	.	M	G	.	.	.	.	.	.	I	.	K	.	.	.	.	.	.	T	S	.	V	.	.	0,20
	PBMCDA6	PBMC	V	.	I	G	.	.	.	.	.	.	I	.	K	.	.	.	.	.	.	T	S	.	V	.	.	20,00
	PBMCDA11	PBMC	V	.	I	G	.	.	.	.	.	.	I	.	K	.	.	.	.	.	.	T	S	.	V	.	.	2,31
	PBMCDA14	PBMC	V	.	I	G	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	T	S	.	V	.	.	0,02
	PBMCRNA1	PBMC	V	.	.	G	.	.	.	.	.	.	I	.	E	.	.	.	.	.	.	T	.	.	E	R	.	0,07
	LNDNA1	LN	V	.	.	G	.	.	.	.	.	D	I	.	E	.	.	.	.	.	.	T	.	.	E	R	.	0,06
	LNDNA2	LN	V	.	.	G	.	.	.	.	.	D	I	.	E	.	.	.	.	.	.	T	.	.	E	R	.	14,18
	LNDNA3	LN	V	.	I	G	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	T	S	.	V	.	.	0,23
	LNDNA6	LN	V	.	I	G	.	.	.	.	.	D	I	.	K	.	.	.	.	.	.	T	S	.	V	.	.	10,72
	LNDNA7	LN	V	.	I	G	.	.	I	.	.	.	I	.	.	.	.	.	.	.	.	T	S	.	A	.	Q	0,06
	LNDNA10	LN	V	.	M	G	.	.	.	.	.	.	I	.	K	.	.	.	.	.	.	T	S	.	V	.	Q	0,35
PID0053	LNRNA5	LN	V	.	I	G	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	T	S	.	A	.	.	0,05
	LNRNA6	LN	V	.	.	G	.	.	.	.	.	.	I	.	K	.	.	.	.	.	.	T	S	.	V	.	.	10,00
	PL3	Plasma	K	.	.	N	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	T	S	D	G	N	Q	0,27
	PL8	Plasma	E	.	.	N	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	T	S	D	G	N	.	17,98
	PBMCDA4	PBMC	K	.	.	N	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	T	S	D	G	N	Q	20,00
	PBMCDA5	PBMC	E	.	.	N	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	T	S	D	G	N	.	20,00
	PBMCDA11	PBMC	E	.	.	N	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	T	S	D	G	N	Q	0,12
PID118	LNDNA3	LN	K	.	.	N	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	T	S	D	G	N	Q	0,02
	LNDNA7	LN	E	.	.	N	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	T	S	D	G	N	Q	0,05
	LNDNA10	LN	K	.	.	N	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	T	S	D	G	N	Q	18,63
	PL6	Plasma	T	.	.	N	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	T	S	.	N	S	N	15,95
PID3011	PL9	Plasma	T	.	.	N	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	T	S	.	N	S	N	20,00
	PBMCDA1	PBMC	T	.	.	S	.	.	.	.	.	.	I	.	.	.	.	.	.	A	.	T	S	.	N	S	N	0,28
	PBMCDA4	PBMC	T	.	.	N	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	T	S	.	N	S	N	19,92
	PL1	Plasma	V	.	.	S	.	.	S	.	.	.	I	.	.	.	.	.	.	.	.	T	.	.	G	S	.	0,16
PID3011	PL2	Plasma	V	.	.	S	.	.	S	.	.	.	I	.	.	.	.	.	.	.	.	T	.	.	G	S	.	18,27
	PBMCDA1	PBMC	V	.	.	S	.	.	S	.	.	.	I	.	.	.	.	.	.	.	.	T	.	.	G	S	.	18,51
	PBMCDA5	PBMC	V	.	.	S	.	.	S	.	.	.	I	.	.	.	.	.	.	.	.	T	.	.	G	S	.	18,98
	PBMCDA7	PBMC	V	.	.	S	.	.	S	.	.	.	I	.	.	.	.	.	.	.	.	T	.	.	G	S	.	0,38
	LNDNA8	LN	V	.	.	S	.	.	S	.	.	.	I	.	.	.	.	.	.	.	.	T	.	.	G	S	.	0,04
	LNDNA14	LN	V	.	.	S	.	.	S	.	.	.	I	.	.	.	.	.	.	.	.	T	.	.	R	S	.	20,00

Table 4.5: Sequence segments representing the V3 glycan. This table lists the amino acid stretches extracted from the full-length HIV-1 *env* sequence alignment and highlights the important amino acid differences observed for each defined epitope. The N-linked glycans are shaded in grey, and the corresponding neutralisation IC₅₀ titers are shown alongside each table. Plasma clones are shown in red, PBMC shown in blue and lymph node clones are shown in green

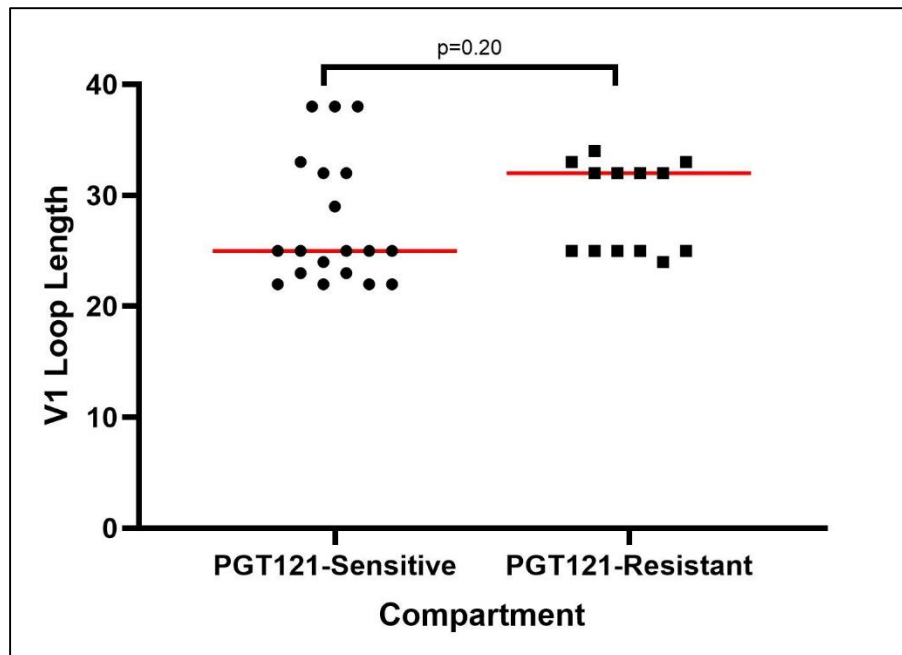


Figure 4.7: Comparison of the V1 loop lengths between PGT121-sensitive and resistant HIV-1 *env* clones to the V3 glycan from PL, PBMC and LN compartments.

Chapter 5: Discussion

In this study we investigated viral diversity, amino acid differences, variable loop lengths, the number of PNGs and the differences in neutralisation sensitivity between plasma (PL), PBMCs, and lymph nodes (LN) derived compartments. HIV-1 *env* sequences intermingled and showed partial compartmentalisation between plasma, PBMC and lymph node compartments. Plasma sequences tended to have a slightly longer V1 loop lengths and more potential N-linked glycosylation sites (PNGS) in the V4 loop, compared to PBMC and LN clones, suggesting a marginally denser glycan shield. Overall, these findings suggest broadly similar glycan shielding across compartments, with only subtle differences in the variable loops that may fine-tune epitope exposure rather than indicate strong compartmentalization. Analysis of the CD4 binding site and the V1V2 loop had discordant mutations, while the V3 glycan, MPER, gp120/gp41 interface, fusion peptide and silent face regions were largely conserved in all three compartments. HIV1 Env clones generated from all three compartments had high neutralisation sensitivity to the CD4bs VRC07-523LS and N6, V2 apex bnAbs (CAP256-VRC26.25, PGDM1400 and PG9), MPER (10E8), and silent face (SF12). Although high resistance was largely seen towards VRC01, PGT121 and N123-VRC34. Overall, viruses derived from plasma consistently displayed the highest resistance to most bnAbs, while PBMC and LN clones remained sensitive to most bnAbs.

Our findings are consistent with earlier studies, which reported that circulating plasma viruses are enriched for immune-selected escape variants, often bearing mutations in the CD4 binding loop, V2 apex stabilizing residues, and the glycan shield (Mandizvo et al., 2022). Similarly, our group reported that VRC07-523LS & N6.LS showed broad neutralization; while VRC01, PGT121 and PGT151 had moderate neutralisation coverage. Interestingly, our study noted similar findings, except for the highest PGDM1400 neutralisation coverage across all three compartments (Mandizvo et al., 2022). The smaller sample size in this analysis likely increased variability and may have amplified differences between the compartments. In keeping with the findings from Jeewanraj et al, differences seen may also be attributed to the percentage of escape mutations that differed by <50% between the compartments (Jeewanraj et al., 2023). Our data is also consistent with existing literature indicating that HIV can establish distinct viral populations in anatomical niches, where immune pressure, antiretroviral penetration and cellular targets may differ (Lorenzo-

Redondo et al., 2016). Our findings are also consistent with recent studies that reported increasing resistance of viruses to bnAbs such as VRC01, PGT121 and 10E8 (Mkhize et al., 2023). Our findings suggest that the combination of PGDM1400 (V2 apex), VRC07-523LS (CD4bs), and SF12 (silent face) could be highly effective in eliminating both peripheral blood, and reservoir Subtype C infections. The compartment-specific neutralization patterns observed in this dataset closely mirror trends described in earlier studies examining HIV diversity and antibody sensitivity across lymphoid tissues, blood-derived cells and plasma. Similar to previous reports, viruses isolated from the lymph node tended to exhibit enhanced sensitivity to multiple bnAb classes, including CD4bs, V2 apex, and V3 glycan antibodies (Nyanhete et al., 2021, Wang et al., 2022). Prior studies of lymphoid reservoirs have shown that follicular and germinal-center viral populations often maintain less immune-evasive glycan profiles and display reduced escape from antibody pressure, consistent with the lower sensitivity seen here (Bronnimann et al., 2018, Wang et al., 2022). This aligns with findings that lymphoid tissue may harbour more “ancestral” or less escape-driven variants compared to peripheral compartments. This reduced immune pressure allows the persistence of variants that remain susceptible to potent bnAbs such as PGDM1400, CAP256-VRC26.25 and N6 consistent with the findings of this study.

The differences in variable loop lengths and PNGS could be due to selective pressures that lead to insertions and deletion of amino acids due to immune escape from the strain-specific neutralizing antibody responses. However, the V3 loop was highly conserved, possibly because this region interacts with the CCR5/CXCR4 coreceptor. These selective pressures could act differently on cell-associated proviruses (archived in infected cells sampled as PBMC) versus actively replicating, freely circulating virus (plasma). Archived proviruses in blood cells may accumulate or retain glycan/loop profiles that alter antigenicity or neutralisation sensitivity, while plasma virus is more directly shaped by current humoral pressures and replication fitness; similar compartment-specific selection and limited compartmentalization have been reported in lymphoid tissues and blood in multiple cohorts (Ho et al., 2008, Gaube et al., 2020, Pardons et al., 2025). The higher resistance observed in plasma support the established notion that viruses circulating in plasma frequently contains immune-selected escape pathways, whereas tissue-resident or cell-associated reservoirs retain greater sensitivity. Similarly, the highest resistance observed in the fusion-peptide bnAbs was also reported in previous studies. However, there is limited data on the sensitivity of subtype C variants to the silent face-SF12 antibody. This study reports a high neutralisation

sensitivity of plasma, PBMC and lymph node viruses to the SF12 mAb, and suggest that this antibody could have potential in immunotherapy.

Neutralisation sensitivity also showed minor differences in IC_{50} titres across compartments for the panel of broadly neutralising antibodies tested, despite some trends in sensitivity for particular variants (for example, PBMC-derived viruses occasionally showing slightly lower sensitivity to PGT151). Together, these results suggest that the overall structural and antigenic features of the Env glycoprotein were broadly similar across PL, PBMC and LN compartments, even though individual viral variants exhibited modest phenotypic variation. This indicates that, despite the distinct cellular environments in which viruses replicate or persist, the fundamental features of the Env glycoprotein that influence antibody recognition such as loop architecture and glycan shield density may be maintained across compartments. Consequently, viruses circulating in plasma appear to broadly reflect the antigenic characteristics of viruses present in lymphoid tissues and circulating blood cells (Page 68). These findings also imply that strong compartment-specific selective pressures on Env may be limited in this cohort, at least with respect to the structural features examined. Instead, viral evolution within individuals may be primarily driven by systemic immune pressures, such as circulating antibodies, rather than localized tissue-specific factors.

Importantly, the lack of significant differences does not necessarily indicate complete homogeneity between compartments, but rather suggests that any observed trends or minor variations were insufficient to produce measurable compartment-specific divergence in this dataset. This supports the idea that viral populations across compartments remain functionally similar with respect to neutralisation sensitivity, which has implications for studies evaluating antibody responses and for the interpretation of plasma-derived viral phenotypes as representative of broader intra-host viral populations.

Overall, the findings reinforce established evidence that amino acid variations within bnAb epitopes influences the antibody neutralisation susceptibility. Lymph nodes compartment had the most neutralisation sensitive viral populations; while PBMC showed moderate neutralisation and plasma had the most escape-adapted variants. However, amino acids variation across compartments suggests that immune pressure may drive diversification in this region. This is one of the few studies to report differences in neutralisation sensitivity in the lymph node compartment as most studies focus on plasma. These bnAbs can support HIV cure strategies by limiting the

persistence of viral reservoirs and enabling immune-mediated clearance of infected cells. In HIV cure studies, bnAbs can neutralize the virus released during latency reversal or treatment interruption, thereby preventing re-seeding of the reservoir. While simultaneously binding Env expressed on reactivated infected cells and recruiting Fc-dependent effector functions such as antibody-dependent cellular cytotoxicity or phagocytosis. When used in combinations that reduce viral escape and engineered for enhanced Fc activity, bnAbs have been shown in animal models to reduce levels of replication-competent virus and delay viral rebound. Although they cannot eliminate transcriptionally silent proviruses on their own, bnAbs are valuable adjuncts to latency-reversing, immune-enhancing, or gene-based interventions aimed at shrinking the inducible HIV reservoir.

However, key limitations of this study include; the small sample size and the potential influence of varying stages of HIV infection among participants, which may introduce heterogeneity into the results. Viral populations in chronic infection are characterized by the accumulation of escape mutations that confer greater neutralisation resistance. Furthermore, advanced infection may be associated with immune dysfunction and reduced CD4⁺ T cell counts, which could impair the generation or effectiveness of neutralizing antibodies. These differences across infection stages may therefore confound the interpretation of neutralisation breadth and potency, as observed outcomes could partly reflect infection stage rather than intrinsic properties of the antibodies or viral epitopes under investigation. While neutralisation assays are mostly done in vitro and real-world in vivo efficacy may differ, they provide controlled, quantitative information that cannot be easily obtained in vivo and provide a standardized, reproducible benchmark for future comparison. Reliance on plasma alone risks missing compartment-specific *env* features that modulate neutralisation, therefore, integrated PBMC/LN sampling should be performed regularly in various populations. Incomplete RNA sampling from PBMC and LN compartments and the lack of LN samples for PID 118 did not allow for a thorough comparison of the compartments. Future work should combine NGS sequencing across PL, PBMC and LN samples at multiple longitudinal points and similar clinical characteristics.

Although the number of participants included in this study was limited ($n = 4$), extensive viral sampling across PL, PBMC and LN compartments enabled detailed intra-participant comparisons. While the findings provide insight into compartment-specific viral evolution in HIV-1 subtype C

infection, the small cohort size limits the ability to generalize these observations to the broader subtype C-infected population and they should therefore be interpreted as exploratory and hypothesis-generating.

Conclusion and implications for HIV cure strategies and bnAb trials

Taken together, these findings suggest that the optimal combination of bnAbs across blood, PBMC and lymph node compartments would be a triple combination consisting of PGDM1400/ CAP256-VRC26.25 (V2 apex), N6/ VRC07-523LS (CD4bs), and SF12 (silent face) /PGT151 (gp120-gp41 interface). While high resistance was observed against the V3 glycan supersite (PGT121), MPER (10E8) and the fusion peptide (N123.VRC34). The triple combination covers the three most conserved and structurally critical epitopes of the HIV envelope, minimizes overlap in escape pathways, and retains potency even in the more resistant plasma compartment. However, substantial variations in the V2 apex, and the V3 glycan were observed between the compartments. CD4 binding site, silent face and interface bnAbs offer complementary neutralization breadth, their epitopes are quite conserved, and minor variations were observed in different compartments.

In summary, this study suggests that a triple combination of VRC07-523LS, PGDM1400 and SF12 may be effective in preventing HIV-1 subtype C. These bnAbs could be used in immunotherapy to eliminate or reduce viral load in people living with HIV, and reduce the size of viral reservoirs in lymph nodes, and PBMC compartments. Successful therapeutic interventions need both neutralisation breadth against diverse *env* phenotypes and penetration into multiple anatomical compartments to avoid leaving escape variants unchecked. Amino acid signatures that mediate resistance to specific bnAbs within specific epitopes were identified and provide insight into the selection of bnAbs for clinical testing, vaccine design/ therapeutic strategies as well as the prediction of neutralisation sensitivity testing. The data also highlights the need for tissue-penetrating broadly neutralizing antibodies or latency-reversing agents with lymphoid access.

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Chapter 7: Supplementary information

Supplementary Table 7.1: HIV *env* primer sequences used in the first and second round of amplification of the *env*

Primer name	Primer sequence
OFM19	5' GCA CTC AAG GCA AGC TTT ATT GAG GCT TA 3'
VIF1	5' GGG TTT ATT ACA GGG ACA GCA GA 3'

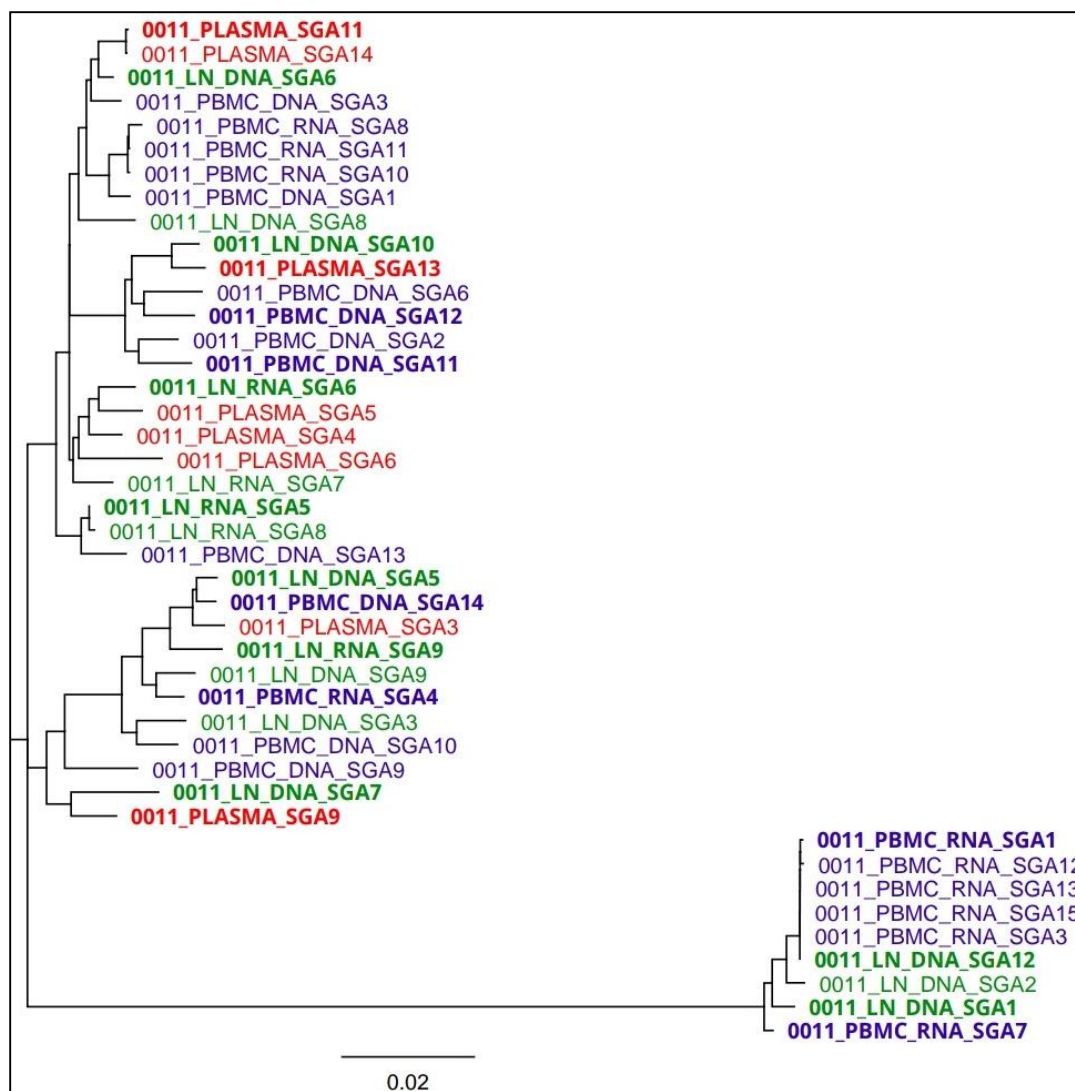
<i>env1A</i>	5' CAC CGG CTT AGG CAT CTC CTA TGG CAG GAA GAA 3'
<i>env1M</i>	5' TAG CCC TTC CAG TCC CCC CTT TTC TTT TA 3'
F-1 inverse Primer	5' CTG CTC GCT TCG CTA CTT GGA 3'
R-1 inverse Primer	5' CGG CAC CTG TCC TAC GAG TTG 3'

Supplementary Table 7.2: HIV-1 *env* primer sequences used in the Sanger sequencing of the TOPO clones generated after second round amplification.

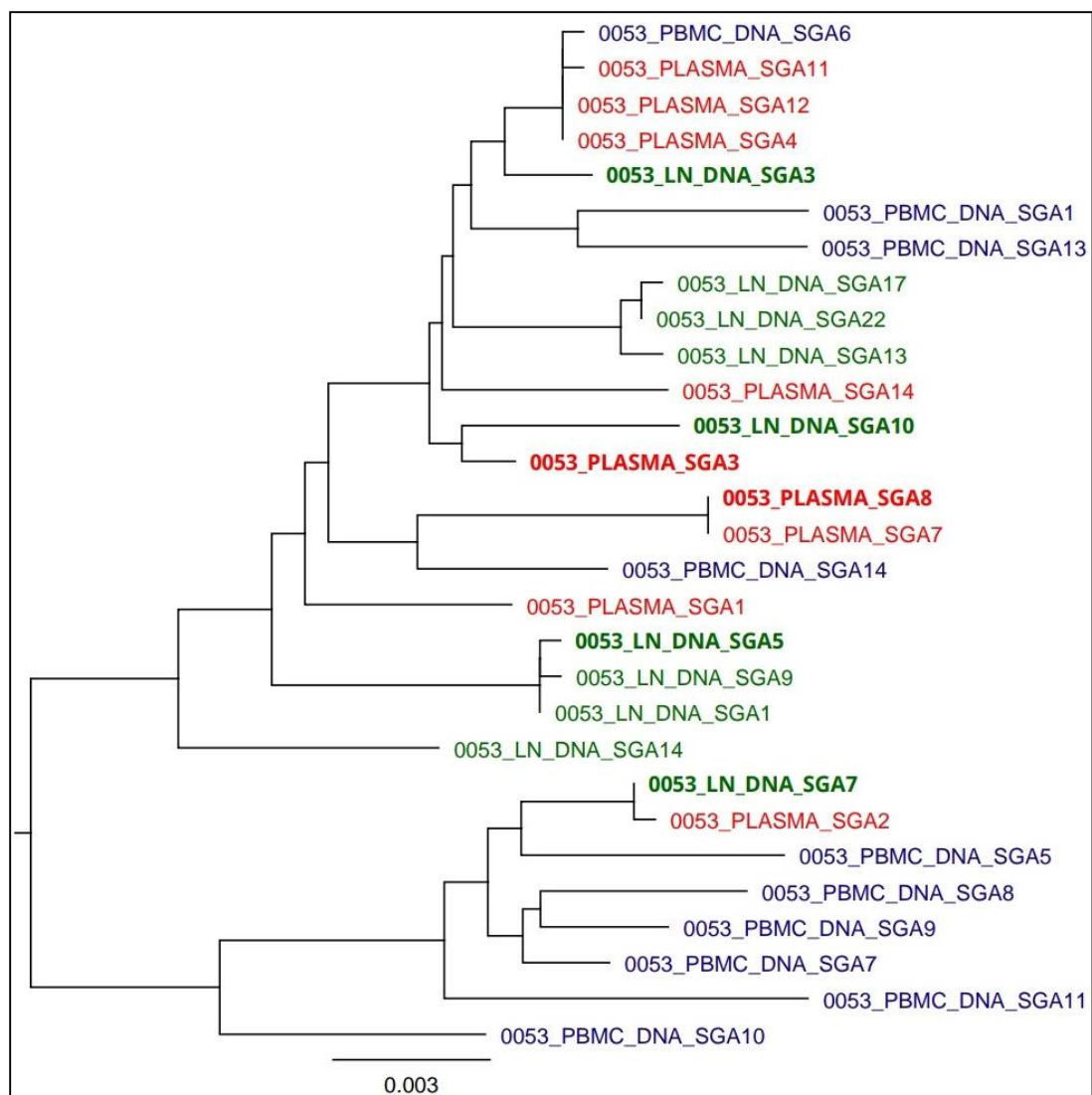
Primer name	Primer sequence	Conditions
ef00	5' AA GAG CAG AAG ACA GTG GCA ATG A 3'	Initial denaturation at 96°C for 1 minute, 25
ed31	5' CCTCAGCCATTACACAGGCCTGTCCAAAG 3'	
sq13f	5' TATATAAATATAAAGTGGTAGAAATTAAGC 3'	

sq14	5' ACTCACGGTCTGGGGCATTA 3'	cycles of denaturation at 96°C for 10 seconds, primer annealing at 50°C for 5 seconds, primer extension at 50°C for 4 seconds and a final hold at 4°C
sq6rc	5' GAATTGGGTCAAAAGAGACCTTTGGA 3'	
sq5.5rc	5' CTAGGAGCTGTTGATCCTTTAGGTAT 3'	
sq3r	5' GCTATGGTATCAAGCAGACTAATAGCACTC'	
<i>envM</i>	5' TAGCCCTTCCAGTCCCCCCTTTTCTTTTA 3'	

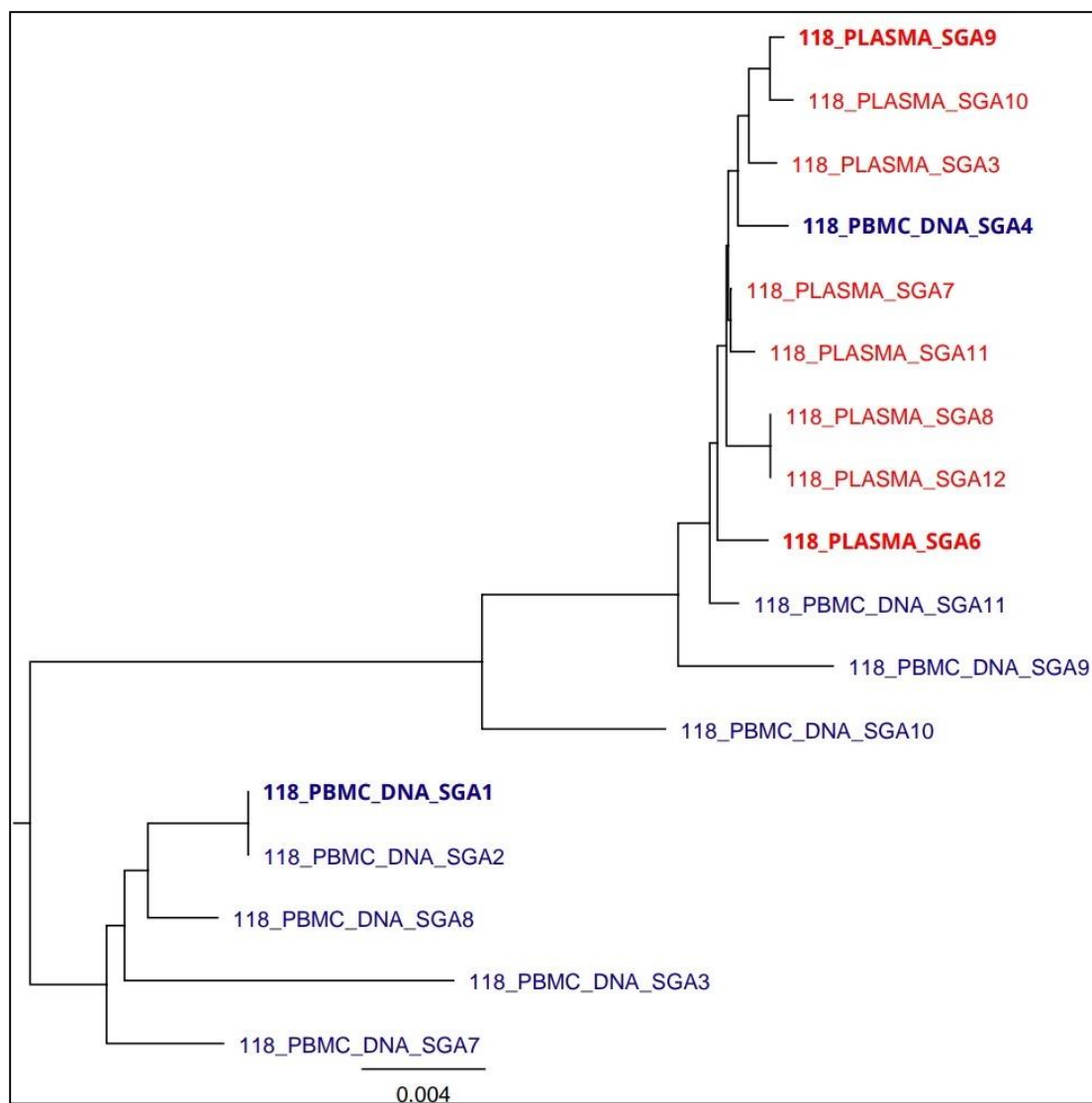
Supplementary figures 7.1 A-D:



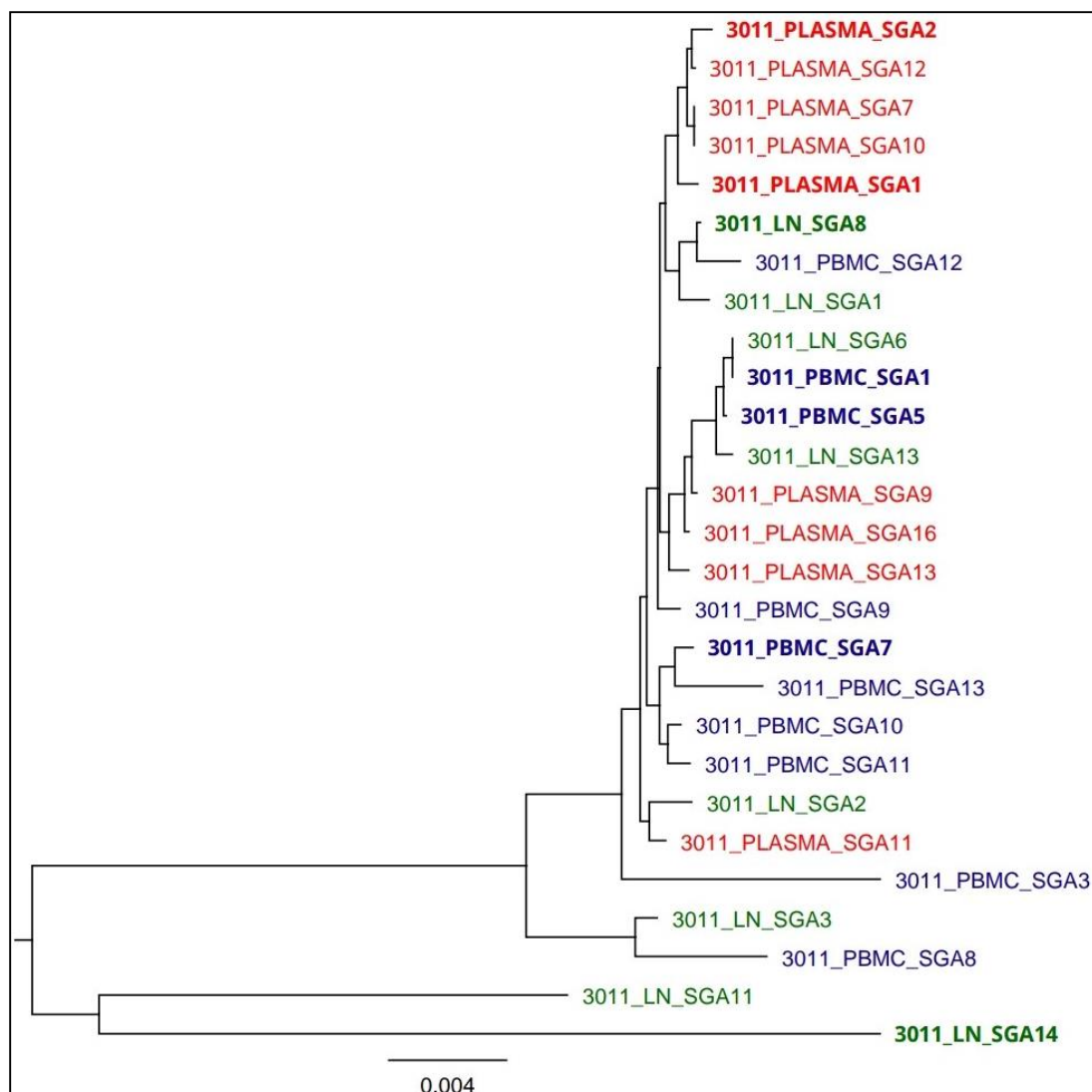
Supplementary Figure 7.1 A: Maximum-Likelihood Tree of SGA *env* Sequences from Participant 0011



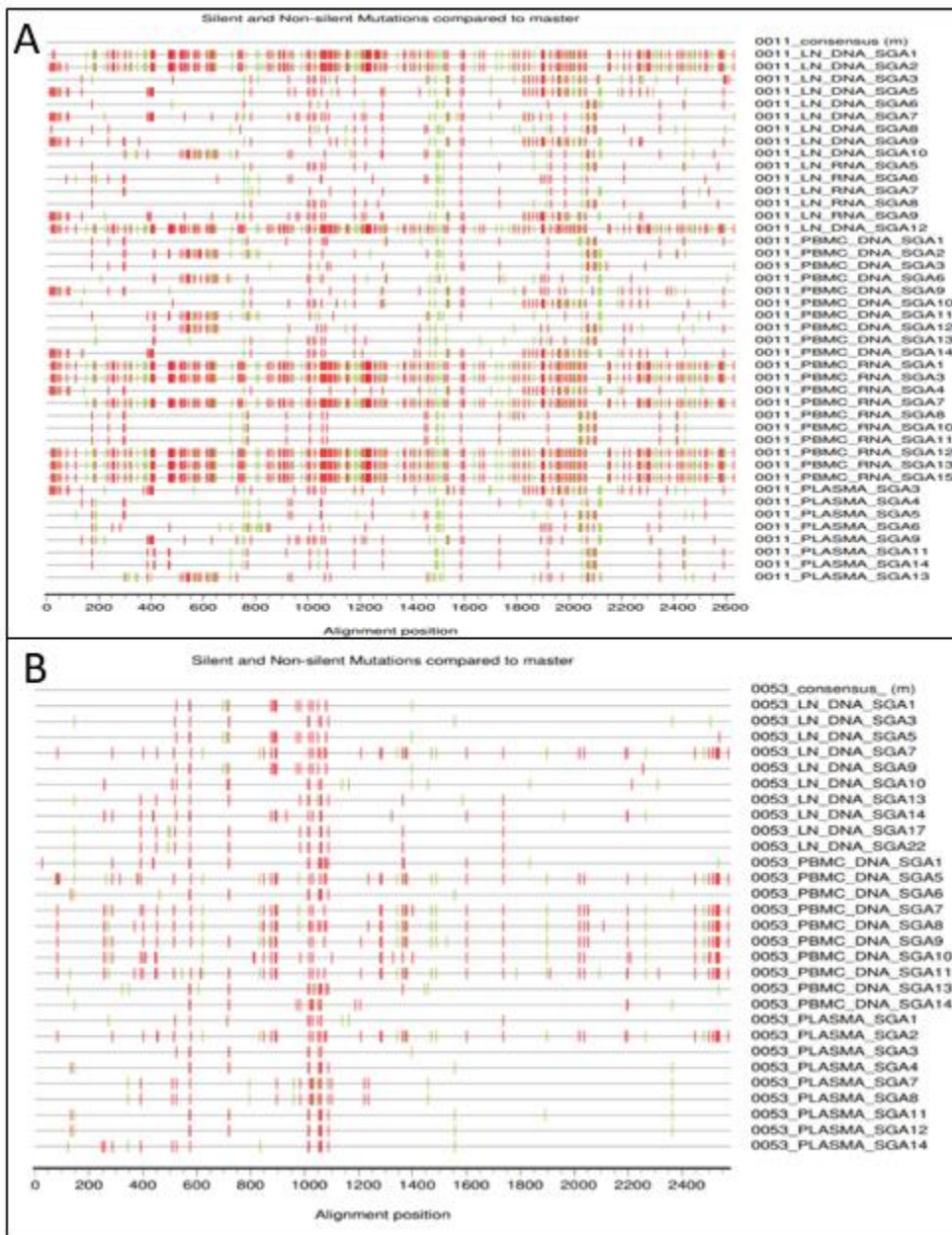
Supplementary Figure 7.1 B: Maximum-Likelihood Tree of SGA *env* Sequences from Participant 0053

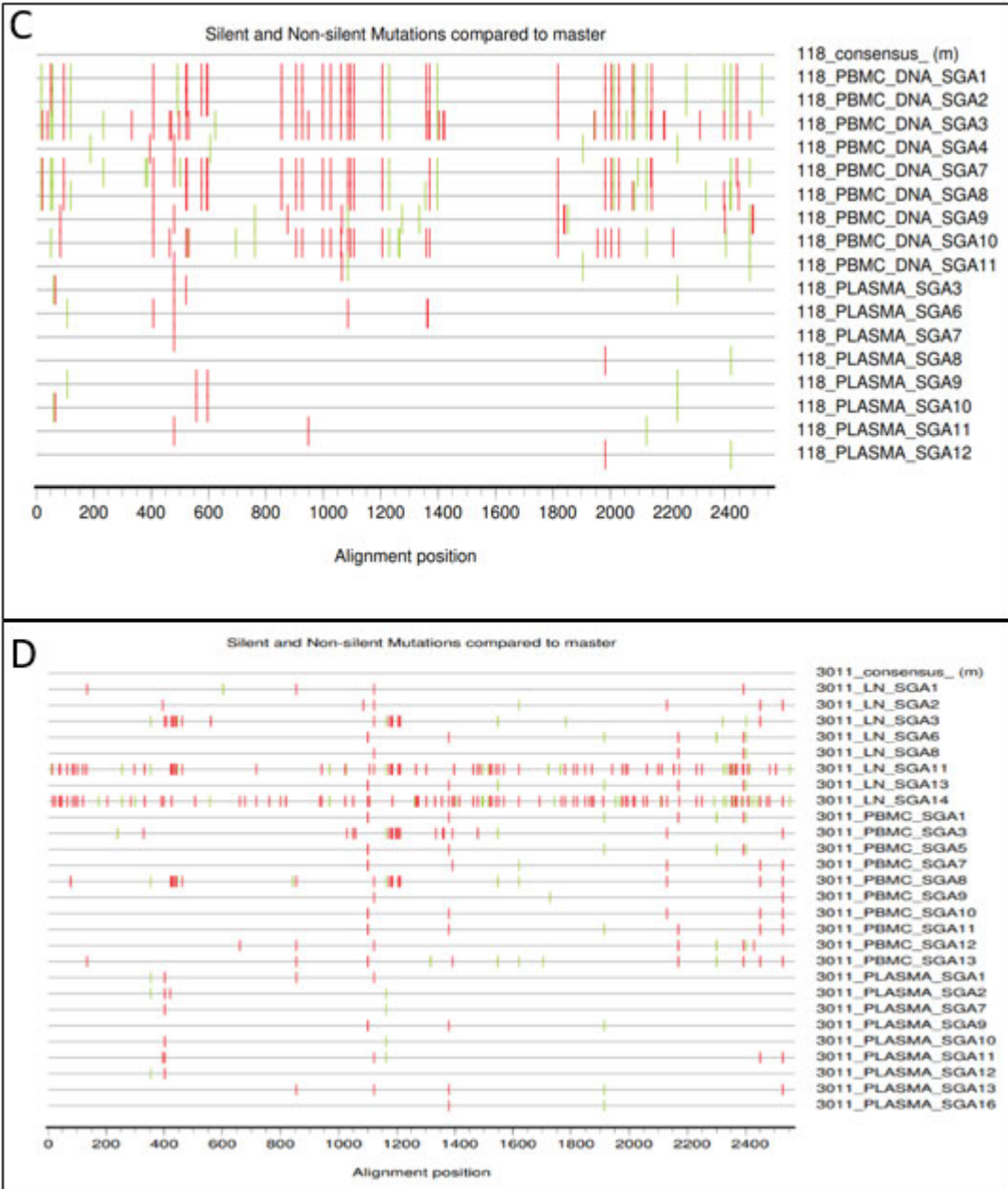


Supplementary Figure 7.1 C: Maximum-Likelihood Tree of SGA *env* Sequences from Participant 118



Supplementary Figure 7.1 D: Maximum-Likelihood Tree of SGA *env* Sequences from Participant 3011





Supplementary figure 7.2 A-D: Highlighter plots generated for each PID showing silent and non-silent mutations. Highlighter plots illustrate nucleotide/amino acid changes in *env* sequences from PL, PBMC and LN compartments relative to the participant-specific consensus. Each horizontal line represents an individual sequence, and colored tick marks denote substitutions, insertions, and deletions at corresponding positions along the envelope gene.

Supplementary Tables 7.3 A-D: Mutation tables showing the amino acid residues and neutralisation IC₅₀ titers per epitope

PID	HXB2 numbering		65	240	262	270	277	281	300	301	336	346	432	440	448	616	637	662	664	665	667	668	671	674	742	788	837	gp120gp41
			V	T	N	V	F	A	N	N	A	A	K	S	N	N	N	E	D	K	A	S	N	N	R	R	C	PGT151
PID0011	PL9	Plasma	.	H	.	I	L	.	G	.	T	S	R	A	T	.	.	A	.	.	K	N	S	D	S	.	G	0,01
	PL13	Plasma	.	H	.	I	L	.	G	.	K	S	R	A	T	.	.	A	.	.	K	N	S	D	S	.	G	0,19
	PBMCDA6	PBMC	.	L	.	I	L	.	G	.	K	S	R	A	T	.	.	A	.	.	K	N	S	D	S	.	G	0,01
	PBMCDA11	PBMC	.	N	.	I	L	.	G	.	K	S	R	A	T	.	.	A	.	.	K	N	S	D	S	.	G	0,05
	PBMCDA14	PBMC	.	H	.	I	L	.	G	.	T	S	R	A	T	.	.	A	.	.	Q	N	S	S	S	.	G	20,00
	PBRNA1	PBMC	.	H	.	I	L	I	G	.	S	K	R	A	.	.	.	A	.	S	N	N	.	S	.	K	.	20,00
	LNDNA1	LN	.	H	.	I	L	I	G	.	S	K	R	A	.	.	.	A	.	S	N	N	.	S	.	K	.	0,01
	LNDNA2	LN	.	H	.	I	L	I	G	.	S	K	R	A	.	.	.	A	.	S	N	N	.	S	.	K	.	20,00
	LNDNA3	LN	.	H	.	I	L	.	G	.	T	S	R	A	T	.	.	A	.	.	Q	N	S	S	S	.	G	0,02
	LNDNA6	LN	.	H	.	I	L	.	G	.	K	S	R	A	T	.	.	A	.	.	K	N	S	D	S	.	G	0,32
	LNDNA7	LN	.	H	.	I	L	.	G	.	T	S	R	A	T	.	.	A	.	.	K	N	S	D	S	.	G	7,97
	LNDNA10	LN	.	H	.	I	L	.	G	.	K	S	R	A	T	.	.	A	.	.	K	N	S	D	S	.	G	0,03
	LNRNA5	LN	.	H	.	I	L	.	G	.	T	S	R	A	T	.	.	A	.	.	K	N	S	D	S	.	G	11,00
	LNRNA6	LN	.	H	.	I	L	.	G	.	A	S	R	A	T	.	.	A	.	.	K	N	S	D	S	.	G	0,01
PID0053	PL3	Plasma	.	N	.	I	L	V	.	.	N	K	Q	A	.	.	.	A	.	S	N	N	.	S	K	.	.	20,00
	PL8	Plasma	.	N	.	I	L	V	.	.	G	K	Q	A	.	.	.	A	.	S	N	N	.	S	K	.	.	0,02
	PBDNA4	PBMC	.	N	.	I	L	V	.	.	N	K	Q	A	.	.	.	A	.	S	N	N	.	S	K	.	.	0,02
	PBDNA5	PBMC	.	N	.	I	L	V	.	.	E	K	R	A	.	.	.	A	.	S	N	N	.	S	K	.	.	20,00
	PBDNA11	PBMC	.	N	.	I	L	V	.	.	E	K	R	A	.	.	.	A	.	S	N	N	.	S	K	.	.	11,95
	LNDNA3	LN	.	N	.	I	L	V	.	.	N	K	Q	A	.	.	.	A	.	S	N	N	.	S	K	.	.	0,02
	LNDNA7	LN	.	N	.	I	L	V	.	.	E	K	R	A	.	.	.	A	.	S	N	N	.	S	K	.	.	0,81
	LNDNA10	LN	.	N	.	I	L	V	.	.	N	K	Q	A	.	.	.	A	.	S	N	N	.	S	.	.	.	0,01
PID118	PL6	Plasma	.	N	.	I	L	.	.	.	E	K	R	E	.	.	.	A	.	S	K	N	.	.	.	.	.	20,00
	PL9	Plasma	.	N	.	I	L	.	.	.	E	K	R	E	.	.	.	A	.	S	K	N	.	.	.	.	.	0,43
	PBDNA1	PBMC	.	N	.	I	L	.	S	.	E	R	R	E	.	.	.	A	.	S	G	N	.	D	.	.	.	0,04
	PBDNA4	PBMC	.	N	.	I	L	.	.	.	E	K	R	E	.	.	.	A	.	S	K	N	.	.	.	.	.	0,01
PID3011	PL1	Plasma	.	S	.	I	L	T	S	.	S	K	R	K	S	.	.	A	.	S	N	N	.	S	.	.	.	12,04
	PL2	Plasma	.	S	.	I	L	.	S	.	S	K	R	K	S	.	.	A	.	S	N	N	.	S	.	.	.	0,52
	PBDNA1	PBMC	.	S	.	I	L	.	S	.	S	K	R	K	S	.	.	A	.	S	N	N	.	S	.	.	.	0,01
	PBDNA5	PBMC	.	S	.	I	L	.	S	.	S	K	R	K	S	.	.	A	.	S	N	N	.	S	.	.	.	0,02
	PBDNA7	PBMC	.	S	.	I	L	.	S	.	S	K	R	K	S	.	.	A	.	S	N	N	.	S	.	.	.	20,00
	LNDNA8	LN	.	S	.	I	L	.	S	.	S	K	R	K	S	.	.	A	.	S	N	N	.	S	.	.	.	20,00
	LNDNA14	LN	.	S	.	I	L	.	S	.	S	K	Q	K	S	.	.	A	.	S	N	N	.	S	.	.	.	0,03

Table 7.3 A: Conserved amino acid signatures in the gp120/gp41 Interface

These table list the amino acid stretches extracted from the full-length alignment and highlights the amino acid differences observed for the epitope. Glycans are shown in grey and the corresponding heatmap is shown alongside each table.

PID	HXB2 numbering		268	270	277	281	440	446	662	667	671	672	673	674	676	680	683	702	726	837	MPER
			E	V	F	A	S	S	E	A	N	W	F	N	T	W	K	L	G	C	10 E 8
PID0011	PL9	Plasma	.	I	L	.	A	N	A	K	S	.	.	D	S	.	.	.	.	G	2,46
	PL13	Plasma	.	I	L	.	A	N	A	K	S	.	.	D	S	.	.	.	.	G	2,31
	PBMCDNA6	PBMC	.	I	L	.	A	N	A	K	S	.	.	D	S	.	.	.	.	G	5,81
	PBMCDNA11	PBMC	.	I	L	.	A	N	A	K	S	.	.	D	S	.	.	.	.	G	7,80
	PBMCDNA14	PBMC	.	I	L	.	A	N	A	Q	S	.	.	S	.	.	.	.	.	G	20,00
	PBMCRNA1	PBMC	.	I	L	I	A	K	A	N	.	.	.	S	.	.	.	.	.	.	2,30
	LNDNA1	LN	.	I	L	I	A	R	A	N	.	.	.	S	.	.	.	.	.	.	8,00
	LNDNA2	LN	.	I	L	I	A	I	A	N	.	.	.	S	.	.	.	.	.	.	3,23
	LNDNA3	LN	.	I	L	.	A	N	A	Q	S	.	.	S	.	.	.	.	.	G	6,97
	LNDNA6	LN	.	I	L	.	A	N	A	K	S	.	.	D	S	.	.	.	.	G	2,60
	LNDNA7	LN	.	I	L	.	A	N	A	K	S	.	.	D	.	.	.	.	.	G	0,19
	LNDNA10	LN	.	I	L	.	A	N	A	K	S	.	.	D	S	.	.	.	.	G	20,00
	LNRNA5	LN	.	I	L	.	A	N	A	K	S	.	.	D	.	.	.	.	.	G	0,30
	LNRNA6	LN	.	I	L	.	A	N	A	K	S	.	.	D	S	.	.	.	.	G	2,63
PID0053	PL3	Plasma	.	I	L	V	A	I	A	N	.	.	.	S	.	.	R	.	E	.	5,11
	PL8	Plasma	.	I	L	V	A	I	A	N	.	.	.	S	.	.	R	.	E	.	3,40
	PBMCDNA4	PBMC	.	I	L	V	A	I	A	N	.	.	.	S	.	.	R	.	E	.	2,74
	PBMCDNA5	PBMC	.	I	L	V	A	I	A	N	.	.	.	S	.	.	.	.	E	.	0,18
	PBMCDNA11	PBMC	.	I	L	V	A	I	A	N	.	.	.	S	.	.	.	.	E	.	3,29
	LNDNA3	LN	.	I	L	V	A	I	A	N	.	.	.	S	.	.	R	.	E	.	0,06
	LNDNA7	LN	.	I	L	V	A	I	A	N	.	.	.	S	.	.	.	.	E	.	18,21
	LNDNA10	LN	.	I	L	V	A	I	A	N	.	.	.	S	.	.	R	.	E	.	2,47
PID118	PL6	Plasma	.	I	L	.	E	T	A	K	.	.	.	.	S	.	Q	.	E	.	10,29
	PL9	Plasma	.	I	L	.	E	T	A	K	.	.	.	.	S	.	Q	.	E	.	8,27
	PBMCDNA1	PBMC	.	I	L	.	E	T	A	G	.	.	.	D	S	.	.	.	E	.	4,85
	PBMCDNA4	PBMC	.	I	L	.	E	T	A	K	.	.	.	.	S	.	Q	.	E	.	8,93
PID3011	PL1	Plasma	.	I	L	T	K	N	A	N	.	.	.	S	.	.	.	.	.	.	7,54
	PL2	Plasma	.	I	L	.	K	N	A	N	.	.	.	S	.	.	.	.	.	.	4,30
	PBMCDNA1	PBMC	.	I	L	.	K	N	A	N	.	.	.	S	.	.	.	.	.	.	4,82
	PBMCDNA5	PBMC	.	I	L	.	K	N	A	N	.	.	.	S	.	.	.	.	.	.	0,18
	PBMCDNA7	PBMC	.	I	L	.	K	N	A	N	.	.	.	S	.	.	.	.	.	.	2,06
	LNDNA8	LN	.	I	L	.	K	N	A	N	.	.	.	S	.	.	.	.	.	.	0,19
	LNDNA14	LN	.	I	L	.	K	N	A	N	.	.	.	S	.	.	.	.	.	.	0,52

Table 7.3B: Minor differences in amino acid signatures identified in the MPER region

These table list the amino acid stretches extracted from the full-length alignment and highlights the amino acid differences observed for the epitope. Glycans are shown in grey and the corresponding heatmap is shown alongside each table.

PID	HXB2 numbering		512	513	514	515	516	517	518	519	520	521	522	523	524	525	526	527	528	529	530	531	532	533	534	535	536	537	538	539	Fusion peptide
			A	V	G	I	G	A	L	F	L	G	F	L	G	A	A	G	S	T	M	G	A	A	S	M	T	L	T	V	
PID0011	PL9	Plasma	.	.	.	.	.	.	V	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	20,00
	PL13	Plasma	.	.	.	L	.	.	M	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	20,00
	PBMCDNA6	PBMC	.	.	.	M	.	.	M	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	20,00
	PBMCDNA11	PBMC	.	.	.	L	.	.	M	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	0,02
	PBMCDNA14	PBMC	.	.	.	.	.	.	V	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	20,00
	PBMCDNA1	PBMC	.	.	.	.	.	.	V	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	A	20,00
	LNDNA1	LN	.	.	.	.	.	.	V	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	A	20,00
	LNDNA2	LN	.	.	.	.	.	.	V	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	A	20,00
	LNDNA3	LN	.	.	.	.	.	.	V	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	20,00
	LNDNA6	LN	.	.	.	V	.	.	M	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	20,00
	LNDNA7	LN	.	.	.	.	.	.	V	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	0,22
	LNDNA10	LN	.	.	.	L	.	.	M	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	20,00
	LNRNA5	LN	.	.	.	V	.	.	M	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	20,00
PID0053	LNRNA6	LN	.	.	.	L	.	.	M	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	20,00
	PL3	Plasma	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	A	.	20,00
	PL8	Plasma	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	A	.	20,00
	PBMCDNA4	PBMC	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	20,00
	PBMCDNA5	PBMC	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	0,05
	PBMCDNA11	PBMC	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	20,00
	LNDNA3	LN	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	A	.	20,00
PID118	LNDNA7	LN	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	20,00
	LNDNA10	LN	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	A	.	20,00
	PL6	Plasma	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	0,16
	PL9	Plasma	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	20,00
PID3011	PBMCDNA1	PBMC	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	20,00
	PBMCDNA4	PBMC	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	20,00
	PL1	Plasma	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	20,00
	PL2	Plasma	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	0,23
	PBMCDNA1	PBMC	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	20,00
	PBMCDNA5	PBMC	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	0,07
	PBMCDNA7	PBMC	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	20,00
PID3011	LNDNA8	LN	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	20,00
	LNDNA14	LN	.	.	R	.	.	.	V	.	.	K	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	20,00

Table 7.3C: Amino acid residues in the Fusion peptide

These table list the amino acid stretches extracted from the full-length alignment and highlights the amino acid differences observed for the epitope. Glycans are shown in grey and the corresponding heatmap is shown alongside each table.

PID	HXB2 numbering		262	263	264	295	296	297	448	449	450	Silent Face
			N	G	S	N	C	T	N	I	T	SF12
PID0011	PL9	Plasma	.	.	.	V	.	I	T	.	.	0,12
	PL13	Plasma	.	.	.	V	.	M	T	.	.	0,03
	PBMCDNA6	PBMC	.	.	.	V	.	I	T	.	.	0,28
	PBMCDNA11	PBMC	.	.	.	V	.	I	T	.	.	0,07
	PBMCDNA14	PBMC	.	.	.	V	.	I	T	.	.	0,03
	PBMCRNA1	PBMC	.	.	.	V	.	.	.	.	.	0,11
	LNDNA1	LN	.	.	.	V	.	.	.	.	.	0,03
	LNDNA2	LN	.	.	.	V	.	.	.	.	.	0,02
	LNDNA3	LN	.	.	.	V	.	I	T	.	.	0,31
	LNDNA6	LN	.	.	.	V	.	I	T	.	.	0,26
	LNDNA7	LN	.	.	.	V	.	I	T	.	.	0,18
	LNDNA10	LN	.	.	.	V	.	M	T	.	.	0,03
	LNRNA5	LN	.	.	.	V	.	I	T	.	.	20,00
	LNRNA6	LN	.	.	.	V	.	.	T	.	.	0,01
PID0053	PL3	Plasma	.	.	.	K	.	.	.	.	.	0,02
	PL8	Plasma	.	.	.	E	.	.	.	.	.	0,08
	PBMCDNA4	PBMC	.	.	.	K	.	.	.	.	.	0,02
	PBMCDNA5	PBMC	.	.	.	E	.	.	.	.	.	15,35
	PBMCDNA11	PBMC	.	.	.	E	.	.	.	.	.	0,15
	LNDNA3	LN	.	.	.	K	.	.	.	.	.	0,03
	LNDNA7	LN	.	.	.	E	.	.	.	.	.	0,01
	LNDNA10	LN	.	.	.	K	.	.	.	.	.	0,09
PID118	PL6	Plasma	.	.	.	T	.	.	.	.	.	0,33
	PL9	Plasma	.	.	.	T	.	.	.	.	.	0,06
	PBMCDNA1	PBMC	.	.	.	T	.	.	.	.	.	0,01
	PBMCDNA4	PBMC	.	.	.	T	.	.	.	.	.	0,06
PID3011	PL1	Plasma	.	.	.	V	.	.	S	.	.	0,03
	PL2	Plasma	.	.	.	V	.	.	S	.	.	0,25
	PBMCDNA1	PBMC	.	.	.	V	.	.	S	.	.	0,01
	PBMCDNA5	PBMC	.	.	.	V	.	.	S	.	.	0,01
	PBMCDNA7	PBMC	.	.	.	V	.	.	S	.	.	0,03
	LNDNA8	LN	.	.	.	V	.	.	S	.	.	0,05
	LNDNA14	LN	.	.	.	V	.	.	S	.	.	0,02

Table 7.3D: Amino acid residues in the Silent face

These table list the amino acid stretches extracted from the full-length alignment and highlights the amino acid differences observed for the epitope. Glycans are shown in grey and the corresponding heatmap is shown alongside each table.

Appendix

BREC approval:



07 May 2025

Miss Keyura Velan (219001879)
School of Laboratory Medicine & Medical Science
Medical School

Dear Miss Velan,

Protocol reference number: BREC/00006367/2023

Project title: Evaluation of neutralization sensitivity in HIV-1 Subtype C between lymph nodes, peripheral blood mononuclear cells and plasma derived viruses

Degree: Masters

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved: 24 April 2025

Expiration of Ethical Approval: 23 April 2026

I wish to advise you that your application for recertification for the above study has been **noted and approved** by a subcommittee of the Biomedical Research Ethics Committee (BREC). The start and end dates of this period are indicated above.

If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

The committee will be notified of the above approval at its next meeting to be held on 10 June 2025.

Yours sincerely



Ms A Marimuthu
(for) Prof S Singh
Chair: Biomedical Research Ethics Committee

Biomedical Research Ethics Committee
Chair: Professor S Singh
UKZN Research Ethics Office Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Email: BREC@ukzn.ac.za
Website: <http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx>

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