



DELINEATING THE ONTOGENY OF AN N332-DIRECTED ANTI-HIV-1 BROADLY NEUTRALISING ANTIBODY LINEAGE.

Thamara Naidoo

1317469

Supervisors: Dr Dale Kitchin (Senior Medical Scientist, Antibody Immunity Research Unit (AIRU), National Institute for Communicable Diseases (NICD)) and Professor Penny Moore (Head of AIRU, NICD and Full Research Professor, University of the Witwatersrand)

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Declaration

I, Thamara Naidoo, declare that this Research Report is my own, unaided work. It is being submitted for the Degree of Masters of Science (Medicine) in Vaccinology at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

A handwritten signature in black ink, appearing to read 'Thamara Naidoo', is positioned above a horizontal line.

(Signature of candidate)

25th day of May 2023 in Parktown, Johannesburg, South Africa.

Abstract

Broadly neutralising antibodies (bNAbs) develop in chronically HIV-1 infected individuals and can neutralise a variety of HIV-1 Env strains, making them an important element contributing to the design of HIV-1 vaccines. By understanding and delineating the long affinity maturation pathways that bNAb lineages follow against evolving viruses, it is hoped that this process will be able to be replicated in uninfected people with a series of vaccine immunogens. Investigating the ontogeny of a N332-directed bNAb lineage will provide insights into the key somatic hypermutations necessary for the development of neutralisation breadth. CAP255.G3 is an N332-directed bNAb isolated from CAP255, a participant in the CAPRISA 002 cohort, at 149 weeks post-infection (wpi). Six key antibody intermediates were selected from the CAP255.G3 lineage arm between 17 and 47 wpi, based on their position on a phylogenetic tree and because they contained mutations that were present in broad members of the lineage. We hypothesized that these intermediates would exhibit breadth similar to CAP255.G3. The intermediates were tested against a panel of eight autologous and six heterologous pseudoviruses using a TZM-bl neutralisation assay. The sequence identity of the first heavy chain complementarity-determining region (CDRH1) of CAP255.G3 differed from the closest intermediate and therefore, a CDRH1 chimera was constructed to determine if the CDRH1 from CAP255.G3 affected neutralisation activity. Later intermediates from 39 and 47 wpi, had moderate neutralisation activity against the autologous pseudoviruses, but only the intermediate closest to CAP255.G3 had limited neutralising activity against the heterologous pseudoviruses. Although the CDRH1 chimera had increased neutralisation activity against the heterologous viruses relative to the intermediates, it was less broad and potent than CAP255.G3. This indicates that additional affinity maturation between 47 and 149 wpi, at sites outside of the CDRH1, was required for the development of neutralisation breadth in the CAP255.G3 lineage arm.

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Table of Contents

Declaration	ii
Abstract	iii
Acknowledgments	iv
List of Figures.....	vii
List of Tables	viii
List of Abbreviations	ix
1. Introduction.....	1
1.1 The burden of the HIV-1 pandemic	1
1.2 An HIV-1 vaccine: a necessity and on-going challenge.....	1
1.3 The HIV-1 envelope trimer	3
1.4 Antibodies and their importance in HIV-1 vaccine development	4
1.5 The development of bNAbs.....	6
1.6 The ontogeny of CAP255 bNAbs	8
1.7 Aims and objectives	10
2. Materials and Methods	11
2.1 Preparation of antibody expression vectors	11
2.2 HIV-1 Env-pseudotyped virus production	15
2.3 Pseudovirus neutralisation assays	15
2.4 Data analysis.....	16
3. Results	17
3.1 CAP255 lineage antibody intermediates from CAP255.G3 arm	17
3.2 Neutralisation of autologous pseudoviruses.....	19
3.3 Neutralisation of heterologous pseudoviruses.....	21
4. Discussion	26
5. Conclusion.....	31
References	32
APPENDIX A: CAPRISA permission letter	39

APPENDIX B: Human Research Ethics Committee (HREC) letter for blanket ethics	40
APPENDIX C: HREC certificate	42
APPENDIX D: Plagiarism declaration	43
APPENDIX E: Turn it in report.....	44

List of Figures

Figure 1. HIV-1 trimer showing the conserved sites of vulnerability targeted by bNAbs	5
Figure 2. Phylogenetic tree for the heavy chain variable region of the broad N332-directed CAP255 antibody lineage	9
Figure 3. Phylogenetic trees showing overlapping neutralisation profiles of CAP255.C5 and CAP255.G3	10
Figure 4. Amino acid sequence alignment of the heavy chain variable region for the CAP255 lineage UCA, mature CAP255.G3 bNAb and CAP255.G3 arm intermediates	18
Figure 5. Neutralisation activity of members of the CAP255.G3 lineage arm against autologous PSVs	20
Figure 6. Neutralisation breadth of members of the CAP255.G3 lineage arm against the heterologous PSVs.....	23
Figure 7. Neutralisation activity of the UCA, six lineage intermediates, CDR-H1 chimera and CAP255.G3 against the panel of 14 PSVs.....	24

List of Tables

Table 1. Synthetic oligonucleotide sequences used to amplify the CAP255.G3 CDRH1 insert and the linearized Int F (47 wpi) backbone.....	14
Table 2. PCR cycling parameters for amplification of the CAP255.G3 CDRH1 insert and Int F (47 wpi) linearized backbone.....	14
Table 3. Table showing SNPs in CAP255.G3 compared to Int F (47 wpi) with the potential to have an impact on neutralisation activity and breadth.....	25

List of Abbreviations

AID: Activation-induced deaminase

AIDS: Acquired immunodeficiency syndrome

AA: Amino acid

AMP: Antibody mediated prevention

ANOVA: Analysis of variance

ART: Antiretroviral therapy

bNAb: Broadly neutralising antibody

CAPRISA: Centre for the AIDS Programme of research in South Africa

CD4bs: CD4 binding site

CDR: complementarity-determining region

COVID-19: Coronavirus disease 2019

ddNTPs: dideoxynucleosides

DMEM: Dulbecco's modified eagle medium

DNA: Deoxyribonucleic acid

EM: Electron microscopy

Env: Envelope glycoprotein

FBS: Fetal bovine serum

FWR: Framework region

GC: Germinal centre

GM: Growth media

HCW: Healthcare worker

HEK: Human embryonic kidney

HIV-1: Human immunodeficiency virus type 1

HPTN: HIV prevention trials network

HVTN: HIV vaccine trials network

IAVI: International AIDS vaccine initiative

IC₅₀: Half maximal inhibitory concentration

IMGT®: the international ImMunoGeneTics information system ®

Indels: insertions and deletions

Int: Intermediate

LB: Luria broth

mAb: Monoclonal antibody

MPER: Membrane proximal external region

MWCO: Molecular weight cut-off

nAb: Neutralising antibody

NHP: Non-human primate

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

PEI-MAX: Polyethylenimine hydrochloride

PrEP: Pre-exposure prophylaxis

PSV: Pseudovirus

RLU: Relative luminescence units

RPM: Revolutions per minute

RT: Reverse transcriptase

SHM: Somatic hypermutation

SNP: Single nucleotide polymorphism

SSA: Sub-Saharan Africa

TCID₅₀: median tissue culture infectious dose

UCA: Unmutated common ancestor

UNAIDS: United nations programme on HIV and AIDS

Wpi: weeks post-infection

1. Introduction

1.1 The burden of the HIV-1 pandemic

The human immunodeficiency virus type 1 (HIV-1) is the causative agent of acquired immunodeficiency syndrome (AIDS) (Ho and Bieniasz, 2008; HIV/AIDS, 2022). HIV-1 infection results in a significant decline in the ability of the immune system to fight off opportunistic infections, leading to progressive immunodeficiency and can result in death if left untreated (HIV/AIDS, 2022). HIV-1 has claimed the lives of 40.1 million [33.6 – 48.6 million] people worldwide since 1981 (HIV/AIDS, 2022). Currently, there are 38.4 million people estimated to be living with HIV-1 with approximately 1.5 million [1.1 – 2.0 million] new infections occurring in 2021 (HIV/AIDS, 2022). In Sub-Saharan Africa (SSA), which is the most affected region worldwide, the current number of people living with HIV-1 is 25.6 million [23.4 – 28.6 million] (HIV/AIDS, 2022). Nearly 40 years after first being identified, HIV-1 remains a global public health emergency and prevention and treatment interventions are required to end the long-lasting pandemic.

1.2 An HIV-1 vaccine: a necessity and on-going challenge

Reduction of the risks involved in acquiring HIV-1 infection include a combination of limiting exposures to risk factors and implementing prevention measures, including the use of pre-exposure prophylaxis (PrEP) (HIV/AIDS, 2022). An example involves the use of the long-acting injectable, cabotegravir for high-risk individuals and more commonly, regular use of condoms to prevent transmission (HIV/AIDS, 2022). Treatment methods for infected individuals involve combinations of antiretroviral therapy (ART), necessary to suppress viral replication (Angel and Tomaras, 2020; HIV/AIDS, 2022). When an individual is virally suppressed on ART, they are unable to transmit HIV-1 to a sexual partner, hence an imperative element of HIV-1 prevention includes increasing access to testing facilities and treatment regimens (Angel and Tomaras, 2020). Since 2020, when Coronavirus disease 2019 (COVID-19) was officially declared a public health emergency, high-risk individuals and infected patients were reluctant to visit healthcare facilities to obtain PrEP or ART due to social distancing and the threat of being exposed to COVID-19 with an already compromised immune system (Chenneville *et al.*, 2020). In addition, most healthcare workers (HCW) were diverted to providing care in COVID-19 treatment wards, resulting in fewer HCW available to tend to HIV-1-infected patients (Chenneville *et al.*, 2020). Another challenge involves disruptions in the international manufacturers supply chain of the

ARTs used in Africa, owing to the closure of international borders due to the COVID-19 pandemic (Uwishema *et al.*, 2022). This has resulted in reduced access to treatment for new and existing HIV-1-infected individuals (Uwishema *et al.*, 2022). Collectively, this has impacted treatment regimens for all individuals which results in an incline in viral loads of infected individuals. The long-term impact of this is yet to be investigated, but it was predicted that the United Nations Programme on HIV and AIDS (UNAIDS) 90-90-90 target will be delayed due to the COVID-19 pandemic (Jamieson and Kellerman, 2016; *UNAIDS report: COVID-19 pandemic derails 2020 HIV targets*, 2020). Furthermore, the constraints involved with prevention and treatment measures include lack of access to healthcare in low-resource areas and accumulative costs involved with life-long PrEP for high-risk individuals, and ART for HIV-1-infected individuals (Angel and Tomaras, 2020). Finally, non-compliance to treatment regimens by the user hinders the ability to suppress viral loads in infected individuals and leads to the continuous spread of HIV-1 (Angel and Tomaras, 2020). Therefore, managing HIV-1 involves successful suppression of viral loads, but requires effort from the healthcare systems to provide proper care in the midst of the COVID-19 pandemic, including users ensuring they maintain healthy lifestyles.

Modelling studies have shown that even with the effective implementation of PrEP and ART, new infections will continue to occur, and the costs involved with prevention and treatment continue to be a burden, especially in low-resource settings like SSA (Freedberg *et al.*, 2015; Adamson *et al.*, 2017). Ultimately, these studies suggest that an effective HIV vaccine is required to end the HIV-1 pandemic. Many vaccines for various disease-causing pathogens, such as measles, hepatitis C and SARS-CoV-2 elicit neutralising antibodies (nAbs), and these have been shown to be a correlate of protection in vaccinated individuals (Green *et al.*, 2001; Law *et al.*, 2013; Verbeke *et al.*, 2021). Thus, it would be essential for an efficacious HIV-1 vaccine to elicit nAbs. However, the HIV-1 envelope glycoprotein (Env) is the only neutralising antibody target on the HIV-1 protein surface and this comes with several challenges (Munro and Mothes, 2015). The HIV-1 Env trimer has great genetic diversity and a surrounding glycan shield to prevent nAbs from neutralising the virus. An HIV-1 vaccine that provides sterilising immunity is essential, as HIV-1 integrates into the genome of host cells, however, this has proven to be a significant challenge (Haynes *et al.*, 2023).

1.3 The HIV-1 envelope trimer

The HIV-1 Env is a trimer of heterodimers consisting of gp120 and gp41 subunits (Munro and Mothes, 2015). The HIV-1 Env trimer contains a region called the CD4 binding site (CD4bs) which facilitates entry of the virus into host cells expressing CD4 (Ward and Wilson, 2015). Cells that express CD4 on their surface include monocytes, macrophages, dendritic cells and T helper cells (Ward and Wilson, 2015). Entry into the target cells occurs via recognition of CD4 cellular receptors via the gp120 receptor-binding domain of the HIV-1 Env (Munro and Mothes, 2015). After binding to CD4, a conformational change occurs in the HIV-1 Env allowing for co-receptor binding to CCR5 or CXCR4, and subsequently causes viral and cellular membrane fusion (Munro and Mothes, 2015; Ward and Wilson, 2015). The HIV-1 Env trimer exhibits several characteristics that allows it to escape neutralisation by antibodies. The HIV-1 Env trimer is surrounded by a poorly immunogenic, host-derived glycan shield, which blocks the access of nAbs to the underlying HIV-1 Env protein surface (Munro and Mothes, 2015; MacLeod *et al.*, 2016; Moyo, Kitchin and Moore, 2020). During infection the *env* gene encoding for the HIV-1 Env trimer protein acquires high levels of intrahost genetic diversity, due to antibodies elicited against the original transmitted founder virus that exerts selective pressure on the HIV-1 Env (Wu *et al.*, 2012). Moreover, the high mutation rates and heterologous recombination occurring during the reverse transcription process, mediated by reverse transcriptase (RT), makes the HIV-1 Env antigenically diverse and allows the HIV-1 Env to escape neutralisation (Munro and Mothes, 2015; Moore, 2018; Van Duyne *et al.*, 2019).

This ultimately selects for viral escape of HIV-1 Env mutants generated by RT, and is a continuous process which occurs when new antibodies are formed to combat the mutated viruses, commonly referred to as the “arms race” (Wu *et al.*, 2012). Therefore, it is necessary to discover strategies to overcome the ability of the HIV-1 Env to escape neutralisation. There are conserved sites on the HIV-1 Env that are functionally vital structures and are less susceptible to mutations caused by the antibody selective pressure (Burton and Hangartner, 2016). These conserved sites exist because the virus would incur a fitness cost if these sites gained mutations (Burton and Hangartner, 2016). Broadly neutralising antibodies (bNAbs) are nAbs that have been isolated from chronically infected HIV-1 individuals and have shown promise in facilitating neutralisation of the HIV-1 Env by targeting the relatively conserved sites on the HIV-1 epitope (Burton and Hangartner, 2016; Deshpande *et al.*, 2016).

1.4 Antibodies and their importance in HIV-1 vaccine development

Antibodies are proteins that are produced by B cells and bind foreign substances, such as antigens (Roth, 2014). A process known as V(D)J recombination aids in the development of antibodies, forming a diverse repertoire of B cell receptors that have the ability to recognise various antigens and in turn, leads to the development of specific antibodies produced by plasma cells (Roth, 2014). An antibody has a basic Y-shaped structure which consists of two heavy chains and two light chains, which include variable regions that bind to specific antigens (Roth, 2014). Antibodies also contain constant regions which determine the antibody class or isotype, such as IgG, IgA, IgM or IgD, and this region determines the antibody function (Roth, 2014). There are five main functions of antibodies which include neutralisation, opsonization, complement activation, antibody-dependent cell-mediated cytotoxicity (ADCC) and immunoregulation (Roth, 2014). Specifically, the neutralisation function of antibodies in this paper refers to the ability of HIV-1 nAbs to block infection by binding to the HIV-1 Env protein, and prevent the virus from binding to the CD4 receptor and co-receptors on potential host cells.

Once an individual acquires HIV-1 infection, their immune systems produce nAbs against the constantly evolving virus and these are referred to as autologous or “self” antibodies. However, the host’s immune system is unable to develop an effective immune response to the rapidly mutating virus and leads to antibody-antigen co-evolution, where the virus mutates in response to the host’s immune response of developing new antibodies (Bonsignori et al., 2017). Over the course of a few months or years, the co-evolution can result in the development of bNAbs which have shown to be effective against multiple variants of the HIV-Env virus (Bonsignori et al., 2017). Generally, bNAbs develop in 15-30% of chronically HIV-1-infected individuals and these antibodies have the ability to neutralise autologous viruses and develop breadth by neutralisation of cross-clade viruses (Gray *et al.*, 2011; Moore, Williamson and Morris, 2015). The bNAbs that have been isolated by several researchers have enabled the development of numerous strategies targeting the various relatively conserved epitopes namely the CD4bs, the V2-apex, the V3-glycan high-mannose patch (also referred to as N332-supersite), the silent face centre, the fusion peptide, the gp120-gp41 interface and the membrane-proximal external region (MPER) (Fig. 1) (Garces *et al.*, 2014; MacLeod *et al.*, 2016; Barnes *et al.*, 2018; Sacks *et al.*, 2019; Moyo, Kitchin and Moore, 2020). The V3-glycan/N332- supersite of vulnerability is one

of the most common epitopes targeted by bNAbs in HIV-1-infected individuals (MacLeod *et al.*, 2016) and is the epitope focused on in this study. Targeting the relatively conserved epitopes on the HIV-1 Env guides vaccine design involving bNAbs, which has led to previous trials shifting focus from other investigated strategies to bNAbs.

The only human HIV-1 vaccine trial that showed moderate levels of protection was the RV144 Thai trial, where the correlate of protection was not bNAbs, but rather non-neutralising antibodies (Rerks-Ngarm *et al.*, 2009). However, when this trial was replicated in South Africa (HVTN702), there was no protective efficacy, probably because many variables were changed (Moodie *et al.*, 2022). In non-human primate (NHP) challenge studies, passive immunization of uninfected animals with bNAbs provided sterilizing immunity as the antibodies were directly administered to the animals (Moldt *et al.*, 2012; Barouch *et al.*, 2013). The Antibody Mediated prevention (AMP) studies together with the HIV Prevention Trials Network (HPTN) and HIV Vaccine Trials Network (HVTN) conducted a human trial where VRC01 was administered intravenously, in a process also known as passive immunization (Corey *et al.*, 2021).

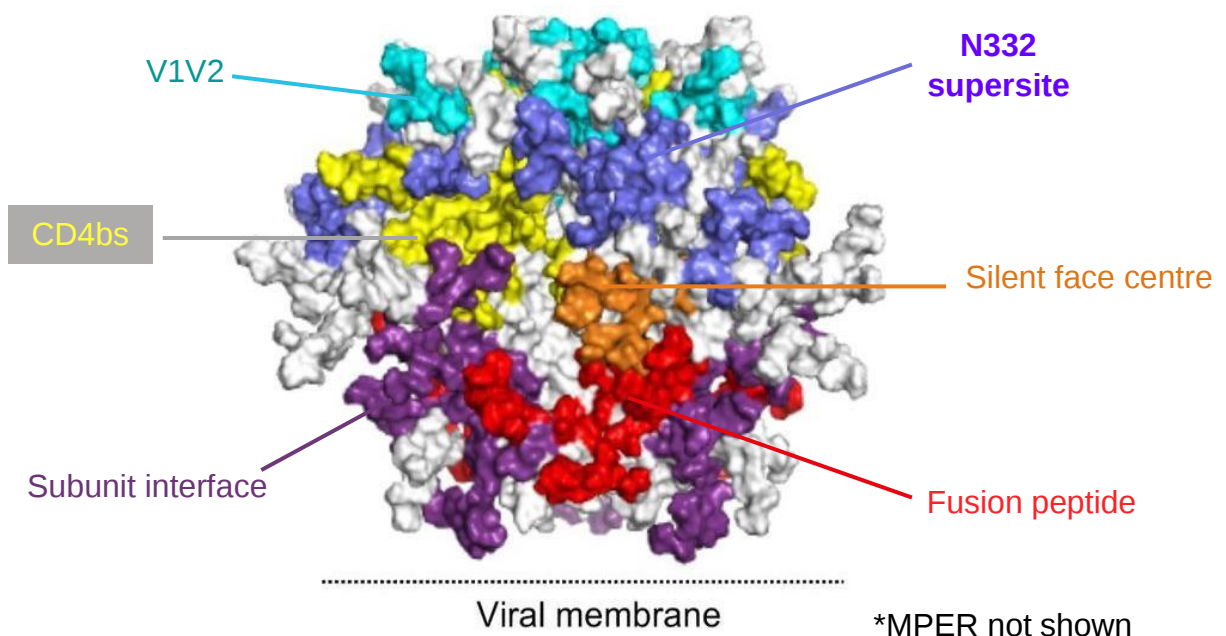


Figure 1. HIV-1 trimer showing the conserved sites of vulnerability targeted by bNAbs. Figure obtained from Chuang *et al.*, 2019.

The trial names were HVTN 703/HPTN 081 and HVTN 704/HPTN 085 and differed in their populations, involving high-risk individuals and in HIV prevalent populations. Overall conclusions were that VRC01 successfully blocked HIV strains classified as highly sensitive to the bNAb (Corey *et al.*, 2021). This trial showed that antibody-mediated prevention of HIV is achievable, as it is the first evidence of a bNAb reducing the risk of acquiring HIV through sexual intercourse (Corey *et al.*, 2021). Together, this information provides a strong rationale that bNAbs may be the correlate of protection for a future, effective HIV-1 vaccine.

One vaccine strategy that recently gained traction involved priming immunogens which utilized a germline-targeting vaccine strategy (Jardine, Kulp, *et al.*, 2016). The study investigated the VRC01-class bNAbs, which are broad and potent, and the vaccine immunogen itself is a self-assembling nanoparticle which presents 60 copies of the HIV gp120 engineered outer domain, germline-targeting version 8 (eOD-GT8) manufactured to have an affinity for VRC01-directed bNAb germline precursors (Jardine, Kulp, *et al.*, 2016; Leggat *et al.*, 2022). To date, eOD-GT8 is the most successful germline targeting immunogen developed. Precursor B cells present at low frequencies were engaged by eOD-GT8 and caused them to proliferate and resulted in expanded lineages, where 97% of the participants who received this immunogen developed VRC01-class derived precursor B cell receptors (Leggat *et al.*, 2022).

The development of the eOD-GT8 immunogen with the ability to engage VRC01 precursors is a breakthrough in the developmental processes of HIV-1 bNAb vaccines. However, the design and implementation of further booster immunogens are required to drive these responses to achieve neutralisation breadth. Furthermore, it is unclear of the mechanisms required to engage precursors for other bNAb specificities i.e., V3-directed bNAbs, and ways to drive these to breadth. Therefore, these are few of the various factors we can learn and apply from studying the development of broad lineages in HIV-1 infected individuals.

1.5 The development of bNAbs

Lineages of bNAbs originate from a naïve B cell precursor, also called an unmutated common ancestor (UCA) (MacLeod *et al.*, 2016; Doria-Rose and Landais, 2019). The development of neutralisation breadth occurs through affinity maturation processes occurring from the UCA, against the constantly co-evolving and escaping autologous virus (Doria-Rose and Landais, 2019). Some antibody lineages gain the ability to tolerate the escape mutations of the autologous virus and gain neutralisation breadth

simultaneously (MacLeod *et al.*, 2016; Doria-Rose and Landais, 2019).

There are several features that bNAbs have in common, including insertions and deletions (indels), the possession of long third heavy chain complementarity-determining regions (CDRH3s) and high levels of somatic hypermutation (SHM) (Doria-Rose and Landais, 2019). The long CDRH3s of some bNAbs have the potential to enhance affinity for glycans by increasing surface interactions between the glycan and bNAb, enabling the CDRH3 to reach the HIV-1 Env (Barnes *et al.*, 2018). Whereas another function includes penetration of the glycan shield to allow bNAbs access to the underlying HIV-1 Env protein surface, and facilitate binding to the neutralising epitopes (Walker *et al.*, 2011; Ward and Wilson, 2015).

The process of SHM occurs in the germinal centres (GCs) of B cells, which is an imperative part of the antibody response (Victora and Nussenzweig, 2012; DeFranco, 2016). Through the GC reaction, antigen-specific B cells undergo cellular proliferation and mutagenesis of their antibody genes which results in the generation of memory B cells and plasma cells that express BCRs and antibodies, respectively, with enhanced affinity for their cognate antigen (DeFranco, 2016). This mechanism of introducing mutations in the antigen-binding sites of B cells, causing the B cells to proliferate and produce high-affinity antibodies is under the influence of the enzyme activation-induced deaminase (AID), and the selection in the GCs against antigens ultimately results in affinity maturation (Victora and Nussenzweig, 2012). Therefore, complex affinity maturation pathways may be required for bNAb development resulting in their common characteristics. However, some bNAbs (PCDN-33A) with significant breadth (50% breadth against a panel of 110 pseudoviruses) did not contain high levels of SHM and no indels (MacLeod *et al.*, 2016), while another study showed that mutations in the framework regions (FWR), that flank the CDRs, are important for breadth and potency, suggesting that not all features of previously described bNAbs are required for development of neutralisation breadth (Klein *et al.*, 2013). Furthermore, a recent study found that lineage members, which were referred to as “off-track” members had high sequence identity to broad members of the lineage, but lack breadth, highlighting the fact that not all antibodies in the lineage with similar features as broad lineage members develop breadth (Bhiman *et al.*, 2015; Sacks *et al.*, 2019). Several factors have been shown to be associated with the development of bNAbs, such as high viral load, low CD4⁺ count, and HIV-1 subtype C superinfection (Landais *et al.*, 2016). However, it is uncertain if these factors contribute to bNAb development in all, or only

a fraction of infected individuals developing bNAbs.

Many studies have utilized X-ray chromatography and electron microscopy (EM) to investigate specific epitope directed bNAbs and gain insights into the structure and how the bNAb physically interacts with the HIV-1 Env trimer and surrounding glycans (Doores *et al.*, 2014; Longo *et al.*, 2016). Some bNAbs interact with the glycans to reach the underlying epitope, while some have long protruding CDR appendages that allow the bNAb to reach the underlying epitope, without glycan interaction. However, these differences vary among diverse epitope-directed bNAbs. This depends on the specificities of the bNAb in the lineage, where some bNAbs from VRC01-directed lineages require the use of the same variable gene and similar size residues from first light chain CDR (CDRL1) and third light chain CDR (CDRL3) for example (Umotoy *et al.*, 2019). Other bNAbs from N332-directed lineages require dependence on glycans N332 or N301 and longer-than-average lengths of CDRH3 (Longo *et al.*, 2016). Therefore, understanding the key characteristics common in already isolated bNAbs, allows researchers to investigate antibody specificities for particular epitope/glycan-directed lineages and apply these to germline-targeting immunogens for future vaccine design and immunization strategies (Umotoy *et al.*, 2019).

1.6 The ontogeny of CAP255 bNAbs

From the CAPRISA 002 acute infection cohort conducted in KwaZulu-Natal, recruited participants who exhibited significant neutralisation breadth were previously identified by plasma screening (Gray *et al.*, 2011). CAP255 was a broad participant and the bNAbs isolated were able to neutralise a cross-clade, heterologous pseudovirus panel (Kitchin *et al.*, *unpublished data*). The CAP255 lineage utilizes the VH4-34*02 germline gene and diverged into two N332-directed bNAb arms namely, CAP255.C5 and CAP255.G3 (Fig. 2). An interesting finding was that CAP255.G3 and CAP255.C5 have high overlapping neutralisation profiles (Fig. 3), but have low sequence identity indicating they have followed different affinity maturation pathways (Kitchin *et al.*, *unpublished data*). This project investigated the ontogeny of the CAP255.G3 arm, by looking at which SHMs were important for breadth development. The focus was placed on the heavy chain as this was previously shown to be most important for mediating breadth (Kitchin *et al.*, *unpublished data*). Additionally, key SHMs were identified from the antibody intermediates isolated at different time points post-infection and compared to mature bNAb, CAP255.G3, to determine which mutations were responsible for the development of breadth in the lineage.

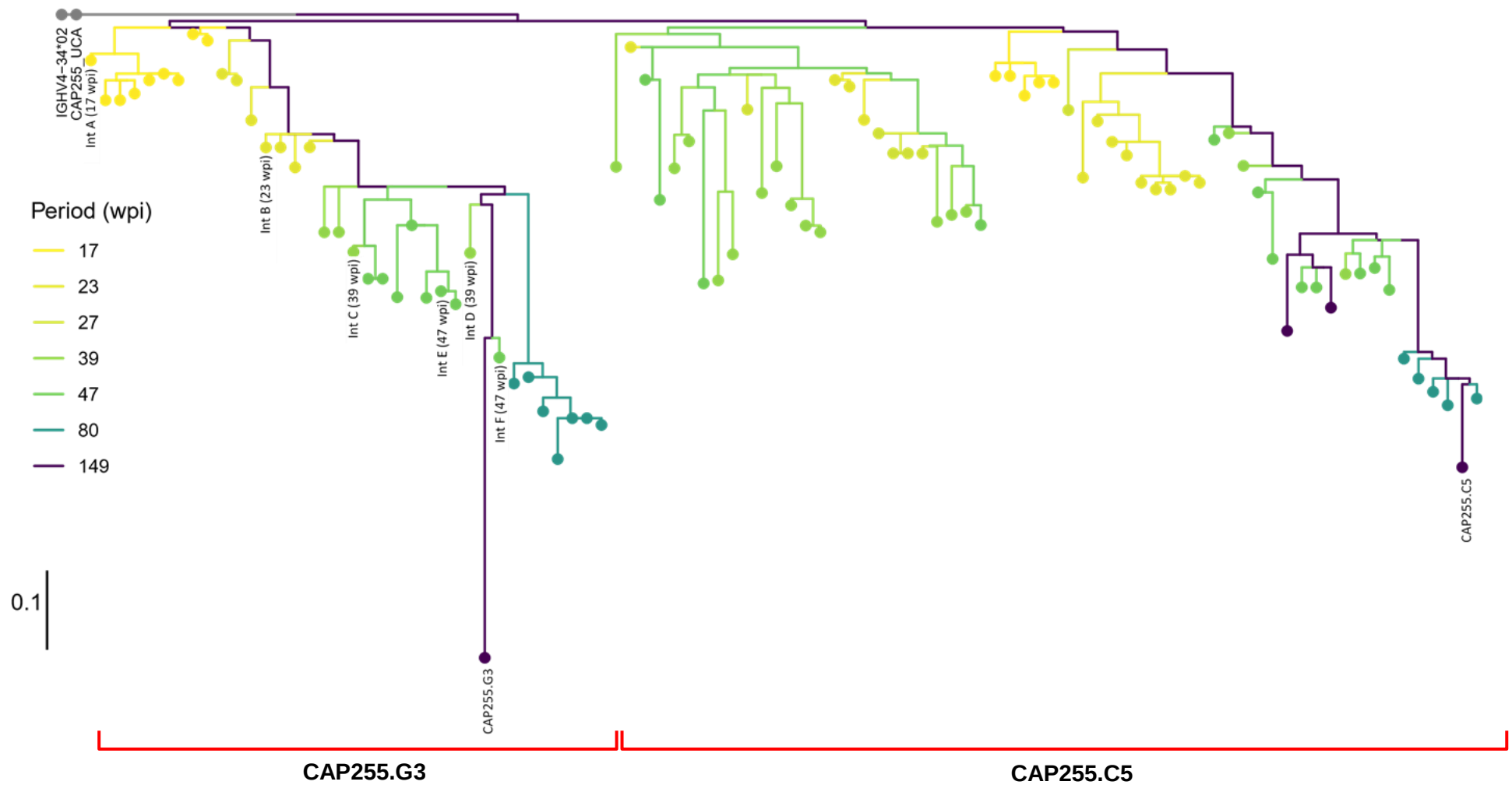


Figure 2. Phylogenetic tree for the heavy chain variable region of the broad N332-directed CAP255 antibody lineage. The CAP255.G3 and CAP255.C5 arms of the lineage are shown with the antibody intermediates selected for neutralisation assays, indicated with labels on the CAP255.G3 arm.

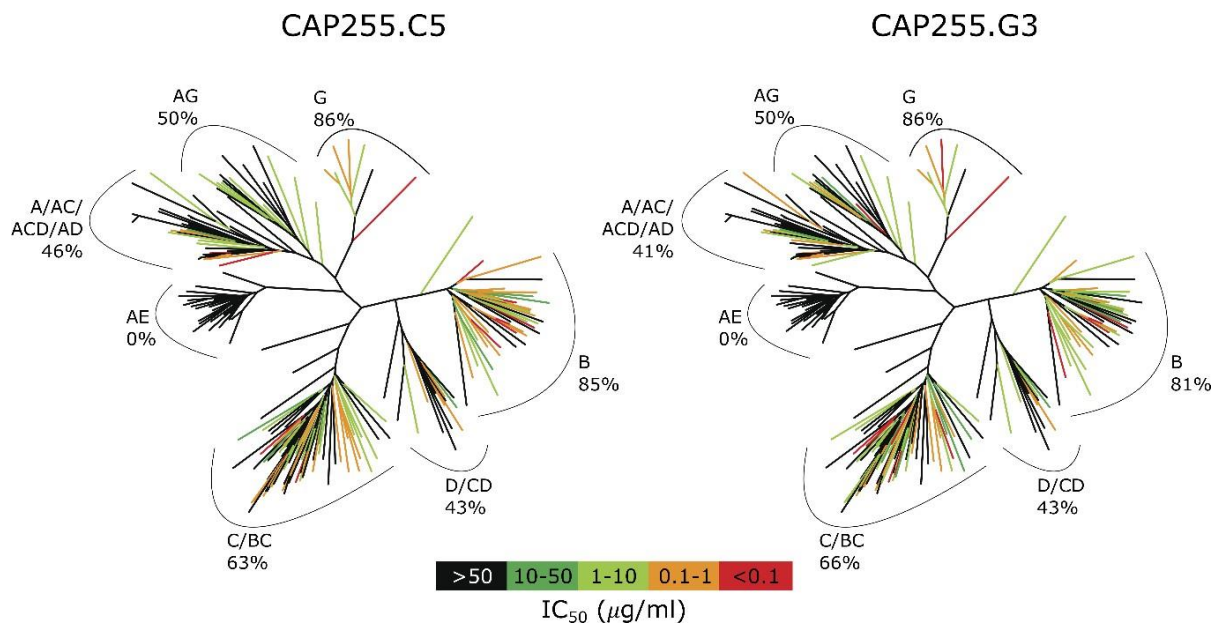


Figure 3. Phylogenetic trees showing overlapping neutralisation profiles of CAP255.C5 and CAP255.G3. Figure obtained from Kitchin *et. al*, unpublished data.

1.7 Aims and objectives

The aim of this project was to delineate the ontogeny of the CAP255.G3 arm of an N332-directed broadly neutralising antibody lineage from participant CAP255, and identify the key somatic hypermutations that conferred neutralisation breadth.

Specific objectives:

1. To clone antibody heavy chain variable gene regions identified from longitudinal deep sequencing, into mammalian expression vectors.
2. Briefly identify key HIV-1 envelope variants that engaged the bNAb lineage intermediates and drove the lineage toward neutralisation breadth.
3. To assess which somatic hypermutations were present in the mature bNAb that were essential for the development of neutralisation breadth.

2. Materials and Methods

2.1 Preparation of antibody expression vectors

Representative antibodies were identified from longitudinal deep sequencing data (Kitchin *et. al*, *unpublished data*) for the CAP255 N332-specific bNAb lineage. Unpaired heavy and light chains (V(D)J and VJ regions, respectively) were previously sequenced for CAP255 broad lineage members at six time points: 17, 23, two 39 and two 47 weeks' post-infection (wpi). The UCA was isolated at 17 wpi, the earliest time-point where clonally related lineage members were observed, and contained no SHMs in the variable (V) and joining (J) regions. Therefore, this transcript was chosen as a proxy for the UCA heavy chain sequence from the longitudinal deep sequencing data. Previously identified sequencing recovered clonal relatives at 80 wpi from the CAP255.C5 lineage arm, but no clonally related transcripts were found between 47 wpi and 149 wpi that belonged to the CAP255.G3 arm. The lack of any intermediate transcripts belonging to the CAP255.G3 arm between 47 and 149 wpi is clearly shown by the long branch length for CAP255.G3 in the phylogenetic tree (Fig. 2). The antibody intermediates were compared to the UCA to identify SHMs gained over time which may have contributed to neutralisation breadth in this lineage. As the native pairing of these heavy chain sequences were unknown, each intermediate was paired with the light chain from the mature, broad CAP255.G3 antibody. Briefly however, heavy and light chain intermediate sequences belonging to the broadly neutralising CAP255 lineage were selected from each longitudinal time point based on two conditions: (i) their position in the lineage phylogenetic tree made them good representatives of lineage members at that time point, and (ii) they had somatic mutations that may be implicated in breadth development due to their presence in later broadly neutralising antibody lineage members.

Mammalian codon optimized UCA constructs, CAP255.G3 heavy and light chain constructs and the six antibody intermediates were synthesized by GenScript (USA). These constructs were digested using restriction sites *Age I* and *Sal I* for the heavy chains and *Age I* and *Xho I* for the light chains and gel extracted for subsequent subcloning. The gel extracted plasmids were subcloned into CMVR expression vectors (NIH HIV Reagent Program, Division of AIDS, NIAID) containing either full length IgG1 or lambda constant region gene sequences using the Roche rapid ligation kit, with T4 ligation buffer. XL10-Gold ultracompetent cells (Agilent Technologies) were transformed with the subcloned constructs and single colonies were selected from

kanamycin agar plates and used to inoculate overnight Luria broth (LB) cultures. Plasmids were purified from the overnight broth cultures using the ZymoPURE II plasmid Maxiprep kit (Zymo Research) as per the manufacturer's instructions. Sanger-dideoxy sequencing was used to ensure the cloning was successful by confirming the identity and orientation of the insert. Sanger sequencing is a DNA sequencing technique that functions by utilizing DNA polymerase enzyme to manufacture a new strand of DNA. The DNA to be sequenced is amplified using a nucleoside mixture that includes fluorescently labelled dideoxynucleosides (ddNTPs) in a polymerase chain reaction (PCR), and this results in termination of the growing DNA at different points. The DNA fragments are subsequently separated based on size using gel capillary electrophoresis. A laser is then used to detect which ddNTPs terminated the chain and shows the base at that position (Crossley et al., 2020).

2.1.1 Expression of monoclonal antibodies

Human embryonic kidney 293F (HEK293F) suspension cell cultures were seeded at 1×10^6 cells/ml in a final volume of 400 mL FreeStyle expression media (Thermo Fischer Scientific) and incubated overnight with shaking (225 RPM), at 37°C, 8% CO₂ and 70% humidity. Freshly prepared polyethylenimine hydrochloride (PEI-MAX) (Polysciences) was added to 10 mL OptiPRO™ SFM serum free culture media (ThermoFischer Scientific) and incubated for 5 minutes. In a separate Falcon tube, 200µg of purified heavy chain and 200µg of purified light chain plasmid DNA were added to an additional 10 mL OptiPRO™ SFM. The DNA-OptiPRO mixture was directly filter-sterilized into the PEI-MAX-OptiPRO mixture through a 0.22 µm filter and incubated at room temperature for 20 minutes. Thereafter, this mixture was added to the HEK293F cells and left to incubate further for another 20 minutes at room temperature. The cells were then incubated for six days at 37°C, 8% CO₂ with shaking (225 RPM). The process was repeated for the six antibody intermediates, which were paired with the mature CAP255.G3 light chain. The UCA was paired with CAP255.G3 light chain and CAP255.G3 was paired with its native chain and followed the procedure above to express the mAbs. The mature CAP255.G3 light chain was used to keep this variable constant and to give the antibody intermediates the best chance of matching neutralisation activity of the mature CAP255.G3 bNAbs

2.1.2 Purification of monoclonal antibodies

Protein A affinity chromatography was used to purify the mAbs from cell supernatants.

After six days, the entire transfection culture was centrifuged at 4 500 RPM at 4°C for 30 minutes and the supernatant was filter-sterilized through a 0.22 µm vacuum filter. For a 400 mL culture volume, 5 mL of protein A beads were loaded onto a 14 cm, 20 mL Econo-pack column (Bio-Rad) and the resin settled to ~2.5 mL. The filter-sterilized supernatant was then loaded onto the column at a rate of 0.5-1mL/minute. Once all the supernatant had passed through, the column was washed with 10 column volumes of 1X phosphate-buffered saline (PBS) and eluted with 12.6 mL of elution buffer (0.1 M glycine, 0.15 M NaCl, pH 2.5 adjusted using 1N HCl) directly into a 15 mL tube containing 1.4 mL neutralisation buffer (1 M Tris pH 8.0 adjusted using 1N HCl). Thereafter, the antibody was transferred to PBS through dialysis and concentrated to 1 mg/mL using a 10 000 molecular weight cut-off (MWCO) Viva Spin Concentrator. The concentration of the antibody was confirmed by Nanodrop ND-1000 spectrophotometer and stored in 1 mL aliquots at -80°C.

2.1.3. Production of the CDRH1 chimera

Following analysis of the aligned sequences of the UCA, against the intermediates and mature bNAbs CAP255.G3 in AliView, specifically in the CDRs, there were many mutations observed in the first heavy chain CDR (CDRH1) of CAP255.G3. Since there were no intermediates sampled after 47 wpi that had similarities to the CAP255.G3 CDRH1, a chimeric heavy chain 47-week intermediate was made to assess the impact on neutralisation breadth. This was carried out by replacing residues LTCVVYGTSTFSD of the native Int F (47 wpi) CDRH1 sequence with the corresponding residue, LTCVVQQT VYTG, from CAP255.G3. The CDRH1 CAP255.G3 insert and Int F (47 wpi) backbone were amplified and linearized respectively, to ensure the native CDRH1 from Int F (47 wpi) plasmid was excluded. Synthetic oligonucleotide primers were designed for the CAP255.G3 CDRH1 insert and Int F (47 wpi) backbone and ordered from Inqaba Biotech (Table 1). The Invitrogen™ Platinum™ SuperFi™ II PCR Master Mix (ThermoFischer, USA) was used to amplify the CAP255.G3 CDRH1 insert and the Int F (47 wpi) backbone according to the PCR cycling parameters in Table 2. Gibson cloning was performed using the NEBuilder® HiFi DNA Assembly Cloning kit to clone the CAP255.G3 CDRH1 insert into the Int F (47 wpi) backbone and was placed in the thermocycler at 50°C for 15 minutes. Once the chimeric sequence was confirmed with the successful introduction of the CDRH1 insert using Sanger sequencing as explained above, the mAb was expressed and tested in neutralisation assays as described below.

Table 1. Synthetic oligonucleotide sequences used to amplify the CAP255.G3 CDRH1 insert and the linearized Int F (47 wpi) backbone.

Primer name	Nucleotide sequence
CDRH1Linear_FWD_Int F	CTA TTG TTG GTC TTG GAT CAG GCA G
CDRH1Linear_RVS_Int F	CAT GTC AGG CTC AGG GTC TC
CAP255.G3.CDRH1_RVS	CTG CCT GAT CCA AGA CCA ACA ATA G
CAP255.G3.CDRH1_FWD	GAG ACC CTG AGC CTG ACA TG

Table 2. PCR cycling parameters for amplification of the CAP255.G3 CDRH1 insert and Int F (47 wpi) linearized backbone.

	Cycles	Temperature (°C)		Time (seconds/minutes)	
		CAP255.G3 CDRH1 insert	Int F (47 wpi) backbone)	CAP255.G3 CDRH1 insert	Int F (47 wpi) backbone)
Initial denaturation	1	98	98	30 seconds	30 seconds
Denaturation	30	98	98	10 seconds	10 seconds
Annealing		55	60	10 seconds	10 seconds
Extension		72	72	15 seconds	3 minutes
Final extension	1	72	72	5 minutes	5 minutes
Hold	1	4	4	∞	∞

2.2 HIV-1 Env-pseudotyped virus production

The HIV-1 Env-pseudotyped viruses were prepared by co-transfecting HEK293T cells with two plasmids, one containing the *env* gene and a second backbone plasmid, pSCAP255.G3 Δ *env*, containing the full HIV-1 genome and a non-functional *env* gene, as described by Montefiori (2009). The HEK293T cells were seeded at 2×10^6 cells/10 mL in growth media (GM) consisting of Dulbecco's modified eagle media (DMEM), 10% fetal bovine serum (FBS), 3% HEPES buffer and 0.5% gentamicin and incubated overnight. The next day, 4 μ g of the HIV-1 *env* plasmid and 4 μ g of pSCAP255.G3 Δ *env* backbone plasmid were mixed with serum-free media and freshly prepared transfection agent, PEI_{max} and incubated at room temperature for 30 – 45 minutes. The entire transfection mixture was added to the HEK293T cells and following a 48-hour incubation (37°C & 5% CO₂), the pseudovirus-containing cell culture supernatants were harvested and filtered through a 0.45 μ m nitrocellulose filter. FBS was added to the pseudovirus stocks to a final concentration of 10% v/v, prior to storage at -80°C in 1 mL aliquots. A median tissue culture infectious dose (TCID₅₀) assay was performed as described by Montefiori (2009).

2.3 Pseudovirus neutralisation assays

The neutralisation activities of the different antibodies produced were determined using the TZM-bl pseudovirus (PSV) assay, as previously described by Montefiori (2009). A 3595 flat bottom 96-well plate (Co-Star) was used and the mAbs were added in duplicates in appropriate wells and titrated in a 3-fold serial dilution. The UCA and all the antibody intermediates were diluted to a starting concentration of 50 μ g/mL, while the starting concentration of CAP255.G3 was 5 μ g/mL due to its high potency. Previously produced PSVs were thawed and diluted with GM to result in relative luminescence units (RLUs) of 20 000 – 60 000. The PSVs were added to all wells except the cell controls and the plates were incubated at 37°C, 5% CO₂ for 45 – 90 minutes. Thereafter, freshly trypsinized TZM-bl cells (0.5×10^6 cells/mL) suspended in GM containing 1.4 % diethylaminoethyl dextran (DEAE) were added to each well in the plate and incubated at 37°C, 5% CO₂. After 24 hours, 130 μ L additional GM was added to each well. Following another 24 hours of incubation, 100 μ L of GM was removed and 100 μ L of Bright Glo luciferase reagent (Promega) added to each well. After a 2-minute incubation at room temperature, 150 μ L was transferred to a 96-well black plate (Co-Star) and the relative luminescence was measured using a VICTOR XLight luminometer (PerkinElmer) and used to calculate the half maximal inhibitory concentration (IC₅₀) of the antibodies tested. The IC₅₀ values were calculated using a

custom in-house Excel macro analysis sheet. All neutralisation assays were repeated to produce two sets of IC₅₀ values to determine the mean IC₅₀ for each mAb-PSV combination.

2.4 Data analysis

The MAFFT tool (Kato and Standley, 2013) in AliView (Larsson, 2014) was used to align the UCA, antibody intermediates and CAP255.G3 amino acid (AA) sequences. Maximum likelihood phylogenetic trees were constructed from the sequence alignments using the IgPhyML tool (Hoehn *et al.*, 2019) and the resultant trees were plotted using the ggtree package in R (Yu *et al.*, 2018). A custom in-house Excel macro sheet was used for calculating IC₅₀ values from the neutralisation assays (Microsoft Excel, 2012). All statistical analyses were performed in Prism (v9; GraphPad Software Inc, San Diego, CA, USA). Non-parametric tests including the Kruskal-Wallis analysis of variance (ANOVA) with Dunns corrections were used for multiple comparisons. P-values less than 0.05 were considered to be statistically significant.

3. Results

3.1 CAP255 lineage antibody intermediates from CAP255.G3 arm

The UCA heavy chain variable region originates from the recombination of the unmutated variable (V), diversity (D) and joining (J) germline gene fragments. Comparisons of the UCA heavy chain variable region to those of the lineage intermediates, and CAP255.G3, provides insights into which SHMs acquired through affinity maturation were important for the development of neutralisation breadth in this lineage, which was largely mediated by the heavy chain.

The heavy chain variable region sequence alignments of the UCA, the six antibody intermediates (Int): Int A (17 wpi), Int B (23 wpi), Int C (39 wpi), Int D (39 wpi), Int E (47 wpi), Int F (47 wpi), and the mature bNAb CAP255.G3, were all compared in their AA alignment configurations and the neutralisation sensitivity compared using the IC₅₀ values via TZM-bl neutralisation assays. The sequence alignments for these intermediates, also referred to as mAbs, are shown in figure 4. The CDRH3 between Int F (47 wpi) and CAP255.G3 share high sequence identity, whereas the CDRH1 sequence between the two mAbs differed substantially. During deep sequencing, a CDRH1 similar to that of CAP255.G3 was not recovered in any of the intermediates and this could account for the long terminal branch length of CAP255.G3 in the phylogenetic tree (Fig. 2).

	CDR-H1	CDR-H2
CAP255_UCA	QVQLQCWGAGLLKPSSETLSLTCAVYGGSFSGYYWSWIRQPPGKGLEWIGEINHSGSTNYPNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARG	
Int A (17 wpi)	S.....R.....	
Int B (23 wpi)	S.....K.....N.IS.....	
Int C (39 wpi)	A.L.D.....YR.....R.....N.TS.....	
Int D (39 wpi)	IXX.....N.TS.....	
Int E (47 wpi)	XX..A.....H.....A.....N.TS.....F..T.	
Int F (47 wpi)	R.....V...T...D.....Q.....YI.....T.....N.TS.....I...T.	
Chimera	R.....V.CQTIVYT.....Q.....YI.....T.....N.TS.....I...T.	
CAP255.G3	.IR.E.....R.....T...V.CQTIVYT.....S..Q.....I..A.....N.TS.....H.Y.....T.....T.	

	CDR-H3
CAP255_UCA	STIRYWGQYQLLPFGAFRFDYWGQGTILVTVSS
Int A (17 wpi)	.A.....P.....
Int B (23 wpi)	Q.S.....S.....
Int C (39 wpi)	P...E.SG.....E.....
Int D (39 wpi)	.M.....P...Q.ST.....E.....
Int E (47 wpi)	.L.....P...Q.SG.....EN.....
Int F (47 wpi)	.A.....P...Q.ST.....EF....H.....
Chimera	.A.....P...Q.ST.....EF....H.....
CAP255.G3	.E.....P...Q.ST.....QS.S..H.....

Figure 4. Amino acid sequence alignment of the heavy chain variable region for the CAP255 lineage UCA, mature CAP255.G3 bNAb and CAP255.G3 arm intermediates. Sequences were aligned with MAFFT with the CAP255 UCA as the reference sequence. The dots indicate the same amino acid as the UCA sequence. The CDR-H1, -H2 and -H3 are shown and the region of the CDR-H1 from CAP255.G3 that was cloned into the CDR-H1 chimera is denoted by the red dotted square.

3.2 Neutralisation of autologous pseudoviruses

Autologous or “self” viruses are those that drove affinity maturation of the CAP255.G3 bNAb lineage, as these are the only viruses that the immune system was exposed to. Therefore, by assessing neutralisation of autologous viruses by the various antibodies, we are able to track the affinity maturation path taken by this lineage arm, from the UCA to the CAP255.G3 bNAb. The subset of autologous PSVs were chosen as these were sensitive to CAP255.G3 plasma at 149 wpi and were representative of viral diversity at each time-point. The neutralising activity of the UCA, six intermediates, CDRH1 chimera and CAP255.G3 against selected autologous viruses from 17-51 wpi that were sensitive to CAP255.G3 are shown in figure 5. Neutralisation activity begins from Int D (39 wpi) in the early eight-week PSVs (A-8 wpi and C-8 wpi shown in blue) and only increases gradually from Int F (47 wpi) for all the other autologous PSVs (Fig. 5).

Neutralisation of the autologous PSVs was observed with Int D – F, however this was limited to only two of the early 8 wpi viruses (A-8 wpi and C-8 wpi) (Fig. 7A). Int F (47 wpi) had higher neutralisation activity for majority of the autologous PSVs, possibly due to the high sequence identity to CAP255.G3, particularly in the CDRH3. We observed that the neutralisation activity from 17 – 47 wpi in all the intermediates (Int A – F) were not significantly different to the UCA ($p > 0.05$). The CDRH1 chimera had greater neutralisation activity than Int F (47 wpi) and like CAP255.G3, was significantly different to the UCA ($p < 0.01$). However, the neutralisation activity of the CDRH1 chimera did not reach CAP255.G3 levels. The IC₅₀ median values of all the mAbs against the autologous PSVs are shown in figure 7C.

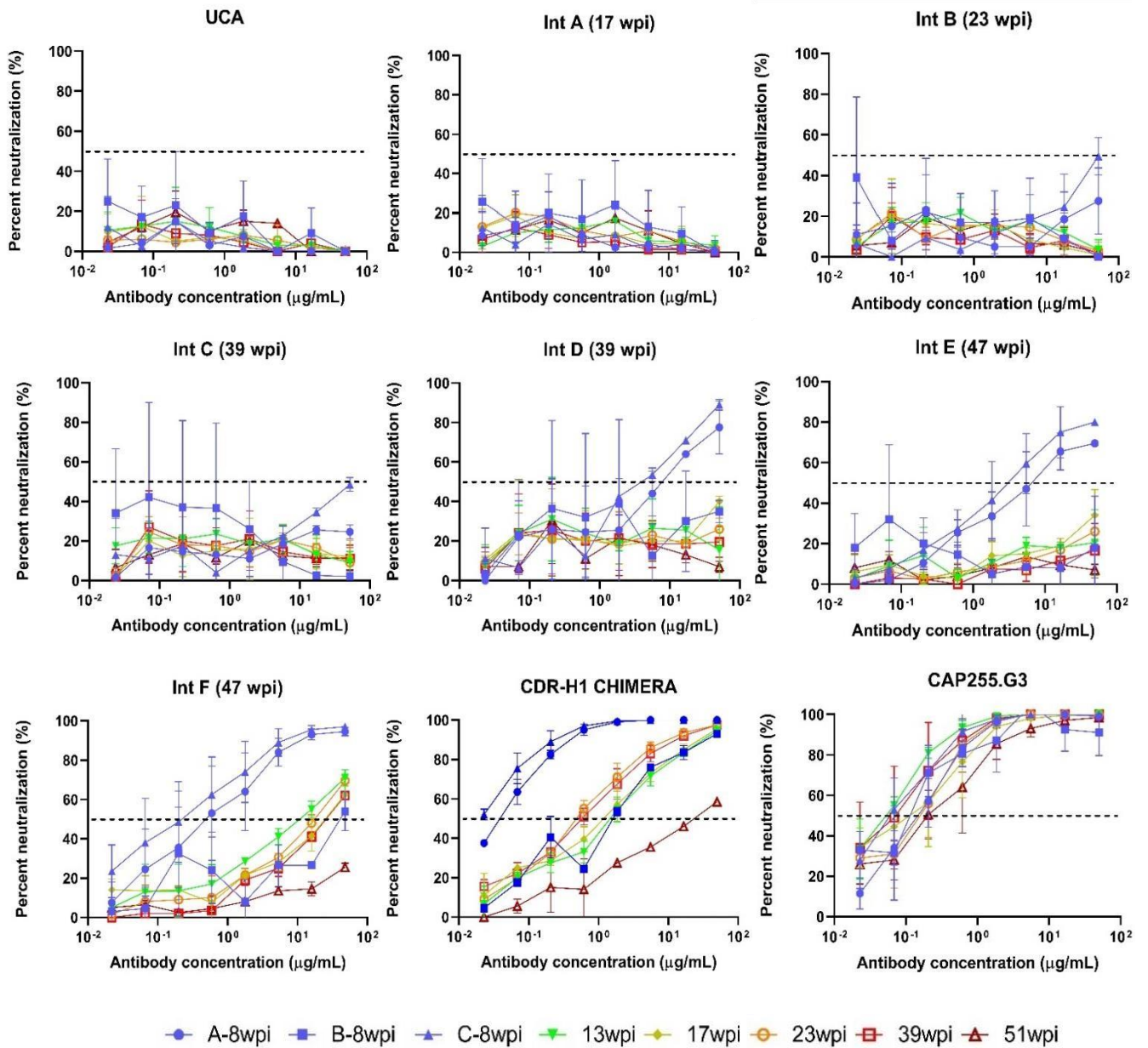


Figure 5. Neutralisation activity of members of the CAP255.G3 lineage arm against autologous PSVs. Neutralisation activity against the early eight-week PSVs is first observed with Int D (39 wpi), with increasing neutralisation activity against later viruses seen with Int F (47 wpi). Neutralisation activity improves against all autologous viruses in the CDR-H1 chimera, after introduction of the CDR-H1 from CAP255.G3 into Int F (47 wpi), but not to CAP255.G3 neutralisation levels.

3.3 Neutralisation of heterologous pseudoviruses

The neutralisation sensitivity of heterologous viruses to bNAb intermediates provides information about how SHMs and affinity maturation leads to the development of neutralisation breadth in the CAP255.G3 arm of the lineage. These heterologous viruses were specifically chosen because they were previously shown to be sensitive to neutralisation by CAP255 149 wpi plasma, which was the time point where CAP255.G3 was isolated. In addition, Gray *et al.* (2011) showed that plasma neutralization activity against these heterologous PSVs developed at different longitudinal time points between 49 and 149 wpi. This makes them ideal candidates for tracking the development of neutralisation breadth in the lineage intermediates. When analysing the neutralisation curves, the UCA had no detectable neutralisation against any heterologous PSVs. While there were no significant differences in neutralisation activity measured by their IC₅₀ median values between the UCA, Int A – F and the CDRH1 chimera ($p > 0.05$) (Fig. 6), there was a significant difference between the UCA and CAP255.G3 ($p < 0.05$). The lack of significant differences between the UCA and all the intermediates indicates the SHMs acquired by these mAbs were not responsible for the development of neutralisation breadth. Discriminating between CAP255.C5 and CAP255.G3 based on neutralisation activity is difficult given their highly overlapping neutralisation profiles (Fig. 3). However, the heterologous PSV, CE703010010.C4, was a virus that could only be neutralised by members of the CAP255.C5 lineage arm and not by members of the CAP255.G3 lineage arm. Therefore, we included this PSV as an internal control in our neutralisation assays to ensure the correct antibody intermediates were used.

Replacing the native Int F CDRH1 with the CAP255.G3 CDRH1 in the CDRH1 chimera, had limited and non-significant impact on neutralisation activity against the heterologous PSVs. However, when analysing the neutralisation curves we observed that Int A – E had no detectable neutralisation against all heterologous PSVs and Int F (47 wpi) had only limited neutralising activity against DU156.12 and TRO.11 PSVs (Fig. 6). The IC₅₀ median values of all the mAbs against the heterologous PSVs showed moderate neutralisation beginning in PSVs DU156.12 and TRO.11, which increased slightly in the CDRH1 chimera, while CAP255.G3 neutralised five of the six heterologous PSVs (Fig. 7C). The difference between the neutralisation breadth of Int F (47 wpi) and CAP255.G3 indicates that breadth developed in this arm of the lineage between 47 wpi and 149 wpi. Upon analysis of the phylogenetic tree, a long

evolutionary branch distance was observed between CAP255.G3 and all other sampled members of the lineage (Fig. 2). This suggests that there were more closely related antibodies to CAP255.G3, with potentially similar levels of breadth, but these have not been sampled through the sequencing conducted to date. Therefore, analysis of the sequence differences between Int F (47 wpi) and CAP255.G3, showed that some single nucleotide polymorphisms (SNPs) may be important for breadth and are shown in table 3. Major contributions involve changes from a polar uncharged, rigid side chain to a polar uncharged side chain (P40S), and from a polar uncharged side chain to a nonpolar, aromatic side chain (S79Y) (Table 3).

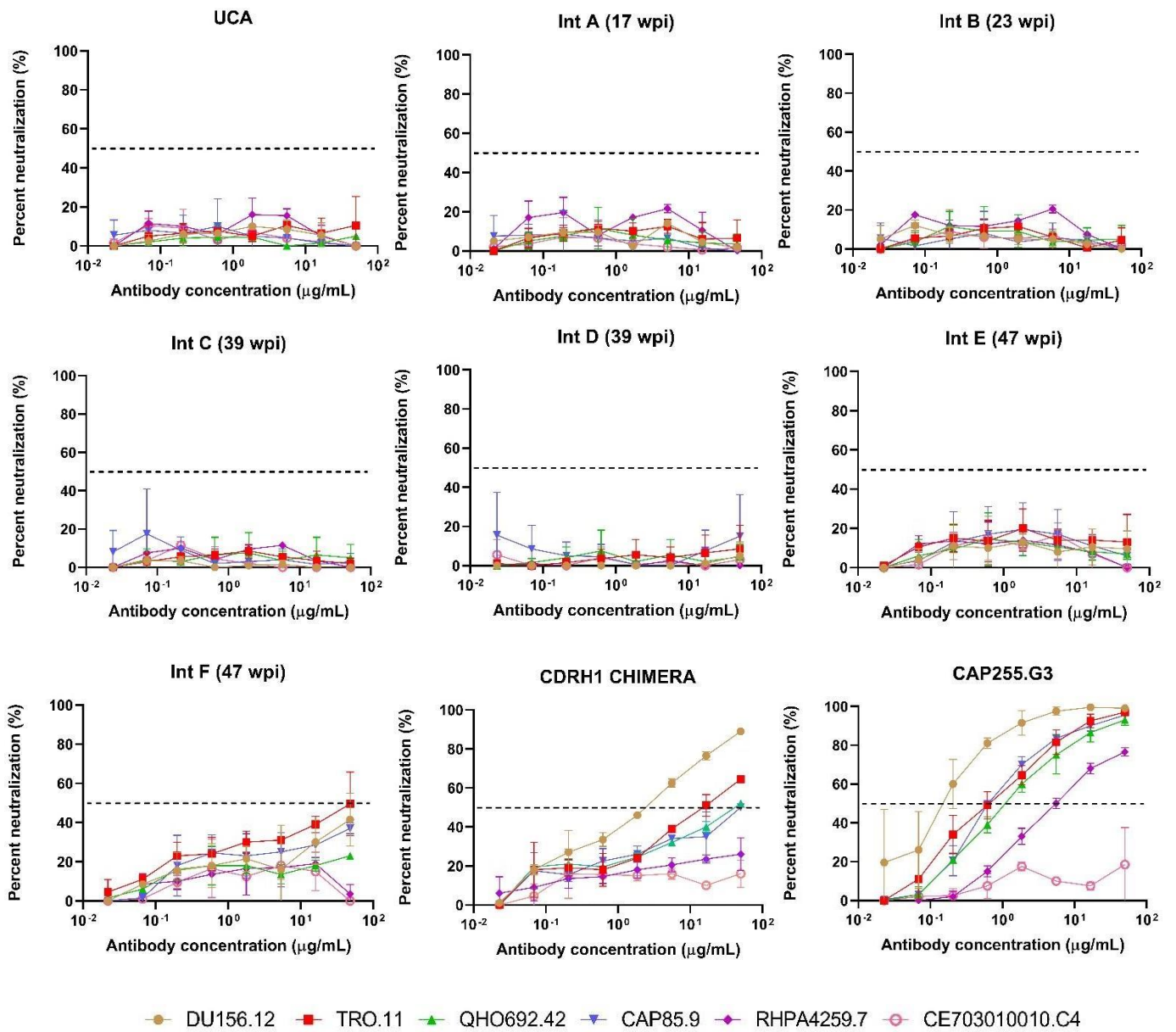


Figure 6. Neutralisation breadth of members of the CAP255.G3 lineage arm against the heterologous PSVs. Int A – E (17 – 47 wpi) have no detectable neutralising activity against any of the heterologous PSVs, whereas Int F (47 wpi) has limited neutralising activity against TRO.11, DU156.12 and CAP85.9. Neutralising activity against the rest of the heterologous PSVs increases after the introduction of the CDR-H1 from CAP255.G3 into Int F (47 wpi), but not to CAP255.G3 neutralisation levels.

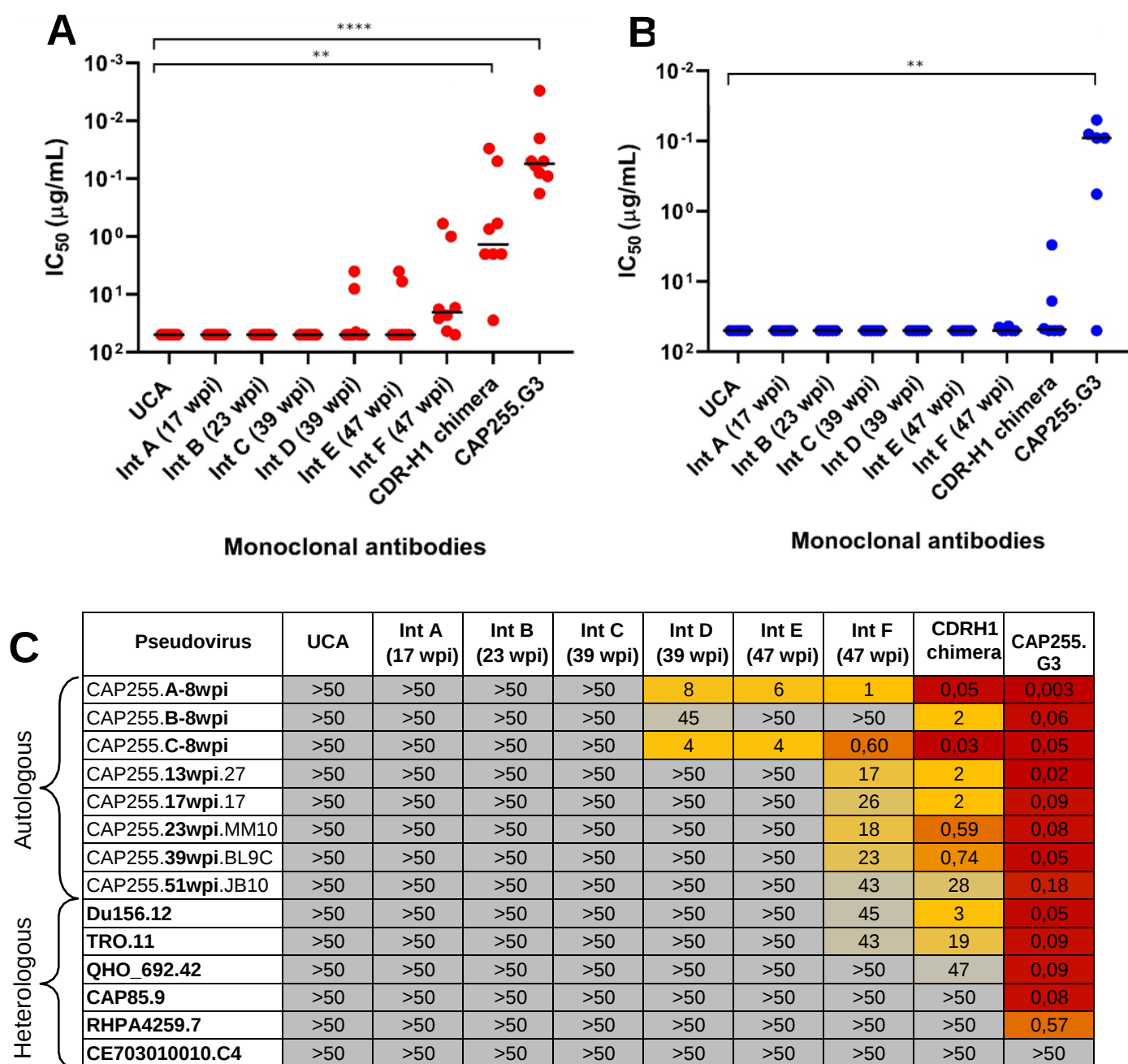


Figure 7. Neutralisation activity of the UCA, six lineage intermediates, CDR-H1 chimera and CAP255.G3 against the panel of 14 PSVs. (A) Antibodies plotted against their IC_{50} median values for autologous PSVs. Median IC_{50} values for each mAb is shown by the black bar. (B) Antibodies plotted against the IC_{50} values for heterologous PSVs. Median IC_{50} values for each mAb is shown by the black bar. (C) Neutralisation data showing the IC_{50} values of the mAbs against the autologous and heterologous PSVs, with warmer colours indicating greater neutralisation activity. All results are the mean of two independent experiments. Statistical analyses were performed using the Kruskal-Wallis test with Dunns correction for multiple comparisons, with ** denoting $p < 0.01$ and **** denoting $p < 0.0001$. Only comparisons where the median IC_{50} value of an antibody was significantly different to the UCA are shown.

Table 3. Table showing SNPs in CAP255.G3 compared to Int F (47 wpi) with the potential to have an impact on neutralisation activity and breadth. The biochemical differences between the residues in CAP255.G3 compared to Int F suggest that when these are knocked-in to Int F, there could be a significant change in function due to changes in paratope structure and/or biochemical nature. Position of SNPs numbered according to the international ImMunoGeneTics information system ® (IMGT®) antibody numbering system.

SNP		AA in Int F (47 wpi)		AA in CAP255.G3	Predicted impact on breadth
Q3R	Q	Polar, with an uncharged side chain	R	Polar basic, with a positively charged side chain	Moderate
Q5E	Q	Polar, with an uncharged side chain	E	Polar acidic, with a negatively charged side chain	Moderate
D31G	D	Polar acidic, with a negatively charged side chain	G	Nonpolar, with an aliphatic side chain	Large
P40S	P	Polar, with an uncharged rigid side chain	S	Polar, with an uncharged side chain	Small
T57A	T	Polar, with an uncharged side chain	A	Nonpolar, with an aliphatic side chain	Large
Q77H	Q	Polar, with an uncharged side chain	H	Nonpolar, with a positively charged side chain	Large
S79Y	S	Polar, with an uncharged side chain	Y	Nonpolar, with an aromatic side chain	Large
A100E	A	Nonpolar, with an aliphatic side chain	E	Polar acidic, with a negatively charged side chain	Large
E119Q	E	Polar acidic, with negatively charged side chain	Q	Polar, with an uncharged side chain	Moderate
F120S	F	Polar, with an aromatic side chain	S	Polar, with an uncharged side chain	Large
G122S	G	Nonpolar, with an aliphatic side chain	S	Polar, with an uncharged side chain	Large

4. Discussion

The HIV-1 pandemic has persisted for over 40 years and an HIV-1 vaccine remains a major priority to combat this virus. Investigating the development of bNAb lineages aids researchers in determining which SHMs are important in contributing to neutralisation breadth, and has the potential to inform vaccine design and immunization strategies (Jardine, Kulp, *et al.*, 2016). This process occurs via the mechanism of directing affinity maturation to positions on bNAbs that are essential for neutralisation breadth and potency, however, bNAbs have not been elicited in animal or human studies to date (Jardine, Kulp, *et al.*, 2016; Leggat *et al.*, 2022). The aim of this study was to delineate the development of an N332-directed anti-HIV-1 bNAb lineage from participant CAP255, whose plasma was previously screened for breadth (Gray *et al.*, 2011). Two bNAbs were previously isolated from CAP255, namely CAP255.C5 and CAP255.G3. This project focused on the ontogeny of the CAP255.G3 arm, where the heavy chain was mostly responsible for mediating neutralisation breadth (Kitchin *et al.*, *unpublished data*). The antibody intermediates that were selected from longitudinal deep sequencing possessed somatic mutations that were present in later bNAb lineage members. This indicates that these antibodies were potentially important intermediates for the development of neutralisation breadth in this clonal lineage.

The antibody intermediates (Int A – F) and mature bNAb CAP255.G3 were compared to the UCA, which contained no SHMs. The intermediates were assayed against a panel of autologous and heterologous PSVs and the IC₅₀ median values between each mAb-PSV combination were compared. No neutralisation activity was observed in the UCA and Int A – C against the panel of autologous PSVs (Fig. 5) and the UCA and Int A – E against the panel of heterologous PSVs (Fig. 6). The median IC₅₀ values of Int A – F against the entire panel of autologous and heterologous PSVs were all non-significantly different to the UCA ($p > 0.05$). Significant differences were only observed between the median IC₅₀ values of the UCA and CAP255.G3 against the autologous and heterologous panels of PSVs ($p < 0.05$) (Fig. 7A and B). Although the neutralisation activity of the intermediates was not significantly different to the UCA, an increase in neutralisation activity was observed from Int D – F against the autologous PSVs (Fig. 5), and from Int F (47 wpi) against the heterologous PSVs (Fig. 6).

Previous investigations of the ontogeny of DH270, a V3-glycan bNAb lineage, showed that the DH270.UCA did not neutralise autologous or heterologous HIV-1 viruses (Bonsignori *et al.*, 2017), consistent with the results from our study. In addition, Bonsignori *et al.* (2017), found that a five AA substitution, four residues in the heavy chain and one residue in the light chain, resulted in heterologous virus neutralisation (Bonsignori *et al.*, 2017). In our study, the differences in median IC₅₀ values showed non-significance between all the intermediates and the UCA, however, the neutralisation curves illustrate initiation of neutralisation activity of autologous PSVs by Int D (39 wpi), and of heterologous PSVs by Int F (47 wpi), possibly due to accumulated mutations present at 39 and 47 wpi. Although mutations accumulate through many rounds of SHM in the intermediates, they may not always impact autologous or heterologous neutralisation. Previous studies have shown that low probability mutations, those that occur where AID activity is rare, and high probability mutations, where AID activity is frequent, accumulate through several rounds of SHM (Wiehe *et al.*, 2018). Their findings showed that some of the improbable mutations were more important in contributing to neutralisation, than the probable mutations (Wiehe *et al.*, 2018). In addition, Sacks *et al.* (2019), compared “off-track” antibodies to bNAbs and found similar percentages of SHM between the “off-track” antibodies and bNAbs, but the bNAbs’ breadth and potency was augmented when compared to the “off-track” antibodies (Sacks *et al.*, 2019). Collectively, this shows that neither the accumulated mutations present in a sequence, nor high sequence identity between antibodies can be attributed to neutralisation capacity, and this needs to be elucidated via neutralisation assays.

The CAP255.G3 bNAb contains 32 differences in AA composition compared to the UCA. However, as mentioned, it is unlikely that all of these mutations were crucial for affinity maturation against the evolving autologous viruses. A previous study utilized a “reductionist” vaccine design approach, where minimally mutated versions of VRC01-class bNAbs were identified, and elicited neutralisation breadth similar to bNAbs containing all mutations (Jardine, Sok, *et al.*, 2016). Additionally, they studied the crystal structure of the bNAbs interacting with the HIV Env trimer to determine the location of SHMs present and if the mutations were distal or proximal to the epitope and ultimately, necessary for neutralisation capacity (Jardine, Sok, *et al.*, 2016). The aim of the present study was to elucidate which specific SHMs were critical for the development of neutralisation breadth in the CAP255.G3 lineage arm.

Int F (47 wpi) was the closest intermediate to CAP255.G3, as shown in the phylogenetic tree (Fig. 2). The long terminal branch length of CAP255.G3 on the phylogenetic tree indicates significant unsampled SHMs between this antibody and the lineage intermediates sampled through sequencing. This indicates that continued affinity maturation between Int F (47 wpi) and CAP255.G3 (149 wpi) resulted in increased neutralisation activity and breadth in this lineage arm. No members of the CAP255.G3 arm were identified between 47 and 149 wpi in the sequencing data, which makes it difficult to determine the specific SHMs important for neutralisation breadth. Int F (47 wpi) differs from CAP255.G3 by 24 AAs and has high sequence identity to CAP255.G3 in the CDRH3. Although Int F (47 wpi) had greater neutralisation activity against the entire PSV panel compared to the earlier intermediates, it does not reach the neutralisation levels of CAP255.G3. Therefore, we aimed to determine which of the 24 AA mutations between Int F (47 wpi) and CAP255.G3 were responsible for the differences in neutralisation activity.

The CDRs of bNAbs have proven to be important for contributing to neutralisation activity, specifically long CDRH3s (Ill *et al.*, 1997; Doria-Rose and Landais, 2019). Therefore, we analysed the CDRs between Int F (47 wpi) and CAP255.G3 and found a seven AA difference in CDRH1, a two AA difference in CDRH2 and a three AA difference in CDRH3 (Fig. 4). Although we did not test the effects of CDRH2 or CDRH3 of CAP255.G3 in isolation, we hypothesized the high sequence identity in these two regions did not contribute to differences in neutralisation and therefore, due to time constraints, we investigated the CDRH1 of CAP255.G3 in isolation. Through the use of recombinant DNA techniques, we replaced six of the seven residues from the CDRH1 of Int F (47 wpi) with those in the CDRH1 of CAP255.G3, which led to the development of the CDRH1 chimera. The omitted residue was a change from AA serine to threonine, where both residues have polar, uncharged side chains and was considered trivial for contributing to neutralisation activity. Thereafter, effect of the CDRH1 chimera against the autologous and heterologous PSVs was assessed in further neutralisation assays.

The CDRH1 chimera showed a moderate increase in neutralisation activity against the autologous PSVs, relative to Int F (47 wpi), but not to CAP255.G3 levels (Fig. 5). Additionally, similar to CAP255.G3, the neutralising activity of the CDRH1 chimera was significantly different to the UCA against the autologous PSVs ($p < 0.05$) (Fig. 7A). The

CDRH1 chimera also had increased neutralisation activity against the heterologous PSVs, relative to Int F (47 wpi) (Fig. 6), indicating affinity maturation in this region contributed to neutralisation breadth. Although the CDRH1 from CAP255.G3 contributed to the development of limited neutralisation breadth, there are additional mutations present in CAP255.G3 that are potentially essential for enhanced neutralisation of the autologous and heterologous PSVs. The SHMs present in the CDRs of bNAbs are generally considered more important than those in the FWRs as they often contribute to neutralisation more than randomly positioned residues (Ill *et al.*, 1997). This is due to the CDR's primary function of binding to the antigen and the FWRs supporting this antibody-antigen interaction (Ill *et al.*, 1997). A previous study investigated a CAP256 sub-lineage which contained high sequence identity antibodies with both broad and narrow neutralisation breadth (Bhiman *et al.*, 2015). This study inferred that individual changes of residues, rather than the sequence identity, had effects on neutralisation breadth (Bhiman *et al.*, 2015). In our study, the CDRH1 of CAP255.G3 contributed moderate neutralisation breadth in this lineage arm. However, future studies could assess whether SHMs in the CDRH2 and CDRH3 regions contributed to the development of neutralisation breadth in the CAP255.G3 arm of the lineage.

A previous study showed that N332-directed bNAbs containing no indels and low SHM were able to achieve broad neutralisation (MacLeod *et al.*, 2016). Another study revealed that an N332-directed bNAb isolated from a paediatric donor, BF520.1, displayed breadth and potency with 3% SHM, but after removal of irrelevant mutations, only 2% SHM was required for the functionality of this bNAb (Simonich *et al.*, 2019). In our study, the heavy chain of CAP255.G3 has 19.7% SHM with 55% neutralisation breadth and median potency of 0.96 µg/mL (IC₅₀) against a panel of 208 PSVs (Kitchin *et al.*, unpublished data). Previous studies of bNAbs suggest that long CDRH3s were described to be important for neutralisation in bNAbs (Ill *et al.*, 1997; Doria-Rose and Landais, 2019), however, our study shows that CDRH1 is important for neutralisation activity. A reinforcing finding from previous literature that coincides with the results of our study is that not all neutralisation breadth stems from SHMs present in the CDRH3 of bNAb lineages (Doores *et al.*, 2014). Our study showed the SHMs present in the CDRH1 of CAP255.G3 resulted in neutralisation activity greater than Int F (47 wpi) against the entire panel of PSVs. This information highlights the variations in N332-directed bNAb characteristics necessary to achieve neutralisation breadth and

highlights that not all mutations are present in the CDRH3s. The sequence analysis of CDRH1, CDRH2 and CDRH3 in CAP255.G3 compared to the antibody intermediates revealed that the largest sequence difference between CAP255.G3 compared to the intermediates was found in the CDRH1. However, it would be beneficial to test the effects of the SHMs in the CDRH2 and CDRH3 on neutralisation breadth, and these should be assessed in future studies.

Establishing the structures of V3-glycan-directed bNAbs bound to their epitope has been valuable for revealing which regions of the bNAb mediate breadth (Garces *et al.*, 2014; Barnes *et al.*, 2018). A beneficial path of investigation would be to determine the crystal structure of CAP255.G3 via X-ray crystallography and EM as implemented by previous studies (Doores *et al.*, 2014; Garces *et al.*, 2015; Barnes *et al.*, 2018). This structure would provide insights to determine the reason that the CDRH1 from CAP255.G3 was important for neutralisation activity against the autologous PSVs and the limited breadth against the heterologous PSVs. There could be an interaction between the CDRH1 and the epitope on autologous PSVs that may be absent in heterologous PSVs or interactions with surrounding glycans that require examination of the structure *in silico*. Gaining further insights into the crystal structure of CAP255.G3 bound to its epitope will assist in elucidating the mechanism of binding, and will contribute to determining shared features with other N332-directed bNAbs. This is essential for vaccine design as immunogens can be constructed to elicit bNAbs with shared characteristics.

5. Conclusion

Investigating the development of a bNAb lineage provides insights into which SHMs present in the bNAb are necessary for neutralisation breadth. In the N332-directed CAP255.G3 lineage arm, the IC₅₀ median values for all antibody intermediates were not significantly different to the UCA, while we observed a significant increase in neutralisation for CAP255.G3 in comparison to the UCA across the panel of autologous and heterologous PSVs. However, an increase in neutralisation activity and breadth was observed from Int F (47 wpi) against the entire panel of PSVs. Since the 47-week intermediates' neutralisation activity was attenuated compared to CAP255.G3, we hypothesized that there were clonally related members between 47 and 149 wpi that were different to the last isolated intermediate that may have had incremental neutralisation activity and breadth with additional SHMs absent at 47 wpi. After analysis of the AA sequence alignments, the CDRH1 of CAP255.G3 contained SHMs that were absent in Int F (47 wpi) and was hypothesized to contribute to neutralisation capacity. The CDRH1 chimera was developed and included the CDRH1 of CAP255.G3 and the Int F (47 wpi) backbone, and contributed moderately to affinity maturation against the autologous PSVs with limited contribution to the development of breadth against the heterologous PSVs. The CDRH3 between CAP255.G3 and Int F (47 wpi) had high sequence identity, with only a three AA difference between these regions, therefore it is unlikely that the CDRH3 contributed to differences in neutralisation activity between these antibodies. However, future studies could identify the SHMs in other CDRs important for breadth by progressively adding mutations present in other CDRs to the CDRH1 chimera. Adding all three heavy-chain CDRs to Int F, instead of specific mutations in the CDRs, would essentially result in production of the CAP255.G3 heavy chain sequence, hence this was not performed in this study. In conclusion, delineating the development of bNAbs provides information that can be utilized in the design of priming and boosting vaccine immunogens, and supplies a plethora of information relating to the levels of SHMs required, including shared features present in N332-directed bNAbs.

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APPENDIX A: CAPRISA permission letter



Generating Knowledge - Impacting Health

Doris Duke Medical Research Institute (2nd floor), 719 Umbilo Road, Private Bag X7, Congella, 4013, Durban, South Africa tel: +27 31 2604555 | fax: +27 31 2604549 | email: caprisa@caprisa.org | www.caprisa.org

5 October 2021

Prof Clem Penny
Chair: Human Research Ethics Committee (Medical)
Phillip V Tobias Health Sciences Building
29 Princess of Wales Terrace
Parktown
Johannesburg
South Africa
2193

Dear Prof Penny,

Re: Thamara Naidoo - MSc Vaccinology study "Delineating the ontogeny of an N332-directed anti-HIV1 broadly neutralising antibody lineage"

In my role as Head of HIV Pathogenesis and Vaccine Research at the CAPRISA and Co-Chair of the CAPRISA 002 Acute Infection Study, I write to confirm that Ms Thamara Naidoo will have access to stored samples from the CAPRISA 002 cohort to carry out the above study for her MSc through the University of the Witwatersrand.

Participants in the CAPRISA 002 study have consented to stored specimens being used for additional studies. The CAPRISA 002 BREC approved Specimen Storage Consent (V4.02) attached here, states that: *"We may test your cells, proteins, other chemicals in your body and genes (DNA). Some of the samples will also be tested to see how your nutritional status may be interacting with HIV infection. Your samples may be analysed in laboratories outside of South Africa"*.

Ms Thamara Naidoo's study will be supervised by Prof Penny Moore and Dr Dale Kitchin and represents an extension of our successful collaboration with Penny's laboratory at the National Institute for Communicable Diseases, Johannesburg.

Please do not hesitate to contact me if there are any queries.

Yours sincerely,

Dr Nigel Garrett

MBBS, MRCP, MSc, PhD
Head of Pathogenesis and Vaccine Research, CAPRISA
Honorary Lecturer in Public Health, University of KwaZulu-Natal
2nd Floor, Doris Duke Medical Research Institute (Private Bag X7)
719 Umbilo Road, Congella, Durban 4013, South Africa
T: +27 (0)31 655 0617 (ECSR) 031 260 4453 (DDMRI) C: +27 (0)76 330 8300
E: nigel.garrett@caprisa.org

Board of Control: B Ntuli (Chair) □ M Rajab (Deputy Chair) □ Q Abdool Karim □ SS Abdool Karim □ AC Bawa □ JH Beare □ JM Frantz □ LP Fried (US) □ ST Harrison
□ TL Jones □ ARDH Moosa □ K Naidoo □ A Nortier □ HW Sherwin □ LV Theron



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APPENDIX B: Human Research Ethics Committee (HREC) letter for blanket ethics

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG	HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
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2022/11/11

Professor P Moore, et al

Centre for HIV & Sexually Transmitted

Infections National Institute for

Communicable Diseases

1 Modderfontein Road

Sandringham

2031

Sent by e-mail to: carolc@nicd.acza

Dear Dr Crowther

Re: Protocol Ref No: M210892

Protocol Title: Viral set point and clinical disease progression: The role of immunological, genetic and viral factors over the course of disease and during antiretroviral therapy

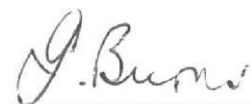
Principal Investigators: Professor P Moore, et al

Thank you for your letter of 2022/11/07.

We note and approve of your progress report and the impressive list of publications. As such, your current ethics clearance remains valid until 2026/09/06, subject to continued satisfactory progress.

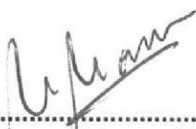
Thank you for keeping us
informed.

Yours Sincerely



Mr I Burns

For the Human Research Ethics Committee (Medical)



Dr M. Nkomo, Co-Chairperson, Human Research Ethics

Dr N Naran, Co-Chairperson, Human Research Ethics Committee (Medical)

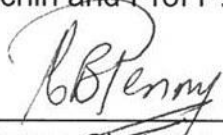
APPENDIX C: HREC certificate

UNIVERSITY OF THE
WITWATERSRAND,
JOHANNESBURG



R14/49 Miss Thamara Naidoo

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL) CLEARANCE CERTIFICATE NO. M211070

NAME: Miss Thamara Naidoo
(Principal Investigator)
DEPARTMENT: Pathology
National Institute for Communicable Diseases (NICD)
PROJECT TITLE: Delineating the ontogeny of an N332-directed anti-HIV-1 broadly neutralizing antibody lineage
DATE CONSIDERED: 29/10/2021
DECISION: Approved unconditionally
CONDITIONS:
SUPERVISOR: Dr D. Kitchin and Prof P. Moore
APPROVED BY: 
Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL: 21/01/2022

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary on the Third Floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report.** The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in **October** and will therefore be due in the month of **October** each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).


Principal Investigator Signature

03/02/2023
Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

APPENDIX D: Plagiarism declaration



PLAGIARISM DECLARATION TO BE SIGNED BY ALL HIGHER DEGREE STUDENTS

I, Thamara Naidoo (Student number: 1317469), am a student registered for the degree of MSc (Med) Vaccinology in the academic year 2023.

I hereby declare the following:

- I am aware that plagiarism (the use of someone else's work without their permission and/or without acknowledging the original source) is wrong.
- I confirm that the work submitted for assessment for the above degree is my own unaided work except where I have explicitly indicated otherwise.
- I have followed the required conventions in referencing the thoughts and ideas of others.
- I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this is not my own unaided work or that I have failed to acknowledge the source of the ideas or words in my writing.
- I have included as an appendix a report from "Turnitin" (or other approved plagiarism detection) software indicating the level of plagiarism in my research document.

Signature:

A handwritten signature in black ink, appearing to read 'Thamara Naidoo', is written over a light blue grid background.

Date: 25/05/2023

APPENDIX E: Turn it in report

ThamaraNaidoo_1317469_MScFinal_20230210.docx

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Fetzer, Ina. "Towards More Potent and More Stable Antibody-Like HIV-1 Entry Inhibitors.", The Scripps Research Institute, 2020

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"Abstracts of the HIV Research for Prevention Meeting, HIVR4P, 17-20 October, 2016, Chicago, USA", AIDS Research and Human Retroviruses, 2016

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Joshi, Vinita. "Delineating HIV-1 Evolutionary Pathways in an Individual with a Broadly Neutralizing Antibody Response", Harvard University, 2020

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