Effect of HIV status and suppression on SARS-CoV-2 disease severity, vaccine response, and evolution

Farina Karim

Supervisor: Prof. Alex Sigal
Co-supervisor: Prof. Yunus Moosa

Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy (Medical Microbiology) in the School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal

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Declaration

I, Ms Farina Karim, declare as follows:

(i) The research proposed in this dissertation, except where otherwise indicated, is my original work.

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(v) Part of this thesis was published in a publication titled: "*HIV status alters disease severity and immune cell responses in Beta variant SARS-CoV-2 infection wave*" with me as the primary author.

(vi) Part of this thesis has been submitted to medRxiv titled: *"Persistent SARS-CoV-2 infection and intra-host evolution in association with advanced HIV infection"*, with me as the primary author. Iven the movement in this field, we decided to keep this paper as a pre-print as it was the first report on persistent intra-host evolution in an individual with advanced HIV infection.

(vii) Part of this thesis has been submitted to a journal for publication titled: "*Variable SARS-CoV-2 evolution and vaccine response in individuals with advanced HIV disease"*, with me as the primary author.

(viii) This dissertation does not contain text, graphics or tables copied and pasted from the internet, unless specifically acknowledged, and the

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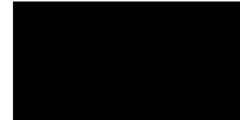
Signed: 

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List of Acronyms

AIDS	Acquired immune deficiency syndrome
ART	Antiretroviral therapy
CD4	Clusters of differentiation 4
CDC	Centers for Disease Control and Prevention
COVID-19	Coronavirus Disease 2019
CTL	Cytotoxic T cells
EFV	Efavirenz
FTC/3TC	Emtricitabine
HCoV	Human Coronavirus
HIV	Human Immunodeficiency virus
ICU	Intensive Care Unit
LVNA	Live virus neutralization assay
MERS-CoV	Middle East respiratory syndrome–related coronavirus
mAb	Monoclonal antibody
mRNA	Messenger RNA
NIH	National Institutes of Health
NRTIs	Nucleoside/nucleotide Reverse Transcriptase Inhibitors
nsp	Non-structural protein
ORF1a	Open reading frame 1a
ORF1b	Open reading frame 1b
PLWH	People living with HIV
PRNT50	Plaque reduction neutralization titre
RBD	Receptor-binding domain
RTC	Replication/transcription complex
SARS- CoV	Severe acute respiratory syndrome coronavirus
SARS- CoV-2	Severe acute respiratory syndrome coronavirus-2

STATS SA	Statistics South Africa
TDF	Tenofovir
VL	Viral load
VOC	Variant of concern
VOI	Variant of Interest
WHO	World Health Organisation

Abstract

SARS-CoV-2 is a global pandemic that has infected 672,115,430 people globally (https://coronavirus.jhu.edu/map.html, accessed 08.02.2023). SARS-CoV-2 is continuously evolving, and new variants pose a continuous threat to curbing this pandemic. Simultaneously, South Africa still struggles to control and manage an enduring HIV pandemic. The synergistic interplay between these two pandemics has necessitated an understanding of how these two viruses interact with each other to tailor an intervention. This thesis investigated the effect of SARS-CoV-2 infection on disease dynamics, differential disease outcomes, vaccine response, as well as SARS-CoV-2 evolution in people living with HIV (PLWH) with different levels of HIV suppression and differing HIV suppression history.

The first study investigated the difference in disease severity amongst PLWH in the first and second infection waves in South Africa. COVID-19. Thereafter, we explored persistent SARS-CoV-2 infection in immunocompromised individuals and intra-host evolution in a case study of a participant with advanced HIV infection. Here, we illustrated that advanced HIV disease may lead to prolonged SARS-CoV-2 infection and shedding of infectious virus and results in intra-host evolution of variant mutations, making intra-host evolution in advanced HIV individuals a particular concern within the South African context. Finally, we observed that effectively controlling HIV through ART facilitates SARS-CoV-2 clearance. The last study widened the observations to five participants with advanced HIV disease and showed that vaccination does induce a potent neutralizing antibody response in this group, but only if HIV viremia is first effectively suppressed with antiretroviral therapy. These findings highlight the importance of suppressing HIV infection in eliciting an effective immune response against, and preventing evolution of SARS-CoV-2.

CHAPTER 1:

Literature Review

1.1. Coronavirus Disease 2019 (COVID-19)

Severe Acute Respiratory Syndrome Coronavirus 2 is in the same family of viruses as SARS-associated coronavirus (SARS-CoV), first identified in February 2003 in Guangdong, China. Between November 2002 and July 2003, SARS-CoV rapidly spread to 29 countries, exhibiting a fatality rate of 9.6% (Kirtipal, Bharadwaj, & Kang, 2020; Machhi et al., 2020). In 2012, following the emergence of SARS-CoV, Middle East respiratory syndrome–related coronavirus (MERS-CoV) was a novel coronavirus first identified in Jeddah, Saudi Arabia and spread to 27 countries with a fatality rate higher than SARS-CoV at 34.4% (Kirtipal et al., 2020; Machhi et al., 2020).

SARS-CoV-2 disease (COVID-19) was first reported on the 31st of December 2019 by the World Health Organisation (WHO) after a cluster of cases presenting with pneumonia-like symptoms were reported in Wuhan City, China. Since the first reported case from Wuhan, SARS- CoV-2 rapidly spread across the world. The first report of COVID-19 in Africa stemmed from Egypt on the 14th February 2020 (https://www.afro.who.int/news/COVID-19-cases-top-10-000-africa, accessed 14.01.2023). The first COVID-19 case identified in South Africa (https://www.nicd.ac.za/first-case-of-covid-19-coronavirus-reported-in-sa/, accessed 14.11.2022) was on the 11th March, 2020 in KwaZulu Natal. The WHO declared SARS-CoV-2, coronavirus disease (COVID-19) a global pandemic on the 11th March 2020 (Anka et al., 2021; Kanwugu & Adadi, 2021).

As of the 8th of February 2023 there have been a total of 672,115,430 people infected globally (https://coronavirus.jhu.edu/map.html, accessed 08.02.2023) COVID-19 (Fig.1) (WHO, 2023), and a total of 6,847,160 attributable deaths. While Africa seems less affected (Fig.1, Fig. 2), logistical and reporting challenges, especially outside of Southern Africa may have led to an underestimate of the COVID-19 burden in this region (Fig. 2).

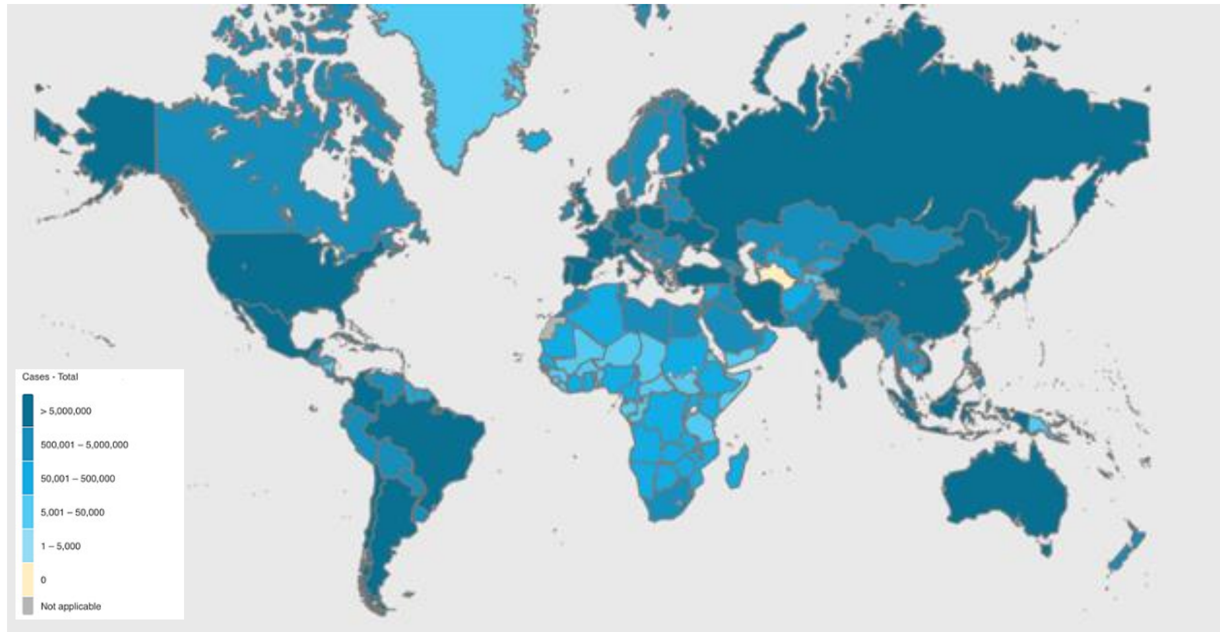

Fig. 1. *World Health Organisation. (2023). COVID-19 (WHO African region) (EPR/HIR). Accessed January 17, 2023, from:* *https://who.maps.arcgis.com/apps/dashboards/0c9b3a8b68d0437a8cf28581e9c063a9*

1.2. COVID-19 in South Africa

On the 30th of January 2020, the South African Ministry of Health established an incident management team in preparation for an anticipated outbreak and local spread of COVID-19. Incident management which prescribes the coordination and mobilisation of resources in response to COVID-19 in South Africa was modelled on WHO's Framework for a Public Health Emergency Operations Centre (WHO, Framework for a Public Health Emergency, 2015). On the 15th of March 2020 a National COVID-19 Command and Control Council (NCCC) was established by the national Cabinet for intergovernmental coordination tasked to take government-wide decisions in response to the COVID-19 pandemic in South Africa (Moonasar D, Pillay A, Leonard E, et al. 2021). With an Incident Management System and Team (IMT) in place from January 2020, together with the NCCC, South Africa made plans to contain the spread of COVID-19 to prevent over-burdening the healthcare system.

This urgent response was fuelled by fears that due to poor access to healthcare, ineffective ambulance systems, and high prevalence of vulnerable populations in South Africa (HIV positive, ART naïve, TB infected) COVID-19 (Abdool Karim, 2020).

On the 15th March 2020, South Africa declared a national state of disaster to prevent the spread of COVID-19 the South African government implemented a national, tiered level of lockdowns including closure of schools and strict travel restrictions. There were four proposed stages to South Africa's response, namely, Preparation (education, surveillance and logistical considerations making space for lab capacity); Primary prevention (social distancing, hand-washing, reduced social gathering volumes, closing of borders and international travelling), Lockdowns to curtail human interaction further, and Surveillance and case-finding (door to door screening, testing, isolation) (Abdool Karim, 2020).

Lockdowns are not unique to South Africa. Numerous other countries also implemented various types of lockdowns to limit the movement and interaction of people across the globe (Giovanetti et al., 2021) to control the transmission of SARS-CoV-2, and prevent the spread of endemic variants (Mohammed, Blebil, Dujaili, & Rasool-Hassan, 2020). They further aimed to prevent further and unnecessary transmission between persons that could result in the virus mutating and creating new variants (Mohammed, Blebil et al. 2020). South Africa's lockdown was one of the strictest lockdowns in the world and served as a temporary measure to slow down transmission and buy time for healthcare facilities to prepare for the pandemic (Campbell et al., 2022). Despite the Lockdown having shown to be effective in curbing the spread of SARS-CoV-2 transmission and keeping community transmission low (Abdool Karim, 2020) it did have negative implications for the South African economy. The efficacy of the lockdown in slowing the spread of the disease has positive consequences for

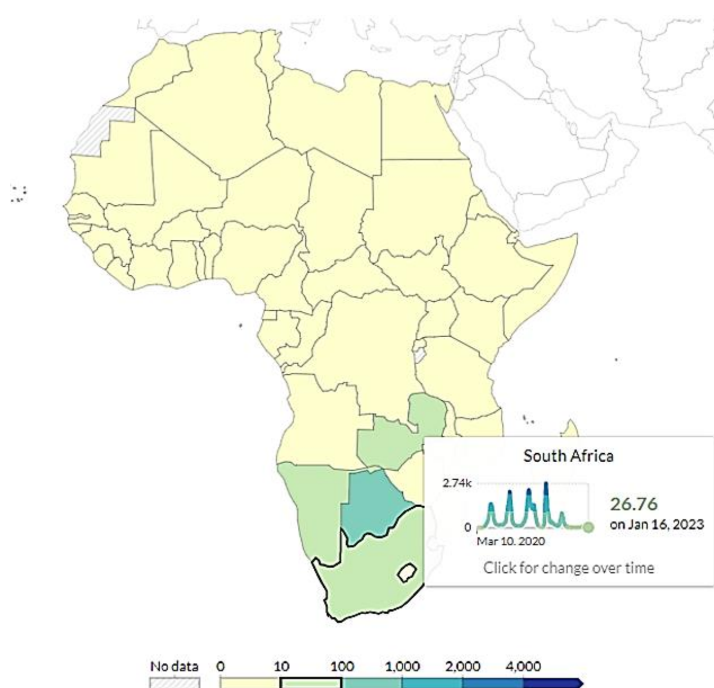

Fig. 2. *Weekly confirmed COVID-19 cases per million people in South Africa, Jan 16th, 2023. Source* https://ourworldindata.org/grapher/weekly-covid-cases-per-million-people?region=Africa&country=~ZAF

minimising the mutation and viral quasispecies of RNA viruses. However, with health resources been focused on the COVID-19 pandemic, HIV care and treatment programmes have been negatively affected by the COVID-19 pandemic (Kanwugu & Adadi, 2021).

1.3. The SARS-CoV-2 virus

Coronaviruses constitute the largest group within the *Nidovirales* (Fig. 3) which are a positive strand RNA virus infecting both vertebrates and invertebrates. Coronaviruses belong to the *Coronaviridae* family, and are zoonotic in origin (P. Masters & Perlman, 2013). As part of the *Coronaviridae* family, genomically and phylogenetically, Coronavirus is constituted by four genera: *Alphacoronavirus, Betacoronavirus, Gammacoronavirus,* and *Deltacoronavirus* (Cui, Li, & Shi, 2019).

Alphacoronaviruses and *Betacoronaviruses* infect only mammals, including humans, whereas *Gammacoronaviruses* and *Deltacoronaviruses* infect mainly warm-blooded vertebrates under the class Aves (Cui et al., 2019; Kirtipal et al., 2020; Leao et al., 2022; Machhi et al., 2020; P. Masters & Perlman, 2013; Wertheim, Chu, Peiris, Kosakovsky Pond, & Poon, 2013; Woo, Huang, Lau, & Yuen, 2010). *Betacoronavirus* family members include severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV), both from zoonotic origins. The SARS-CoV-2 pandemic marked the third zoonotic introduction of what turned out to be a highly pathogenic coronavirus to humans (V'Kovski, Kratzel, Steiner, Stalder, & Thiel, 2021). Bats are said to be natural hosts for both viruses (W. Li et al., 2005), and intermediate hosts facilitate the crossing of viruses between animals and human and can include civet cats and dromedary camels (Wertheim et al., 2013). They are mostly characterised by respiratory illness in humans and gastroenteritis in animals. Human Coronavirus (HCoV) infections can range from the common cold to severe acute respiratory syndrome depending on age and underlying co-morbidities.

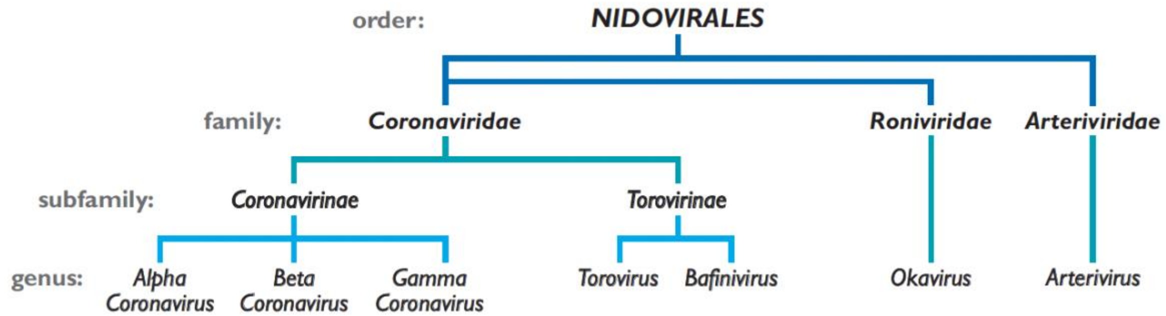

Fig. 3. *Taxonomy of the order Nidovirales (P. Masters & Perlman, 2013)*

1.4. The structure of SARS-CoV-2

Coronaviruses (Fig. 3) are large, enveloped RNA viruses consisting of a ~30kb strand of positive sense RNA genome, with 14 open reading frames (ORFs), encoding 29 viral proteins. They are known to have the largest genome among all RNA viruses (Kirtipal et al., 2020; Lu et al., 2020; Machhi et al., 2020; P. S. Masters, 2006; W. Yang & J. Shaman, 2022; W. Yang & J. L. Shaman, 2022). SARS-CoV-2 consists of four structural proteins: nucleocapsid (N) protein, envelope (E) protein, membrane (M) protein and spike (S) protein, and sixteen non-structural (nsp) proteins (Fig. 4).

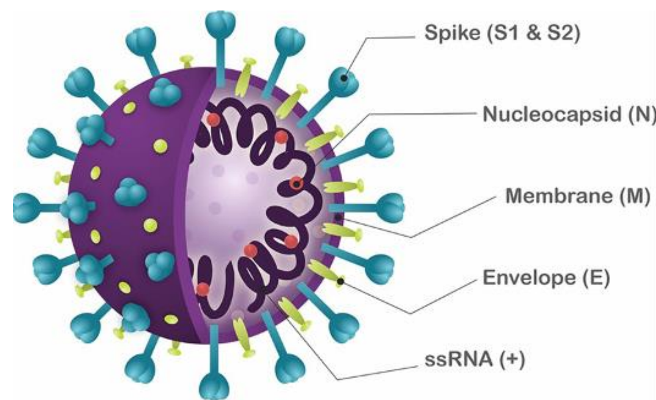

Fig. 4. *A schematic representation of the structure of SARS-CoV-2 (Santos, Grosche, Bergamini, Sabino-Silva, & Jardim, 2020)*

The N protein coats the viral RNA in the virion. The viral membrane consists mainly of the M glycoprotein which binds the nucleic acid genome to the inner surface of the host cell membrane. The M protein assists with viral replication and antiviral immune suppression (Zheng et al., 2020). The S protein facilitates the attachment of the virus to the host receptor angiotensin-converting enzyme 2 (ACE2) and fusion with host cell membrane, which is critical for the entry of SARS-CoV-2 into host target cell (P. S. Masters, 2006; Wang et al., 2020) (Fig. 5).

The 16 nsps are crucial for virus replication and genomic transcription, genomic RNA template reading and synthesis (Guo et al., 2020). Because ORF1a and ORF1b encode 15–16 non-structural proteins (nsp) they influence the viral replication and transcription complex (RTC) and taken together with the Coronaviruses accessory proteins which have limited conservation, they contribute overall to the modulation of host responses to infection (V'kovski, Kratzel, Steiner, 2021).

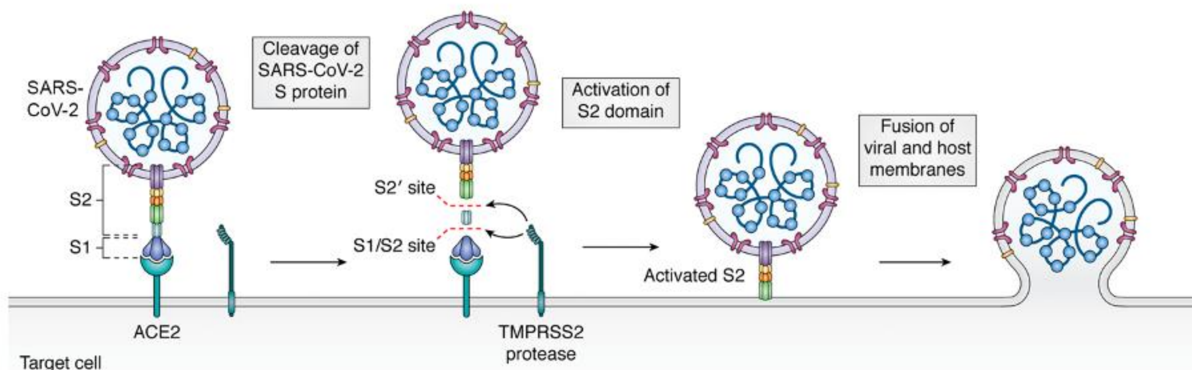

Fig. 5. *Mechanisms of SARS-CoV-2 plasma membrane viral entry (Mourad, Azar, & Azar, 2021)*

1.5. Conserved regions among Betacoronaviruses

SARS-CoV-2 has five highly conserved features between SARS-CoV-2 and other Betacoronavirus (betaCoV) lineages which includes the 3'-UTR, the E gene, ORF10, the 5'-UTR, and nsp10 at 97.4, 95.1, 93.8, 91.1, and 89.7% conservation, respectively (Chan, Choi & Schork 2020). It was further shown that ultra-conserved 5'- and 3'-terminal regions of SARS-CoV-2 are shared amongst betaCoV lineage B genomes, including SARS-CoV and different groups of bat CoVs, albeit genome-wide genetic similarity could be as low as ~79% (Chan & Choi, 2020). The ultra-conserved 5'- and 3'-terminal regions of SARS-CoV-2 increase its viral tropism and pathogenicity, and in conjunction with secondary RNA structures contribute substantially to RNA synthesis (V'kovski, Kratzel, Steiner, 2021). There are two large open reading frames (ORFs; ORF1a and ORF1b) located at the 5′ end which occupy two-thirds of the capped and polyadenylated genome.

SARS-CoV-2 uses the human ACE2 as a cellular entry receptor, and studies have shown that SARS-CoV contact residues that interact with ACE2 were highly

conserved in SARS-CoV-2 (V'kovski, Kratzel, Steiner, 2021). SARS-CoV uses the cell-surface serine protease (TMPRSS2) as a co-receptor, for priming and entry, particularly in the human respiratory tract where TMPRSS2 is expressed, strongly contributing to both SARS-CoV-2 spread and pathogenesis in the lung. Unlike SARS-CoV, SARS-CoV-2 replicates more rapidly in the upper respiratory tract, a region where ACE2 is expressed but which low levels of TMPRSS2, unlike SARS-CoV which primarily targets pneumocytes and lung macrophages in the lower respiratory tract (V'kovski, Kratzel, Steiner, 2021).

1.6. COVID-19 evolution

1.6.1. Global evolution

Coronaviruses' (CoVs) rapid evolution attributable to both the RNA genome and viral recombination (Kirtipal et al., 2020; Leao et al., 2022) create a likely scenario wherein they may emerge in humans through cross-species infections (Woo et al., 2012). SARS-CoV-2, like other Ribonucleic acid (RNA) viruses change over time and mutate rapidly due to viral recombination, high mutation rates, high yields, and short replication times (Siqueira et al., 2021). When a large number of people are infected, the probability of new virus mutations increases. These changes have implications for the transmissibility of the virus, disease severity and vaccine efficacy. When the mutations lead to variants (Fig. 6), they present new challenges in terms of COVID-19 treatment and prevention (Jia & Gong, 2021; Krause et al., 2021; Markarian et al., 2022; Cameron A Smith & Ashby, 2022; C. A. Smith & Ashby, 2023).

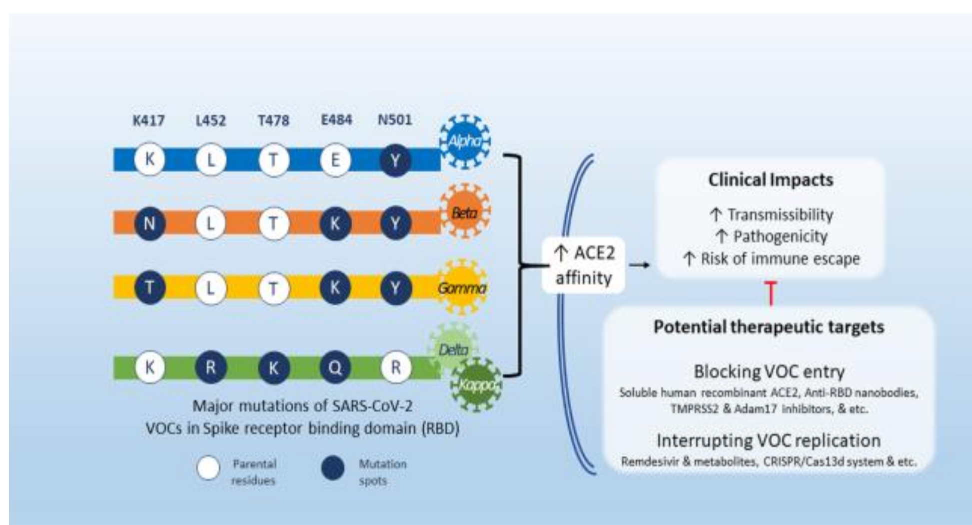

Fig. 6. *Illustrative figure of the mutations of SARS-CoV-2 RBD (Khateeb, Li, & Zhang, 2021)*

To date, South Africa has experienced five distinctive COVID-19 waves, each driven by a new variant (Fig. 7). The first confirmed positive PCR SARS-CoV-2 case was reported in KwaZulu-Natal, South Africa, on the 5th of March 2020, marking the start of the ancestral infection wave where infection was with the original SARS-CoV-2 virus with the D614G mutation. This wave peaked in July 2020, followed by the second infection wave (Beta infection wave with infections by the Beta variant) which peaked in January 2021 (https://www.nicd.ac.za/first-case-of-covid-19-coronavirus-reported-in-sa/, accessed 14.11.2022). In June 2021 South Africa entered the third wave (Delta wave), December 2021 saw the peak of the fourth wave (Omicron BA.1 wave). The fifth wave was the BA.4/BA.5 wave. The Beta and the BA.1 and BA.5 Omicron subvariants were first detected in South Africa. The BA.1 subvariant was first isolated and its antibody immune escape first determined and published by the Sigal laboratory at AHRI (S. Cele, Jackson, et al., 2021). The Beta and BA.5 were also first isolated as live virus in the Sigal laboratory, although our group was not the first to publish on their immune escape (S. Cele, Gazy, Jackson, Hwa, Tegally, Lustig, Giandhari, Pillay, Wilkinson, Naidoo, Karim, Ganga, Khan, Bernstein, Balazs, Gosnell, Hanekom, Moosa, Network for Genomic Surveillance in South, et al., 2021; Khadija Khan et al., 2022).

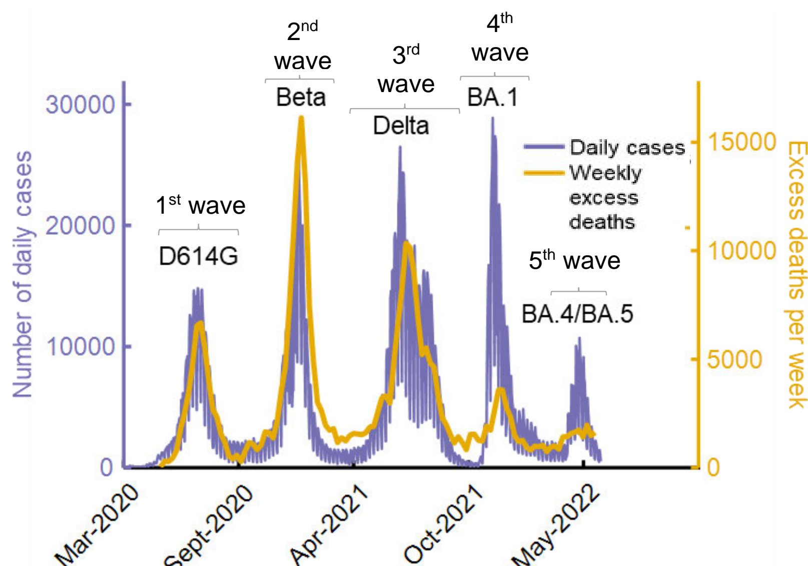

Fig. 7. *SARS-CoV-2 infection waves in South Africa. Predominant infecting variant shown above each wave peak. Adapted from Khan et al., Nat Commun 13, 4686 (2022).*

The second wave was driven by the Beta variant of concern from the 501Y.V2 mutation (lineage B.1.351) sequenced on the 18th December 2020 (S. Cele, Gazy, Jackson, Hwa, Tegally, Lustig, Giandhari, Pillay, Wilkinson, Naidoo, Karim, Ganga,

Khan, Bernstein, Balazs, Gosnell, Hanekom, Moosa, Lessells, et al., 2021; Jia & Gong, 2021). Beta was identified in South Africa on the 18th December 2020 and became the dominant virus lineage in South Africa (Zawilska, Lagodzinski, & Berezinska, 2021). The Beta variant that dominated the second wave is characterized by three substitutions at key residues in the receptor binding domain (RBD) (K417N, E484K and N501Y), and it has three substitutions (L18F, D80A and D215G) and deletion of three amino acid (242-244) in the N-terminal domain and one substitution (A701V) in loop 2 of the spike protein (Table 1). Comparatively, Beta showed significant escape from the neutralising COVID-19 antibodies in individuals who had previously been infected with the ancestral SARS-CoV-2 virus.

The third wave was the Delta wave (B.1.617.2), first identified in India in late 2020. This variant has seven amino acid substitutions (T19R, L452R, T478K, D614G, P681R and D950N) and deletion of two amino acids (157-158) in the N-terminal domain of the spike protein (Table 1). Comparatively, Delta was more highly transmissible compared to the B.1.1.7, showing increased immune escape (Riemersma et al., 2022); This variant was more infectious than B.1.351. due to higher viral loads, higher reinfection rates, longer duration of infection and an ability to escape natural immunity (S. S. A. Karim & Karim, 2021; Suelen H. Qassim et al., 2022; S. H. Qassim et al., 2022). The increased transmissibility of this variant is attributed to L452R mutation in the RBD of the Spike protein, which has been shown to increase transmissibility by increasing viral infectivity and fusion efficacy, thus promoting viral replication (Motozono et al., 2021). Another mutation associated with increased transmissibility of SARS-CoV-2 variants is P681R which appears near the furin cleavage site of the S protein , increasing the efficiency of cleavage and therefore infectivity (Mlcochova et al., 2021).

The fourth wave was driven by the Omicron variant B.1.1.529.1 (BA.1), which was first discovered in South Africa and Botswana in November 2021 (K. Khan, F. Karim, S. Cele, et al., 2022; K. Khan, F. Karim, Y. Ganga, et al., 2022). To date, globally, Omicron variants dominate COVID-19 infections, and more recently, sub-lineages of Omicron have dominated. Omicron was reported about 23 months into the outbreak of the COVID-19 pandemic (S. S. A. Karim & Karim, 2021), a time characterised by vaccine immunity. On the 26th November 2021 Omicron was declared a variant of

concern, had higher reinfection rates than Beta, reduced vaccine effectiveness and demonstrated significant antibody escape. Despite this, however, Omicron was found to be less severe in terms of duration of hospitalisation, decreased mortality and Intensive Care Unit (ICU) admissions (Abdullah et al., 2022; Goga et al., 2022).

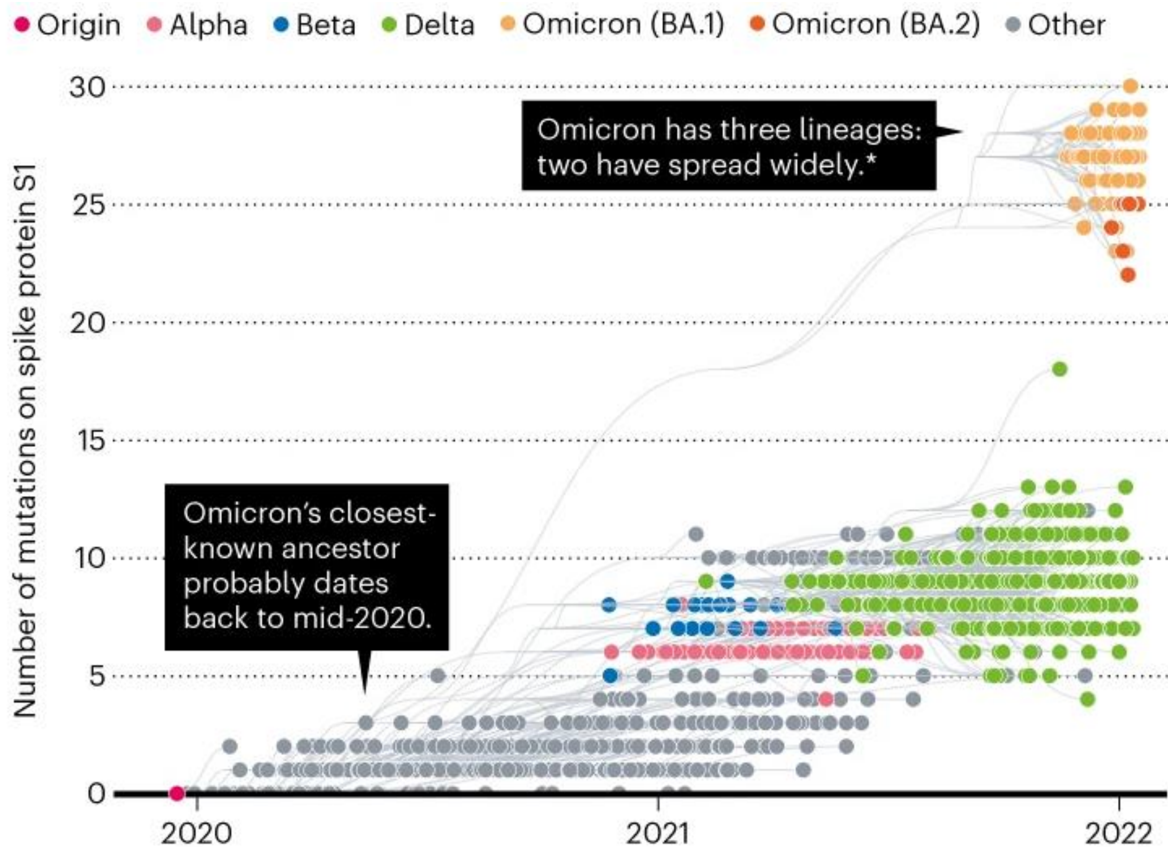

Fig. 8. *Chart illustrating mutations in the S1 subunit of the spike protein (Source: Nature, 2022 https://www.nature.com/articles/d41586-022-00215-2)*

Figure 8 above illustrates the mutations that occurred in the Omicron variant, some changes are common amongst other mutations that contribute to increased ability to bind to ACE2 receptors and avoid neutralising antibodies (Thomas P. Peacock et al., 2022). Omicron has a diminished ability to infect via the plasma membrane route which requires both ACE2 and TMPRSS2 and predominantly uses the endocytic pathway (Meng et al., 2022).

1.6.2. Variant evolution

Major variants emerge abruptly, without intermediate forms. One possibility of how this can happen is reverse zoonosis (Bayarri-Olmos et al., 2021; Fenollar et al., 2021; Griffin et al., 2021; Hale et al., 2022; Hoffmann et al., 2021; Koeppel et al., 2022; Kuchipudi et al., 2022; Meekins, Gaudreault, & Richt, 2021; Ren et al., 2021; Zhou et al., 2022). An animal host is infected by a human, the virus mutates to adapt to the new host, and then re-infects a human host. A second possibility is evolution in long-term infection in immunocompromised individuals (Avanzato et al., 2020; Baang et al., 2021; Sandile Cele et al., 2022; B. Choi et al., 2020; Corey et al., 2021; Hoffman et al., 2021; Jensen et al., 2021; F. Karim et al., 2021; Kemp et al., 2021; Maponga et al., 2022; Thomas P Peacock, Penrice-Randal, Hiscox, & Barclay, 2021; Riddell et al., 2022; Wilkinson et al., 2022). Evolution in immunocompromised people is not unique to SARS-CoV-2 and has been shown in influenza and salmonella infections (Tanner & Kingsley, 2018; Xue, Moncla, Bedford, & Bloom, 2018).

Otherwise healthy people can clear a SARS-CoV-2 infection in around two weeks (Msomi, Lessells, Mlisana, & de Oliveira, 2021). Studies of SARS-CoV-2 infections in immunosuppressed individuals show prolonged viral shedding and the evolution of genomic changes in the SARS-CoV-2 spike protein associated with escape from neutralizing antibodies (Wilkinson et al., 2022). Immune compromise may happen because of long-term uncontrolled HIV infection, termed advanced HIV disease (Sandile Cele et al., 2022; Hoffman et al., 2021; F. Karim et al., 2021; Riddell et al., 2022), defined as a CD4+ T cell count < 200 cells/microliter. About 1 in 10 people living with HIV (PLWH) in South Africa has advanced HIV disease (Carmona et al., 2018; Chihana et al., 2019). The number of PLWH in South Africa is about 8 million as of 2021 (https://www.unaids.org/en/regionscountries/countries/southafrica, accessed 03.01.2023). Therefore, the number of people immunosuppressed because of advanced HIV disease is ~800,000 in South Africa alone.

Table 1. *Variants of Concern Adapted from (J. Y. Choi & Smith, 2021)*

	Lineage/ mutations	Country detected	1st detected	Spike Mutations	Vaccine evasion	Disease severity
Alpha	B.1.1.7	UK	Sep-20	N501Y, D614G, P681H, A570D, P681H, T716I, 5982A, D111H	No	Yes
Beta	B.1.351	RSA	Oct-20	K417N, E484K, N501Y, D614G, A701V, D80A, D215G	Yes	Yes
Delta	B.1.617.2	IND	Dec-20	L452R, T478K, D614G, P681R, T19R, G142D, E156E, D950N	Yes	Yes
	BA.1	RSA/BW	Nov-21	S371L, K417N, N440K, G446S, E484A, Q493R, G496S, N501Y, N856K, N969K, A67V, T95I, G142D, N211I, R214ins, G339D, S371L, S373P, S375F, K417N, N440K, G446S, S477N, T478K, E484A, Q493R, G496S, Q498R, N501Y, Y505H, T547K, D614G, H655Y, N679K, P681H, N764K, D796Y, N856K, Q954H, N969K, L981F	Yes	No
Omicron	BA.2	RSA	Nov-21	T19I, L24S, G142D, V213G, G339D, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, S477N, T478K, E484A, Q493R, Q498R, N501Y, Y505H, D614G, H655Y, N679K, P681H, N764K, D796Y, Q954H, N969K	Yes	No
	BA.4/BA.5	RSA	Nov-21	T19I, L24S, G142D, V213G, G339D, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, L452R, S477N, T478K, E484A, F486V, Q498R, N501Y, Y505H, D614G, H655Y, N658S, N679K, P681H, N764K, D796Y, Q954H, N969K	Yes	No

1.7. Human Immunodeficiency Virus (HIV) and the South African context

In 2021 there were 38.4 million people living with HIV (PLWH) globally with 28.7 million PLWH accessing antiretroviral therapy (ART) (UNAIDS Fact sheet, 2021, (https://www.unaids.org/en/resources/fact-sheet, accessed 14.01.2023). The majority of PLWH are in the low- and middle-income countries.

The South African population is estimated to be 60 million (https://www.statssa.gov.za/?page_id=964, accessed 14.01.2023). An estimated 8 million adults and children are HIV positive, of which 5.5 million PLWH are on ART (https://www.unaids.org/en/resources/fact-sheet, accessed 14.01.2023). This makes South Africa the country with the largest HIV epidemic in the world (Akullian et al., 2021). It was further estimated that in 2021 HIV/AIDS caused 12% of overall deaths in South Africa (https://www.statssa.gov.za/?page_id=964, accessed 14.01.2023).

Approximately 60-70% of known HIV positive cases in South Africa are on ART (https://www.statssa.gov.za/?page_id=964, accessed 14.01.2023). The province of KwaZulu-Natal accounts for the highest HIV prevalence in South Africa with an estimated 2 million people (27%) living with HIV (https://www.unaids.org/en/regionscountries/countries/southafrica, accessed 17.01.2023).

Despite the burden of HIV, efforts to promote test and treat have resulted in a significant decrease in HIV/AIDS related deaths. With the scale up of access to ART, Medical Male Circumcision (MMC) clinics and preventative treatments, HIV incidence has been decreasing (Akullian et al., 2021; Mashishi, Makatini, & Adu-Gyamfi, 2021).

1.7.1. Types of ART

In 2004, South Africa implemented one of the largest public sector ART programs in the world, furthered in 2013 with the release of a third-line ART program in the public sector, promoting a ritonavir-boosted darunavir (DRV/r), raltegravir, and etravirine regimen. The intention of this program was to respond to those patients who have failed both first-line non-nucleoside reverse transcriptase inhibitor–based ART and second-line protease inhibitor (PI)-based ART (Moorhouse et al., 2019). In 2019 clinical guidelines changed from first-line treatment which included Tenofovir (TDF), Emtricitabine (FTC/3TC), and Efavirenz (EFV) to a fixed-dose combination (FDC) of Tenofovir (TFV) disoproxil 300mg, Lamivudine (3TC) 300mg and Dolutegravir (DTG) 50mg (https://www.nicd.ac.za/wp-content/uploads/2019/11/2019-ART-Clinical-Guidelines-25-Nov.pdf, accessed 14.01.2023). South Africa adopted TLD as its preferred first-line regimen, replacing a regimen known as TEE or Tenofovir/Emtricitabine/Efavirenz for most adults and children starting treatment over the age of 10. The second-line regimen is protease inhibitor based, with DRV/r or ritonavir-boosted lopinavir with 2 NRTIs (https://www.nicd.ac.za/wp-content/uploads/2019/11/2019-ART-Clinical-Guidelines-25-Nov.pdf, accessed 14.01.2023).

Despite the drive towards nation-wide ART initiation programs, COVID-19 lockdown restrictions led to a significant disruption in accessing HIV care and treatment with a drop in the number of HIV diagnoses and referral for care and the timeous initiation of ART. There was also a shift in healthcare, with a reallocation of limited health care resources to prioritise COVID-19 (van Staden, Laurenzi, & Toska, 2022). This presented concerns and challenges related not only to initiation and adherence to ART, but also disrupting PLWH's access to medication, exacerbating non-adherence and further complications stemming from treatment disruptions. Individuals who acquire COVID-19 whilst HIV viremic are more susceptible to more severe SARS-

CoV-2 infection and outcomes (https://www.cdc.gov/hiv/basics/covid-19.html, accessed 07.02.2023)

South Africa has a high proportion of PLWH who have advanced HIV disease (CD4 < 200 cells/mm^3) (Freer & Mudaly, 2022) where the negative effect of COVID-19 on health outcomes is exacerbated by treatment disruptions and non-adherence to ART. Immunosuppressed persons are known to be susceptible to both HIV-related and unrelated illnesses and are therefore of particular interest in this study as we examine the interaction between HIV and COVID-19.

1.8. Clash of two pandemics: HIV and COVID-19

1.8.1. Antibody Responses to SARS-CoV-2 and HIV

2HIV and SARS-CoV-2 are both caused by RNA viruses, which means that they have a high mutation rate, evolve rapidly, and thus undermine prevention and treatment efforts (Temin, 1989). However, SARS-CoV-2 does not integrate into the host's DNA (Illanes-Álvarez, Márquez-Ruiz, Márquez-Coello, Cuesta-Sancho, & Girón-González, 2021).

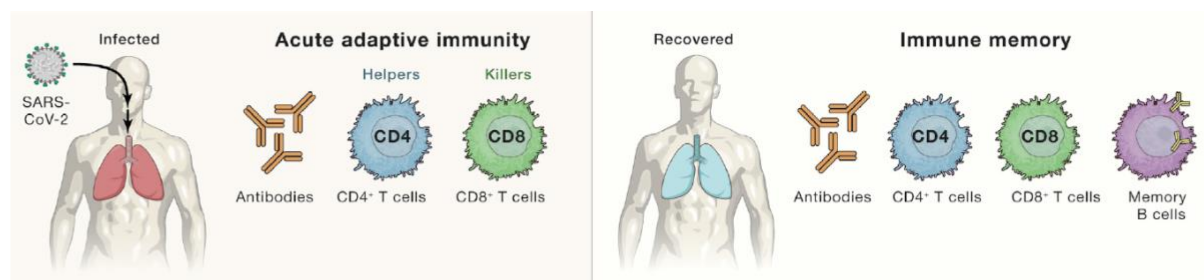

Fig. 9. *The major components of adaptive immunity in viral immune responses. Adapted from Sette & Crotty, 2021.*

The immune system is divided into the innate and the adaptive immune system, each defined by different cell types. The innate immune system responds to the invasion of pathogens within hours by hampering the replication and spread of pathogens and by triggering adaptive immune responses. Innate responses include the restriction of viral replication within infected cells (Sette & Crotty, 2021). Adaptive immune responses, on the other hand, are slower as they are required to select specific clones from a large pool of naïve B and T cells (Sette & Crotty, 2021).

Virus-specific CD4+ T cells, CD8+ T cells, and antibodies produced by B cells constitute the three major components of acute adaptive immunity to a viral infection (Fig. 9). B cells are responsible for the production of antibodies, and together with the CD4+ T helper cells and the CD8+ cytotoxic T cells (CTL) the host is able to control and clear infections. This occurs predominantly by, first, antibodies binding to the SARS-CoV-2 spike protein and preventing its interaction with the cellular ACE2 receptor, a process called neutralization. Second, CTLs kill the infected cells. CD4+ T

helper cells facilitate both processes (Sette & Crotty, 2021). The success of vaccines is premised on the functioning of the adaptive immune system (Sette & Crotty, 2021).

After infection with SARS-CoV-2, most individuals have a short duration of the innate immune response after which they develop neutralising antibodies (Fig. 10) that target the viral spike protein and stop viral infections by recognising epitopes on the surface of the virus and by blocking their entry into the host cell (Crawford et al., 2021; Lagunas-Rangel & Chávez-Valencia, 2021). Serological and neutralisation assays can be used to determine the immune response and characterise antibody responses (Amanat et al., 2020). Neutralising antibodies are present from about 10 days post-symptom onset following COVID-19 infection and peak and wane roughly 4 months post symptom onset (Crawford et al., 2021). Past studies suggest that individuals exhibiting more severe disease have higher levels of neutralising antibodies (Crawford et al., 2021). In individuals infected with HIV, the T cell response, and specifically the CD4+ helper T cell response is impaired, leading to a dysregulation of the adaptive immune responses to other pathogens (Boasso, Shearer, & Chougnet, 2009).

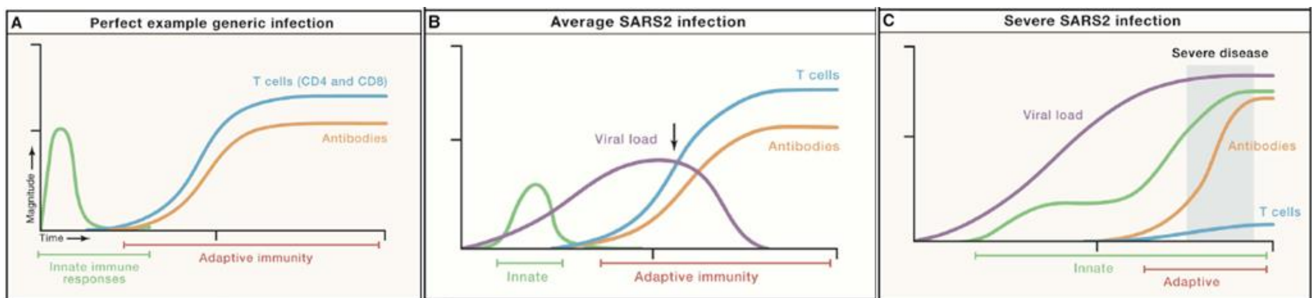

Fig. 10. *An integrated working model of COVID-19 immunology and disease severity (adopted from Sette & Crotty, 2021, p. 863)*

A driving factor of more severe COVID-19 disease is a dysregulated pro-inflammatory response known as a cytokine storm (Chersich et al., 2020). The COVID-19 cytokine storm evokes a sudden and rapid increase in the number of pro-inflammatory cytokines to the site of infection causing lung injury and destruction to human tissue, damaging vascular barriers, alveoli and capillaries, leading to multiple organ failure, and in some cases, death (Ragab, Salah Eldin, Taeimah, Khattab, & Salem, 2020). Because a dysregulated immune response has a more severe outcome in COVID-19,

the possibility exists that dysregulation of immunity by HIV would increase COVID-19 disease severity and reduce immune control of SARS-CoV-2.

1.8.2. The COVID-19 and HIV syndemic

There is conflicting data regarding the clinical outcomes of HIV infected patients co-infected with COVD-19 (Brown, Spinelli, & Gandhi, 2021; Gatechompol, Avihingsanon, Putcharoen, Ruxrungtham, & Kuritzkes, 2021). Several studies (Barbera et al., 2021; Wu, Chen, & Zhang, 2020) have shown that PLWH maybe be at a higher risk for both acquisition and severe disease due to a concomitant COVID-19 infection. Despite this, however, throughout Europe and the United States there is no empirical evidence to suggest that PLWH have a higher risk of severe disease. In South Africa, however, Boulle et al. (Boulle et al., 2020) found a higher mortality rate amongst PLWH coinfected with COVID-19. This could be attributable to factors other than HIV status itself, including HIV suppression level and history (Jones et al., 2021).

Studies have shown that CD4+ T cell concentrations might affect disease severity in HIV-positive individuals. Low CD4+ T cell counts (<200 cells/mm3) indicates advanced HIV disease. Multiple studies showed that PLWH have similar outcomes to HIV negative patients where their CD4+ T cell count is >200, and where their HIV is controlled with ART (Augello, Bono, Rovito, Tincati, & Marchetti, 2023; Budak, Scott, Dhanireddy, & Wood, 2021; Cooper, Woodward, Alom, & Harky, 2020; Danwang, Noubiap, Robert, & Yombi, 2022; Dzinamarira et al., 2022; Karmen-Tuohy et al., 2020; Kowalska et al., 2020; Mellor et al., 2021; Oyelade et al., 2022). Another case series study looking at 14 patients with COVID-19 and HIV co-infection, where only one of the 14 participants was HIV viremic also concluded that they did not observe any differences in the COVID-19 outcomes between PLWH and the HIV negative population. They also found that they were not at a greater risk for severe disease or death (Gudipati et al., 2020).

Those studies that showed HIV as a risk factor for acquiring SARS-CoV-2 infection with a demonstrable increase in disease severity and a higher risk of mortality from COVID-19 were predominantly in PLWH with HIV viremia, with CD+ counts < 200 (Brown et al., 2021; Ssentongo et al., 2021). It was found in South Africa that PLWH

have a higher hazard rate of COVID-19 deaths, whereas researchers in the USA and Europe found that PLWH seem to experience similar outcomes to those without HIV. These differential findings could be attributable to differences inherent to the cohort characteristics researched, including the number of patients on ART, rates of HIV viremia, comorbidities (including active TB and diabetes), but relating predominantly to CD4 counts, and HIV viremia (Brown et al., 2021). A study by Etienne et al (2020) looked at the risk factors underlying severe COVID-19 disease in patients co-infected with HIV and found that 13 out of 19 patients with severe/critical COVID-19 disease were from South Africa and of sub-Saharan origin. Another cohort study conducted in New York found that rates of hospitalisation of HIV viremic HIV infected patients with low CD4 counts coinfected with COVID-19 were higher than those who were not diagnosed with HIV (Tesoriero et al., 2021). These findings suggest that clinical severity of COVID-19 is advanced by HIV infection (Lee et al., 2022) especially where CD4 counts are low, and patients are HIV viremic. These findings suggest an urgent and unmet need to understand disease dynamics in those PLWH who are coinfected with COVID-19, especially where HIV is viremic, and with low CD4 counts.

1.9. Vaccines

The currently approved COVID-19 vaccines in South Africa are the Janssen Ad26.CoV2.S adenovirus vectored vaccine and the and Pfizer-BioNTech BNT162b2 mRNA vaccine. Ad26.CoV2.S is a single dose adenovirus serotype 26 vector based vaccine whilst Pfizer is a mRNA based, two-dose vaccine (Heinz & Stiasny, 2021; Mendonça, Lorincz, Boucher, & Curiel, 2021). The national COVID-19 vaccination programme began on the 17th February 2021 for priority groups which included healthcare workers who were administered Ad26.CoV2.S. The prioritisation of healthcare workers was facilitated through the Sisonke study (SAMRC, 2021). Later in August 2021, the BNT162b2 vaccine was rolled out to the general population.

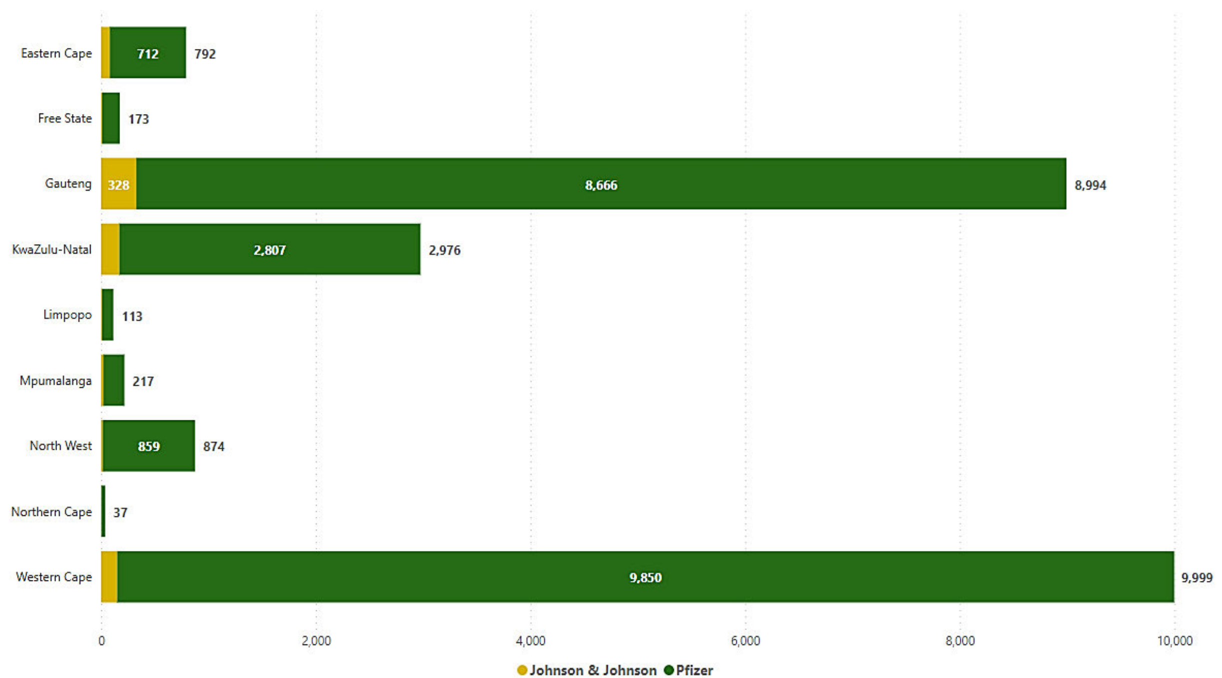

Fig. 11. *Total number of vaccinated immunocompromised individuals in South Africa by province (Source: SACoronavirus https://sacoronavirus.co.za/latest-vaccine-statistics/)*

Only 31.6% (~38 million) of South Africans have been vaccinated, of which 22,512,349 have received only one dose, and 19,315,266 have been fully vaccinated since roll-out (South African Department of Health, 2022). One of the reasons for the relatively low vaccination statistics in South Africa is attributable to vaccine hesitancy. The South African Department of Health (p. 1, 2022). released their 78th South Africa COVID-19 and Vaccines Social Listening Report on the 5th December 2022 detailing the misinformation on social media surrounding COVID-19 vaccines and boosters, calling them “clot shots”. Vaccine hesitancy has been reported to be amongst the top ten threats to global health despite evidence that vaccines are safe, and show no interference with HIV medications (Plummer & Pavia, 2021).

The recommendation from the NIH is that all PLWH get vaccinated, irrespective of their CD4 count or HIV viral load (Duly, Farraye, & Bhat, 2022) but it could be argued that PLWH with low CD4 counts and who are HIV viremic should therefore be prioritised when receiving the COVID-19 vaccine (Chersich et al., 2020). Vaccines help overcome delays in mounting T-cell responses and in triggering an adaptive response by priming the immune system (Grifoni et al., 2020; Juno et al., 2020; Peng et al., 2020).

In South Africa, only 24,175 known immunocompromised individuals have been vaccinated (Fig. 11). There is not much known about the efficacy of vaccines in immunocompromised populations and their relative response to vaccines. It is hypothesised that vaccine response in immunocompromised persons may vary according to their level of immunosuppression and the type of vaccine received (Clara Bessen et al., 2022; C. Bessen et al., 2022; Lauriane Nault et al., 2021; L. Nault et al., 2022). Whilst PLWH who were not HIV viremic responded favourably to vaccines, those PLWH with advanced HIV disease, HIV viremia and with low CD4 counts were at a higher risk of not responding to vaccines (Lauriane Nault et al., 2021; L. Nault et al., 2022).

CHAPTER 2:

HIV status alters disease severity and immune cell responses in the Beta variant SARS-CoV-2 infection wave

The study aimed to determine if PLWH had more severe COVID-19 disease relative to HIV negative participants. Two comparative sub-cohorts were selected, namely participants who were infected in the first and second COVID-19 infection waves. Waves differed in the infecting variant, whereby the first infection wave was ancestral strains and the second wave was dominated by the Beta variant. Findings indicated that PLWH had a higher disease severity compared to HIV uninfected persons in the Beta infection wave as indicated by increased requirement for supplemental oxygen. Higher disease severity was found to be significantly associated with low CD4 + T cell counts. It was found that CD4 + T cell counts recovered after the SARS-CoV-2 infection was cleared.

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RESEARCH ARTICLE

HIV status alters disease severity and immune cell responses in Beta variant SARS-CoV-2 infection wave

Farina Karim[1,2], Inbal Gazy[2,3], Sandile Cele[1,2], Yenzekile Zungu[1], Robert Krause[1,2], Mallory Bernstein[1], Khadija Khan[1,2], Yashica Ganga[1], Hylton Rodel[1,4], Ntombifuthi Mthabela[1], Matilda Mazibuko[1], Daniel Muema[1,2], Dirhona Ramjit[1], Thumbi Ndung'u[1,4,5,6], Willem Hanekom[1,4], Bernadett Gosnell[7], COMMIT-KZN Team, Richard J Lessells[2,3,8], Emily B Wong[1,9], Tulio de Oliveira[2,3,8,10,11], Mahomed-Yunus S Moosa[7], Gil Lustig[8], Alasdair Leslie[1,2,4]*, Henrik Kløverpris[1,2,4,12]*, Alex Sigal[1,2,6]*

[1]Africa Health Research Institute, Durban, South Africa; [2]School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal, Durban, South Africa; [3]KwaZulu-Natal Research Innovation and Sequencing Platform, Durban, South Africa; [4]Division of Infection and Immunity, University College London, London, United Kingdom; [5]HIV Pathogenesis Programme, The Doris Duke Medical Research Institute, University of KwaZulu-Natal, Durban, South Africa; [6]Max Planck Institute for Infection Biology, Berlin, Germany; [7]Department of Infectious Diseases, Nelson R. Mandela School of Clinical Medicine, University of KwaZulu-Natal, Durban, South Africa; [8]Centre for the AIDS Programme of Research in South Africa, Durban, South Africa; [9]Division of Infectious Diseases, Department of Medicine, University of Alabama at Birmingham, Birmingham, United States; [10]Centre for Epidemic Response and Innovation, School of Data Science and Computational Thinking, Stellenbosch University, Stellenbosch, South Africa; [11]Department of Global Health, University of Washington, Seattle, United States; [12]Department of Immunology and Microbiology, University of Copenhagen, Copenhagen, Denmark

***For correspondence:** Al.Leslie@ahri.org (AL); Henrik.Kloverpris@ahri.org (HK); alex.sigal@ahri.org (AS)

Group author details: COMMIT-KZN Team See page 13

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Abstract There are conflicting reports on the effects of HIV on COVID-19. Here, we analyzed disease severity and immune cell changes during and after SARS-CoV-2 infection in 236 participants from South Africa, of which 39% were people living with HIV (PLWH), during the first and second (Beta dominated) infection waves. The second wave had more PLWH requiring supplemental oxygen relative to HIV-negative participants. Higher disease severity was associated with low CD4 T cell counts and higher neutrophil to lymphocyte ratios (NLR). Yet, CD4 counts recovered and NLR stabilized after SARS-CoV-2 clearance in wave 2 infected PLWH, arguing for an interaction between SARS-CoV-2 and HIV infection leading to low CD4 and high NLR. The first infection wave, where severity in HIV negative and PLWH was similar, still showed some HIV modulation of SARS-CoV-2 immune responses. Therefore, HIV infection can synergize with the SARS-CoV-2 variant to change COVID-19 outcomes.

Introduction

HIV is a prevalent infection in KwaZulu-Natal, South Africa (***Kharsany et al., 2018***) which also has a high SARS-CoV-2 attack rate (***Tegally et al., 2021a***; ***Tegally et al., 2021b***). HIV depletes CD4 T helper cells (***Dalgleish et al., 1984***) which are a critical part of the adaptive immune response and

are also the main target of HIV infection. CD4 T cell death occurs after cellular infection with HIV (*Westendorp et al., 1995*), or in bystander or incompletely infected cells due to activation of cellular defense programs (*Doitsh et al., 2010*; *Doitsh et al., 2014*), and is halted and, to some extent, reversed by antiretroviral therapy (ART), even sub-optimal therapy (*Jackson et al., 2018*).

The loss of CD4 T cells leads to dysregulation of many aspects of the immune response, including germinal center formation and antibody affinity maturation, which requires help from the highly HIV susceptible CD4 T follicular helper cells (*Okoye and Picker, 2013*; *Pallikkuth et al., 2012*; *Perreau et al., 2013*). In association with this, HIV also causes B cell dysregulation and dysfunction (*Moir and Fauci, 2013*). Moreover, T cell trafficking, activation, and exhaustion profiles of both CD4 and CD8 subsets are also modulated by HIV infection (*Day et al., 2006*; *Deeks et al., 2004*; *Mavigner et al., 2012*).

Both antibody and T cell responses are critical for effective control and clearance of SARS-CoV-2. More severe COVID-19 disease correlates with lymphopenia and low T cell concentrations (*Lucas et al., 2020*; *Sekine et al., 2020*; *Chen et al., 2020a*), whilst mild disease correlates with a robust T cell response to SARS-CoV-2 (*Grifoni et al., 2020*; *Sekine et al., 2020*; *Rydyznski Moderbacher et al., 2020*; *Mathew et al., 2020*; *Mateus et al., 2020*; *Liao et al., 2020*; *Chen et al., 2020b*). Neutralizing antibodies and associated expansion of antibody secreting B cells (ASC) are elicited in most SARS-CoV-2 infected individuals (*Woodruff et al., 2020*; *Robbiani et al., 2020*; *Quinlan et al., 2020*), and neutralizing antibody titers strongly correlate with vaccine efficacy (*Khoury et al., 2021*; *Earle et al., 2021*), indicating their key role in the response to SARS-CoV-2 infection. In contrast, high neutrophil numbers are associated with more severe disease and an elevated neutrophil to lymphocyte ratio (NLR) is often considered a risk factor for a more severe COVID-19 outcome (*Liu et al., 2020a*; *Liu et al., 2020b*; *Zhang et al., 2020*).

Results from epidemiological studies of the interaction between HIV and SARS-CoV-2 from other locations are mixed. Several large studies observed that disease severity and/or mortality risk is increased with HIV infection (*Western Cape Department of Health in collaboration with the National Institute for Communicable Diseases, South Africa et al., 2021*; *Geretti et al., 2021*; *Bhaskaran et al., 2021*; *Tesoriero et al., 2021*; *Braunstein et al., 2021*; *Jassat et al., 2021a*) while others found no statistically significant differences in clinical presentation, adverse outcomes, or mortality (*Huang et al., 2021*; *Sigel et al., 2020*; *Shalev et al., 2020*; *Vizcarra et al., 2020*; *Stoeckle et al., 2020*; *Dandachi et al., 2021*; *Härter et al., 2020*; *Karmen-Tuohy et al., 2020*; *the Northwell COVID-19 Research Consortium et al., 2020*; *Inciarte et al., 2020*; *Hadi et al., 2020*). Worse outcomes for PLWH tended to be in patients with low CD4 (*Hoffmann et al., 2021a*; *Dandachi et al., 2021*; *Braunstein et al., 2021*) and low absolute CD4 count was a risk factor for more severe disease (*Western Cape Department of Health in collaboration with the National Institute for Communicable Diseases, South Africa et al., 2021*).

HIV is known to interfere with protective vaccination against multiple pathogens (*Avelino-Silva et al., 2016*; *Carson et al., 1995*; *Cooper et al., 2011*; *Fuster et al., 2016*), typically as a consequence of sub-optimal antibody responses. In line with this, results from a South-African phase IIb trial of the Novavax NVX-CoV2373 vaccine, which uses a stabilised prefusion spike protein, showed 60% efficacy in HIV-uninfected individuals. However, overall efficacy dropped to 49% upon inclusion of PLWH (*Shinde et al., 2021*), although it is important to note that the numbers of PLWH in the study were very small. Nonetheless, there were more breakthrough cases in PLWH in the vaccine arm than the placebo arm.

An important consideration in infections in South Africa is the infecting variant, which in the second infection wave peaking January 2021 was predominantly the B.1.351 variant of concern (VOC) now designated as the Beta variant. In the current third infection wave it is predominantly the B.1.617.2 Delta variant. We and others have shown that the Beta variant has evolved the ability to escape neutralization by antibody responses elicited by earlier strains of SARS-CoV-2 or by vaccines based on those strains (*Cele et al., 2021*; *Wibmer et al., 2021*; *Garcia-Beltran et al., 2021*; *Hoffmann et al., 2021b*). Loss of vaccine efficacy of the AstraZeneca ChAdOx vaccine in South Africa was associated with this drop in neutralization capacity (*Madhi et al., 2021*). The second infection wave driven by Beta infections also showed increased mortality of hospitalized cases relative to the first infection wave (*Jassat et al., 2021b*).

What factors contributed to the evolution of the Beta variant in South Africa is yet unclear. One possibility is intra-host evolution in immunosuppressed PLWH with advanced HIV who are unable to

clear SARS-CoV-2 (*Karim et al., 2021*). There is also evidence that variants evolved other adaptations to the host in addition to those in the spike glycoprotein which lead to antibody escape and enhanced transmission. These include evolution of resistance to the host interferon response (*Guo et al., 2021*; *Thorne et al., 2021*), as well as enhanced cell-to-cell transmission (*Rajah et al., 2021*). Changes in the virus may make infection with some variants substantially different in disease course, transmission dynamics, and effect on PLWH relative to ancestral SARS-CoV-2 strains or other variants.

Here, we aimed to determine the effects of HIV on the immune response to SARS-CoV-2 infection in KwaZulu-Natal, South Africa. This is important because we need to better understand COVID-19 disease course and vaccine efficacy in this population, as well as the possible reasons for the emergence of variants which lead to immune escape from neutralizing antibodies. Our results indicate that infections in the Beta variant infection wave led to more severe disease in PLWH relative to HIV-negative participants. Higher severity was associated with a lower CD4 T cell count. Yet, the CD4 count recovered, indicating that these participants may not have had a low CD4 count when first exposed to SARS-CoV-2. In addition, there were changes in the response of immune cell subsets associated with SARS-CoV-2 infection in PLWH relative to HIV-negative participants in the first infection wave, even in the absence of a statistically significant increase in disease severity, indicating that HIV infection may modulate the immune response to SARS-CoV-2.

Results

HIV infection is associated with higher disease severity in the Beta variant infection wave

We initiated a longitudinal observational cohort study to enroll and track patients with a positive COVID-19 qPCR test presenting at three hospitals in Durban, South Africa. Patients presented due to either COVID-19 symptoms or because they were known contacts of a confirmed COVID-19 case.

All participants were initially admitted to a hospital facility, then discharged after varying periods and followed up as outpatients. Enrollment was between June 2020 and May 2021. Participants were followed up weekly for the first month post-enrollment, and at 3-month intervals thereafter. At each study visit, a blood sample and a combined nasopharyngeal and oropharyngeal swab was taken. The purpose of a combined swab was to maximize the detection probability by qPCR of SARS-CoV-2 in the upper respiratory tract. Blood was used to determine HIV status, HIV viral load, and cellular parameters such as the concentration of CD4 T cells and the NLR. We also tested the frequencies of more specific immune cell subsets by flow cytometry (only available for infection wave 1 samples).

Up to May 2021, 236 participants were enrolled in the study, for a total of 986 study visits (*Supplementary file 1*). All participants are assumed to be vaccinated with BCG in infancy in accordance with South African national guidelines. The majority of participants were female, possibly reflecting better linkage to care. Enrollment was a median 11 days post-symptom onset (*Supplementary file 2*). De-identified participant data used here are available as Source Data included in the supplementary materials.

Out of 236 study participants, 93 (39%) were PLWH (*Table 1*) and 89% of study participants were of African descent. PLWH were significantly younger than HIV uninfected participants. Hypertension, diabetes and obesity, known risk factors for more severe COVID-19 disease (*Zhou et al., 2020*; *the Northwell COVID-19 Research Consortium et al., 2020*), were common: Hypertension and obesity were present in 24%, and 42% of study participants respectively, a similar prevalence to that reported in the province of KwaZulu-Natal where this study was performed (*van Heerden et al., 2017*; *Malaza et al., 2012*). Diabetes prevalence in our study was 18%, compared to 13% reported for South Africa (*Federation, 2019*). Hypertension and diabetes were significantly lower in the PLWH group (*Table 1*). 28 or 30% of PLWH were HIV viremic at any point in the study. For individuals on ART, median ART duration was 9 years. ART regimen was determined by liquid chromatography with tandem mass spectrometry (LC-MS/MS) and was predominately efavirenz (EFV) based, with some participants transitioning to a dolutegravir (DTG) based regimen. In addition, there was a small subset of PLWH on a ritonavir boosted lopinavir (LPV/r) as well as other ART combinations. About 12% of PLWH had no detectable ART despite a clinical record of ART, or were ART naive

Table 1. Participant characteristics.

	All (n=236)	HIV- (n = 143, 60.6%)	HIV+ (n=93, 39.4%)	Odds ratio (95% CI)	p-value
Demographics					
Age years, median (IQR)	45 (35–57)	49 (35–62)	41 (35–50)	-	0.003*
Male sex, n (%)	82 (34.7)	48 (33.6)	34 (36.6)	1.1 (0.7–2.0)	0.68
Current smoker, n (%)	13 (5.5)	4 (2.8)	9 (9.7)	3.7 (1.2 – gt_{10})	0.038
Comorbidity, n (%)					
Hypertension#, n=235	57 (24.1)	42 (29.4)	15 (16.1)	0.5 (0.2–0.9)	0.023
Diabetes	42 (17.8)	32 (22.4)	10 (10.8)	0.4 (0.2–0.9)	0.024
Obesity#, n=221	91 (42.3)	64 (47.1)	27 (29.0)	0.6 (0.3–1.0)	0.086
Active TB	10 (4.2)	1 (0.7)	9 (9.7)	>10	0.001
History TB	32 (13.6)	3 (2.1)	29 (31.2)	>10	<0.0001
HIV associated parameters					
HIV viremic, n (% of all HIV)	-	-	28 (30.1)	-	-
Years ART, median (IQR)	-	-	9.4 (3.9–13.2)	-	-
CD4 cells/µL median (IQR) n=221	633 (326–974)	887 (534–1148)	464 (200–702)	-	<0.0001*
CD4/CD8	1.2 (0.8–1.7)	1.6 (1.2–2.1)	0.8 (0.4–1.1)	-	<0.0001*
Disease severity, n (%)					
Asymptomatic	33 (14.0)	25 (17.5)	8 (8.6)	0.4 (0.2–1.0)	0.058
Ambulatory with symptoms	128 (54.2)	80 (55.9)	48 (51.6)	0.8 (0.5–1.4)	0.59
Supplemental oxygen	62 (26.3)	30 (21.0)	32 (34.4)	2.0 (1.1–3.5)	0.024
Death	13 (5.5)	8 (5.6)	5 (5.4)	1.0 (0.3–2.9)	>0.99
COVID-19 treatment, n (%)					
Corticosteroids	74 (31.2)	47 (32.9)	27 (29.0)	0.8 (0.5–1.5)	0.57
Anticoagulants	53 (22.5)	35 (24.5)	18 (19.4)	0.7 (0.4–1.4)	0.43
Symptom, n (%)					
Sore throat	88 (37.3)	55 (38.5)	33 (35.5)	0.9 (0.5–1.5)	0.68
Runny nose	53 (22.5)	30 (21.0)	23 (24.7)	1.2 (0.7–2.3)	0.53
Cough	153 (64.8)	91 (63.6)	62 (66.7)	1.1 (0.7–2.0)	0.68
History of fever#, n=235	58 (24.7)	29 (20.3)	29 (31.2)	1.8 (1.0–3.3)	0.063
Shortness of breath	148 (62.7)	87 (60.8)	61 (65.6)	1.2 (0.7–2.1)	0.49

p-value calculated via 2-sided Fisher's Exact test, except for * which was calculated via Mann-Whitney U test. # Not including pregnancy or unable to be measured.

(***Supplementary file 3***). The absolute CD4 T cell count and the CD4 to CD8 T cell ratio was significantly lower in PLWH relative to HIV negative participants at enrollment. The incidence of active TB and the fraction of participants with a history of TB were much higher in the PLWH group (***Table 1***).

A minority of study participants (14%) were asymptomatic and presented at the hospital because of a close contact with a confirmed COVID-19 case. To include the asymptomatic participants in our analysis, we used time from diagnostic swab as our timescale, which was tightly distributed for symptomatic participants relative to symptom onset at a median of 3 to 4 days apart (***Supplementary file 2***).

The majority of participants in the study (54%) had symptoms but did not progress beyond mild disease, defined here as not requiring supplemental oxygen during the course of disease and convalescence. Twenty-six percent of participants required supplemental oxygen but did not die and 6% of participants died. Our cohort design did not specifically enroll critical SARS-CoV-2 cases. The requirement for supplemental oxygen, as opposed to death, was therefore our primary measure for disease severity.

There was a significant difference in the frequency of participants requiring supplemental oxygen (without subsequent death) between HIV-negative participants and PLWH (21% versus 34% respectively, odds ratio of 2.0 with 95% confidence intervals of 1.1–3.5, ***Table 1***).

To determine if the fraction of participants requiring supplemental oxygen differed between the first infection wave and the Beta variant dominated second infection wave, we compared disease severity between the first infection wave (***Figure 1***, ***Supplementary file 4***), and the second infection wave (***Figure 1***, ***Supplementary file 5***). In the first infection wave, there was no significant difference in the fraction of participants requiring supplemental oxygen between HIV-negative and PLWH participants (***Supplementary file 4***, p=0.5). However, significantly more PLWH required supplemental oxygen in the second wave (***Supplementary file 5***, odds ratio of 4.0 with 95% CI of 1.6–10.4, p=0.005). Comparing within the HIV-negative and PLWH groups, there was only a moderate increase in the fraction of participants requiring supplemental oxygen between SARS-CoV-2 infection wave 1 and infection wave 2 in HIV-negative participants (19% to 25%) which was not significant (***Figure 1***). In contrast, the number of PLWH participants requiring supplemental oxygen more than doubled from 24% to 57% (p=0.0025, ***Figure 1***).

To examine whether the differences in the requirement for supplemental oxygen in PLWH were because of differences in the level of HIV control between waves, we examined the fraction of timepoints where participants showed HIV viremia. We excluded low level viremia and set the threshold at VL > 200 HIV RNA copies/mL (***Ryscavage et al., 2014***). Furthermore, we determined whether ART was detectable in the blood by LC-MS/MS. Second wave participants had approximately twofold higher fraction of timepoints where HIV viremia was detected (***Figure 1—figure supplement 1A***). In agreement with this, the fraction of participants with no detectable ART in the blood was also about twofold higher (***Figure 1—figure supplement 1B***). These observations are consistent

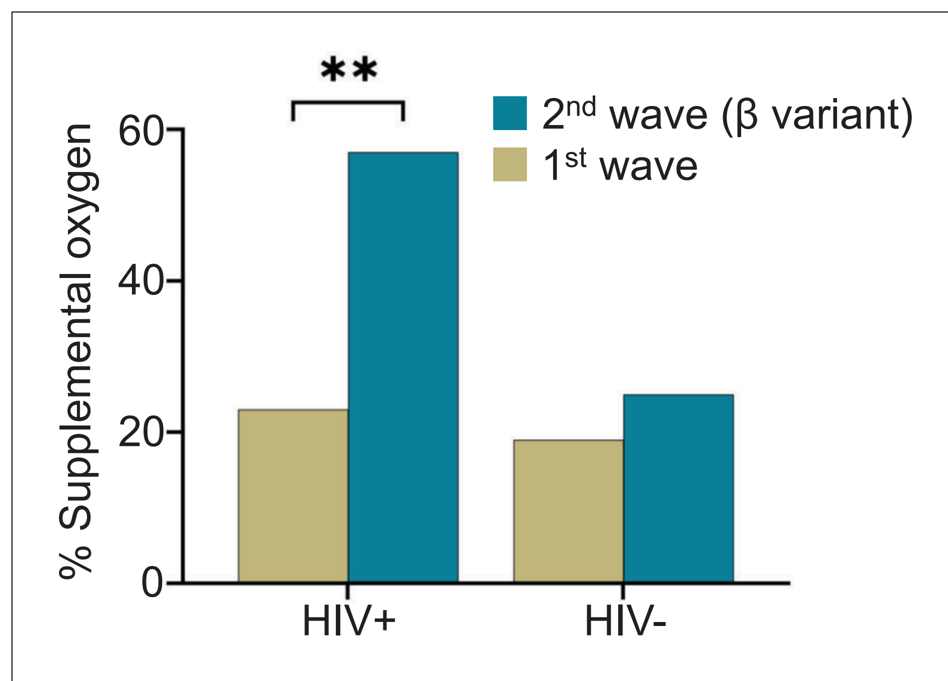

Figure 1. Fraction of PLWH and HIV-negative participants requiring supplemental oxygen during the first and the Beta variant dominated second infection waves. p=0.0025 by Fisher's Exact test.
The online version of this article includes the following figure supplement(s) for figure 1:

Figure supplement 1. Viremia and ART in PLWH in wave 1 versus wave 2.
Figure supplement 2. Effect of ART regimen on disease severity.
Figure supplement 3. Distribution of CD4 counts by HIV status.
Figure supplement 4. Viremia and ART in PLWH requiring versus not requiring supplemental oxygen.
Figure supplement 5. Dependence of time to SARS-CoV-2 clearance on CD4 count and HIV status.

with diminished suppression of HIV in second wave PLWH enrolled in this study. The specific HIV regimen had no discernible effect on disease severity (***Figure 1—figure supplement 2***).

We compared comorbidities and other characteristics between the PLWH and HIV negative participants on supplemental oxygen (***Table 2***). Strikingly, the median age of PLWH on supplemental oxygen was 21 years younger relative to HIV negative (41 versus 62, p=0.003). PLWH had significantly lower frequency of comorbidities which are usually associated with more severe COVID-19 disease: both hypertension (p=0.03) and diabetes (p=0.03) were lower. In contrast, the median CD4 T cell count across all study visits was lower in PLWH (277 versus 339), although this difference did not reach statistical significance (p=0.07). There was no significant difference in the fraction of participants treated with corticosteroids (p=0.2).

Interestingly, when comparing HIV-negative participants requiring supplemental oxygen to those not requiring supplemental oxygen (***Supplementary file 6***), those on supplemental oxygen were significantly older (62 versus 47 years, p=0.002), and had significantly higher frequency of hypertension (p=0.002) and diabetes (p=0.02). This differed from PLWH, where differences in age and comorbidities were not significant between PLWH requiring supplemental oxygen and those not (***Supplementary file 7***), although there was a trend to a higher frequency for hypertension (p=0.1).

HIV viremic participants showed lower CD4 counts relative to HIV suppressed or HIV negative participants (***Figure 1—figure supplement 3***). Surprisingly, there was no difference in either the fraction of HIV viremic timepoints or fraction of timepoints where ART was not detected in the blood between the group of PLWH requiring supplemental oxygen and the no supplemental oxygen group (***Figure 1—figure supplement 4***). We also analyzed the time of SARS-CoV-2 clearance as a function of CD4 count and HIV status and found that while participants with a low CD4 count (<200) showed a trend of longer time to SARS-CoV-2 clearance (p=0.11), HIV viremia had no effect (***Figure 1—figure supplement 5***). Hence, while the PLWH enrolled in the second wave had both worse control of HIV infection and had a higher fraction requiring supplemental oxygen, we did not observe that the PLWH requiring supplemental oxygen had a higher frequency of HIV viremia.

Table 2. Characteristics by HIV status of participants requiring supplemental oxygen.

	All (n=68)	HIV- (n = 35, 51.5%)	HIV+ (n=33, 48.5%)	Odds ratio (95% CI)	p-value
Demographics					
Age years, median (IQR)	51 (38–64)	62 (47–66)	41 (36–56)	-	0.003*
Male sex, n (%)	25 (36.8)	12 (34.3)	13 (39.4)	1.2 (0.5–3.3)	0.80
Current smoker, n (%)	2 (2.9)	1 (2.9)	1 (3.0)	1.1 (<0.1 – >10)	$gt_{0.99}$
Comorbidity, n (%)					
Hypertension	26 (38.2)	18 (51.4)	8 (24.2)	0.3 (0.1–0.8)	0.026
Diabetes	17 (25.0)	13 (37.1)	4 (12.1)	0.2 (0.1–0.8)	0.025
Obesity#, n=57	23 (40.4)	11 (31.4)	12 (36.4)	1.8 (0.6–5.1)	0.42
Active TB	6 (8.8)	1 (2.9)	5 (15.2)	6.1 (0.9 – >10)	0.10
History TB	16 (23.5)	2 (5.7)	14 (42.4)	12.2 (2.7 – >10)	$lt_{0.001}$
HIV associated parameters					
HIV viremic, n (% of all HIV)	-	-	9 (27.3)	-	-
Years ART, median (IQR)	-	-	11.6 (6.1–13.3)	-	-
CD4 cells/µL median (IQR) n=65	309 (170–545)	339 (227–592)	277 (134–461)	-	0.072*
COVID-19 treatment, n (%)					
Corticosteroids	43 (63.2)	25 (71.4)	18 (54.5)	0.5 (0.2–1.3)	0.21
Anticoagulants	31 (45.6)	18 (51.4)	13 (39.4)	0.6 (0.2–1.6)	0.34

p-value calculated via two-sided Fisher's Exact test, except for * which was calculated via Mann-Whitney U test. # Not including pregnancy or unable to be measured.

SARS-CoV-2 has differential effects on CD4 count and the neutrophil to lymphocyte ratio between infection waves in PLWH

We next determined whether the increased disease severity in PLWH in infection wave two was reflected in the cellular immune response to SARS-CoV-2 infection. We therefore examined the CD4 count and NLR, both known to be strongly associated with disease severity. We used a three-point scale for disease severity, where 1: asymptomatic, 2: mild, and 3: supplemental oxygen (at any point in the study) or death. Death was merged with supplemental oxygen because of the small number of participants who died, and was not excluded in any of the subsequent analyses.

As expected, we observed a significant decrease in CD4 T cell count at the highest severity which included disease that required administration of supplemental oxygen and/or resulted in death (***Figure 2A***, see ***Figure 2—figure supplement 1*** for all data points and number of data points per graph).

We then asked whether PLWH in infection wave two showed different CD4 T cell responses to SARS-CoV-2. Since decreased CD4 count could be due to HIV infection alone, we separated the data into timepoints when SARS-CoV-2 was detectable by qPCR and after SARS-CoV-2 was cleared. Upon SARS-CoV-2 clearance, the immune response of convalescent participants should start the return to baseline, and differences due to SARS-CoV-2 should decrease and reflect HIV mediated effects only.

The CD4 counts in PLWH in infection wave 2 were lower during active SARS-CoV-2 infection relative to wave 1 (***Figure 2B***, median 172 versus 420 cells/µL, a decrease of 2.4-fold) and were below the 200 cells/µL clinically used threshold indicating a low CD4 count. However, CD4 counts for PLWH for both wave 2 and wave 1 recovered post-SARS-CoV-2 clearance (408 for wave 2 versus 584 cells/µL for wave 1), consistent with the low CD4 count in PLWH in wave 2 being SARS-CoV-2 induced. CD4 counts for both groups were substantially above the 200 cells/µL threshold after SARS-CoV-2 clearance. HIV-negative participants showed no or minor differences in CD4 counts between waves, although these minor differences showed significance due to the large number of participant timepoints for this group (***Figure 2C***).

The NLR had a remarkably similar pattern. An elevated NLR associated strongly with higher disease severity (***Figure 2D***). PLWH with active SARS-CoV-2 infection in wave 2 showed a twofold increase in the NLR relative to PLWH with active SARS-CoV-2 infection in wave 1 (***Figure 2E***). This difference declined to 1.2-fold once SARS-CoV-2 was cleared, consistent with differences in NLR being SARS-CoV-2 driven and not a result of other pathology in PLWH in wave 2. In contrast, the NLR was lower in HIV negative participants in wave 2 relative to wave 1 in the presence of SARS-CoV-2 (***Figure 2F***).

The observed recovery of the CD4 count may result from improved access to ART due to the hospital visit in wave 2. We therefore checked whether the fraction of HIV viremic participants decreased upon convalescence and whether there was an associated decrease in the number of PLWH with undetectable ART. We observed no significant differences in either viremia or fraction of PLWH with undetectable ART in either wave between timepoints which were SARS-CoV-2 positive and those that were negative (***Figure 2—figure supplement 1***). This indicates that the increase in the CD4 was not due to better linkage to care after the hospital visit but rather due to SARS-CoV-2 clearance.

Differences in the frequencies and associations of immune cell subsets in PLWH and HIV-negative participants

To examine differences in immune cell subset associations between HIV-negative and PLWH participant groups, we conducted detailed phenotyping of immune cells using longitudinal fresh PBMC samples and correlated these to measured phenotypes and clinical parameters in both HIV-negative and PLWH groups (***Figure 3***; see ***Figure 3—figure supplement 1*** for gating strategies). We used established approaches for gating of cell subsets (***Sanz et al., 2019***; ***Khodadadi et al., 2019***). This was only performed for the first wave participants, where cells were available for additional phenotyping by flow cytometry.

For HIV-negative participants, there were significant negative and positive correlations between CD4 T cell parameters, and between these and the CD8 T cell count and phenotypes (***Figure 3***, yellow box). There were negative correlations between CD4 and the CD8 CCR7+ T cell phenotype and

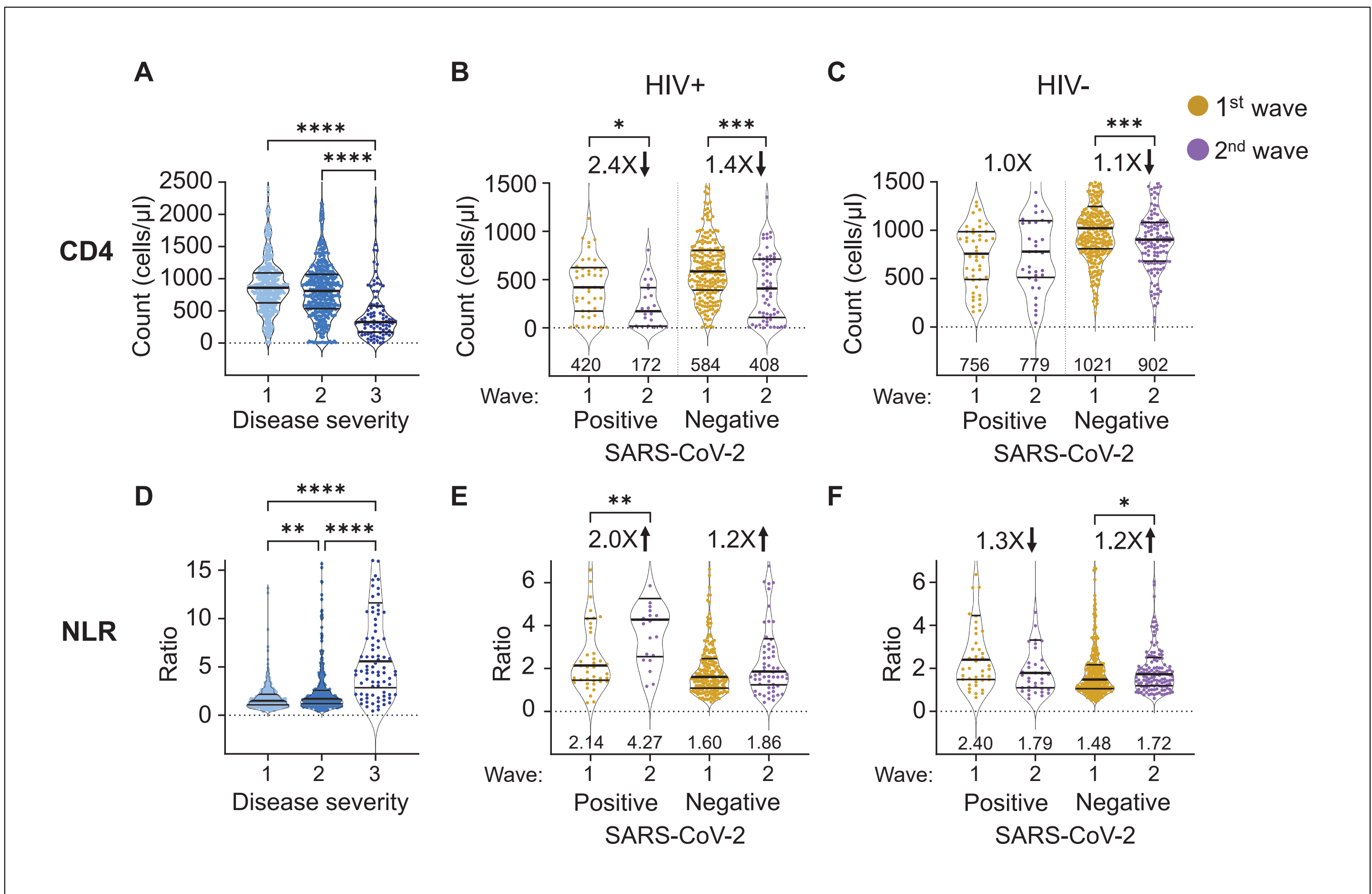

Figure 2. The differential effect of HIV on the CD4 count and neutrophil to lymphocyte ratio between waves. (**A**) The concentration of CD4 T cells in the blood in all participants in all infection waves and at all timepoints as a function of disease severity. Disease severity was scored as 1: asymptomatic, 2: mild, and 3: on supplemental oxygen or death. CD4 counts in PLWH (**B**) and HIV negative (**C**) participants in wave 1 versus wave 2 during active SARS-CoV-2 infection and after SARS-CoV-2 clearance. (**D**) Neutrophil to lymphocyte ratio (NLR) in the blood in all participants in all infection waves and at all timepoints as a function of disease severity. NLR in PLWH (**E**) and HIV negative (**F**) participants in wave 1 versus wave 2 during active SARS-CoV-2 infection and after SARS-CoV-2 clearance. SARS-CoV-2 positive indicates a timepoint where SARS-CoV-2 RNA was detected. Data shown as violin plots with median and IQR, with the median denoted below each plot. Fold-change in the second wave versus first wave is indicated, with arrow denoting direction of change. p-values are * <0.05; ** <0.01; *** < 0.001, **** < 0.0001 as determined by Kruskal-Wallis test with Dunn's multiple comparison correction or by Mann-Whitney U test. Plots scales were restricted to highlight changes close to the median.
The online version of this article includes the following figure supplement(s) for figure 2:

Figure supplement 1. The differential effect of HIV on the CD4 count and neutrophil to lymphocyte ratio between waves - full dataset and number of data points per plot.
Figure supplement 2. No significant increase in control of HIV infection at convalescence relative to active SARS-CoV-2 infection.

CD56+CD16+ NK cells (purple box). The fraction of NK cells positively correlated with the CXCR3 fraction of CD4 T cells, with HLA-DR on CD8 T cells, and with PD-1 on both cell types (purple box). In addition, there were correlations between CD8 T cell count and CD19 B cell parameters, such as fractions of naïve and memory B cells (red box). Interestingly, disease severity as well as the CD4/CD8 ratio showed correlations with B cell parameters, including the frequency of antibody secreting cells (ASC), which were lost in PLWH (orange box).

New correlations arose in PLWH, particularly involving CD8 T cells: CXCR3+ CD8 T cells were negatively correlated with disease severity but positively correlated with the CD4/CD8 ratio and the CD4 T cell count (*Figure 3*, black box). CD8 T cell activation (HLA-DR+) was correlated with several CD19+ B cell phenotypes (green box), and the plasma cell to plasmablast ratio, determined by CD138 expression, correlated with both CD4 and CD8 T cell phenotypes (blue box). In addition,

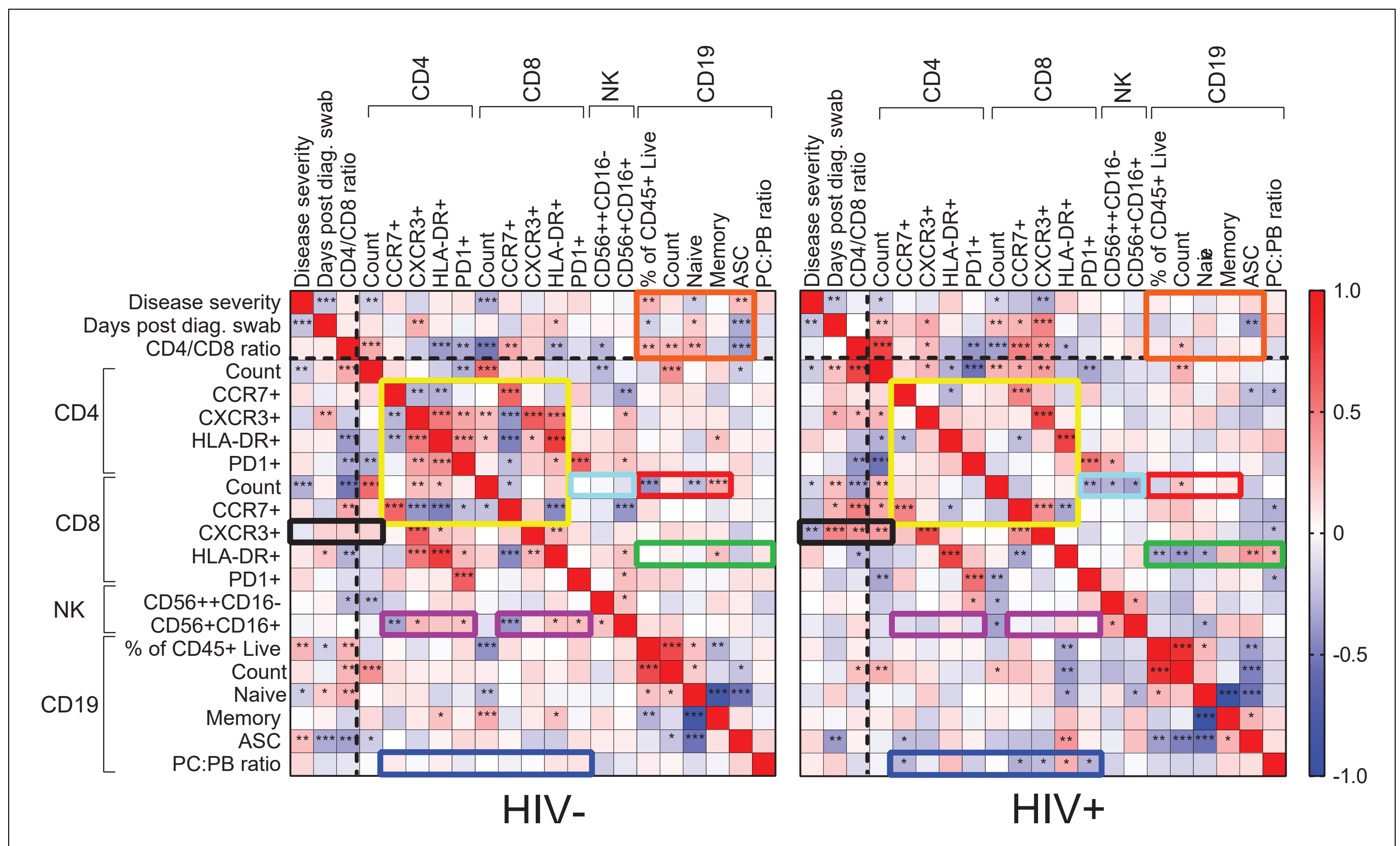

Figure 3. Immune cell and clinical correlates in HIV negative and PLWH groups. Spearman rank correlation values (ρ) are shown from red (1.0) to blue (−1.0). p-values per correlation are *< 0.5; **< 0.01; ***< 0.001. The number of matched pairs for HIV negative participants ranged from 77 to 229 and for PLWH from 48 to 164. Rectangles represent regions where a set of correlations is present in one group and absent in the other. Black dashed lines represent the divide between clinical and cellular parameters.

The online version of this article includes the following figure supplement(s) for figure 3:

Figure supplement 1. Gating strategy.

CD8 T cell count showed negative correlations with CD8 PD-1 and NK cell phenotypes only in PLWH (turquoise box).

Out of the set of markers examined, the combination of PD-1 and HLA-DR expression is linked to T cell activation (***Sauce et al., 2007***; ***Vollbrecht et al., 2010***), while CXCR3 expression is essential to recruitment of T cells to tissues (***Groom and Luster, 2011***). We therefore asked whether these markers showed differences between HIV negative and PLWH in the first infection wave during the time participants were positive for SARS-CoV-2, despite there being no significant differences in disease severity in this wave. In CD8 T cells, we observed a significant decrease in the fraction of CXCR3 expressing cells in the blood compartment in PLWH relative to HIV-negative participants (***Figure 4A***). We also observed an increase in the fraction of PD-1+HLA-DR+ cells (***Figure 4B***). For CD4 cells, there was no significant decrease in the fraction of CXCR3+ cells although a decrease was apparent (***Figure 4C***). Similarly to CD8 T cells, there was an increase in PD-1+HLA-DR+ CD4 T cells in PLWH (***Figure 4D***). There was no difference between PLWH and HIV-negative participants in any cell/marker combination after SARS-CoV-2 clearance.

Discussion

We observed that in our cohort, COVID-19 disease severity was higher in PLWH, consistent with some of the larger epidemiological studies (***Western Cape Department of Health in collaboration with the National Institute for Communicable Diseases, South Africa et al., 2021***; ***Geretti et al.,***

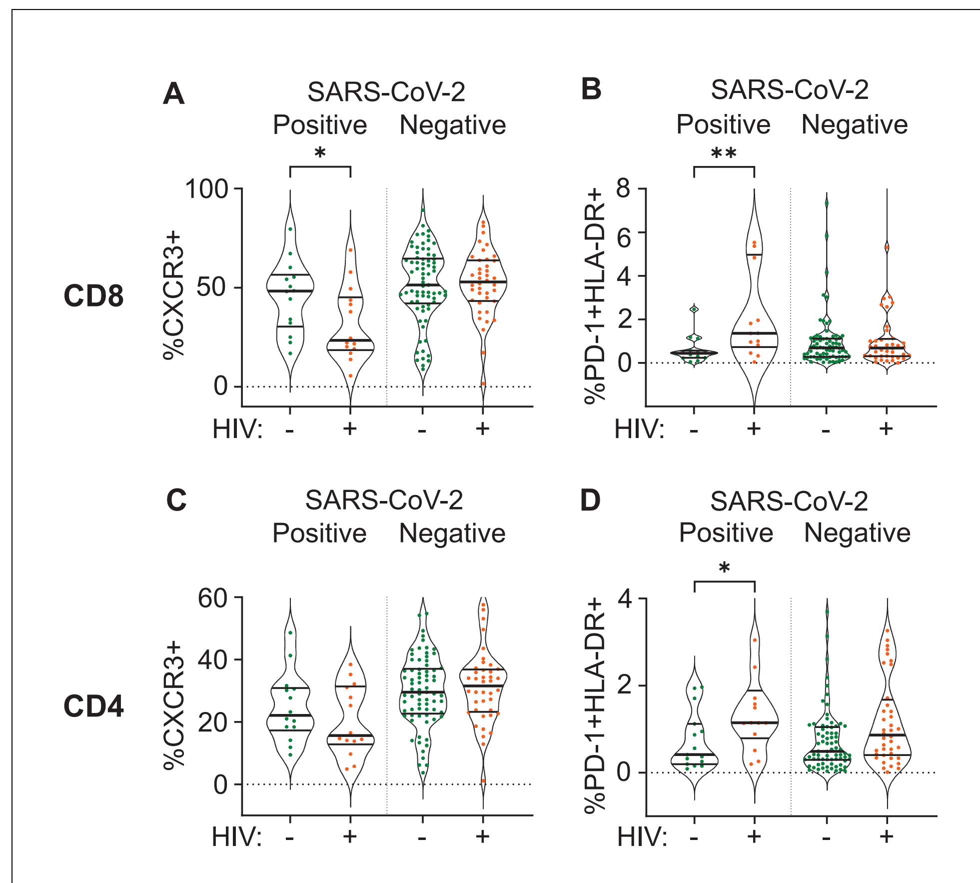

Figure 4. Differences between PLWH and HIV-negative participants in immune cell markers. Percent of CD8 T cells positive for CXCR3 (**A**) or double positive for HLA-DR and PD-1 (**B**). Percent of CD4 T cells positive for CXCR3 (**C**) or double positive for HLA-DR and PD-1 (**D**). Data is composed of 15 participant timepoints which were SARS-CoV-2+HIV-, 14 SARS-CoV-2+HIV+, 40 SARS-CoV-2-HIV+, and 74 SARS-CoV-2-HIV-, where SARS-CoV-2+ indicates SARS-CoV-2 RNA was detected in the upper respiratory tract. p-values for differences between PLWH and HIV-negative participants are * <0.05; ** <0.01; *** < 0.001, **** < 0.0001 as determined by the Mann-Whitney U test.

2021; ***Bhaskaran et al., 2021***; ***Tesoriero et al., 2021***; ***Braunstein et al., 2021***; ***Jassat et al., 2021a***), although in this study differences were detected in the frequency of participants requiring supplemental oxygen and not in mortality. Our cohort may not be a typical 'hospitalized cohort' as the majority of participants did not require supplemental oxygen. We therefore cannot discern effects of HIV on critical SARS-CoV-2 cases since these numbers are too small in the cohort. However, focusing on lower disease severity enabled us to capture a broader range of outcomes which predominantly ranged from asymptomatic to requiring supplemental oxygen. Understanding this part of the disease spectrum could be important since it may indicate underlying changes in the immune response which affect long-term quality of life and response to vaccines.

We observed a higher fraction of PLWH requiring supplemental oxygen relative to HIV negative participants in the second, Beta variant dominated SARS-CoV-2 infection wave in KwaZulu-Natal, South Africa. The odds ratio for requiring supplemental oxygen in the second wave for PLWH was 4.0 relative to HIV negative participants. The 95% confidence intervals were wide at 1.6–10.4, reflecting the relatively small number of participants. However, confidence intervals did not overlap one.

Consistent with HIV infection leading to more severe SARS-CoV-2 infection outcomes in our study is the much younger age of PLWH requiring supplemental oxygen relative to HIV negative participants (41 versus 63 years). PLWH on supplemental oxygen also had lower frequencies of hypertension and diabetes. Age, hypertension, and diabetes are risk factors for more severe COVID-19 disease (***Yang et al., 2020***; ***Guan et al., 2020***; ***Ambrosioni et al., 2021***; ***Jassat et al., 2021a***), and

their absence may indicate that the more severe outcome is driven by another factor, with HIV infection being the simplest explanation.

The cause of the difference between waves in PLWH may be because PLWH enrolled in the second infection wave had worse suppression of HIV with ART: both the fraction of timepoints where viremia was detected and where ART was absent were about twofold higher and indeed were very high at about 40%. We therefore expected that this showed a direct link between HIV viremia and the requirement for supplemental oxygen during COVID-19 disease in PLWH. However, there was no difference in the frequency of viremia between those requiring supplemental oxygen and those not.

Furthermore, the substantial recovery of CD4 T cell counts in PLWH after SARS-CoV-2 clearance in wave 2 may be consistent with the Beta variant having more impact on the CD4 count relative to the ancestral SARS-CoV-2 strain infections in the first wave. A similar pattern was seen in the NLR, which was higher in wave two relative to wave 1 in PLWH with active SARS-CoV-2 infection, but then decreased to similar levels upon convalescence. The role of the Beta variant is supported by data showing extensive evolution, increasing the ability of Beta to escape the interferon response and result in more efficient viral cell-to-cell transmission (***Guo et al., 2021***; ***Thorne et al., 2021***; ***Rajah et al., 2021***). Beta variant hospitalizations also led to more deaths in South Africa (***Jassat et al., 2021b***). Therefore, the effect of the variant on PLWH in addition to HIV suppression status should be considered.

Our data detailing the SARS-CoV-2 response of more defined immune cell subsets in PLWH versus HIV negative participants is limited by the data only being available for the first infection wave. However, even in samples from that wave, there were multiple differences in correlations between cell subsets in PLWH relative to HIV negative participants, which may be another indication of differences in the immune response to SARS-CoV-2. We cannot deduce from these associations whether the differences could have an impact on disease severity. However, the fraction of CXCR3+ CD8 T cells decreased in the blood compartment and PD-1+HLA-DR+ CD8 and CD4 T cells increased. The increase in PD-1+HLA-DR+ T cells indicates T cell activation (***Sauce et al., 2007***; ***Vollbrecht et al., 2010***) which associates with worse COVID-19 outcomes (***Chen et al., 2020b***). CXCR3 plays a key role in T cell homing to sites of inflammation and is activated by interferon-inducible ligands CXCL9, CXCL11, and CXCL10 (IP-10) (***Groom and Luster, 2011***; ***Rodda et al., 2021***). A decrease in CXCR3 indicates either that T cells are less able to home to the site of infection, or that there is more inflammation in PLWH during SARS-CoV-2 infection and therefore more homing of the CXCR3+ CD8 T cells to tissues so that the fraction of CXCR3+ cells left in the blood decreases. Either way, the combination of these changes likely indicates either more pronounced SARS-CoV-2 infection or an impaired response in PLWH despite the similar infection outcomes in this wave.

In summary, PLWH showed increased disease severity mostly restricted to the second infection wave, where the Beta variant was dominant. Increased severity was associated with low CD4 T cell counts and high NLR which stabilized post-SARS-CoV-2 clearance in second wave infected PLWH to close to wave 1 PLWH values, arguing for a synergy between SARS-CoV-2 and HIV to decrease CD4 T cell numbers and increase the NLR rather than the status of HIV infection alone determining these parameters. More work is required to understand how these HIV related immune perturbations influence long-term immunity to SARS-CoV-2 infection and whether vaccine response will be affected.

Materials and methods

Ethical statement and study participants

The study protocol was approved by the University of KwaZulu-Natal Institutional Review Board (approval BREC/00001275/2020). Adult patients (>18 years old) presenting at King Edward VIII, Inkosi Albert Luthuli Central, or Clairwood Hospitals in Durban, South Africa, between June 2020 to May 2021, diagnosed to be SARS-CoV-2 positive as part of their clinical workup and able to provide informed consent were eligible for the study. Written informed consent was obtained for all enrolled participants.

Clinical laboratory testing

An HIV rapid test and viral load quantification was performed from a 4 ml EDTA tube of blood at an accredited diagnostic laboratory (Molecular Diagnostic Services, Durban, South Africa) using the RealTime HIV negative1 viral load test on an Abbott machine. CD4 count, CD8 count, and a full blood count panel were performed by an accredited diagnostic laboratory (Ampath, Durban, South Africa). Depending on the volume of blood which was drawn, the CD8, CD4, and full blood count was not available for every participant, and numbers performed are detailed in the figure legends.

qPCR detection of SARS-CoV-2

RNA was extracted from combined oropharyngeal and nasophryngeal swabs from 140 µl viral transport medium using the QIAamp Viral RNA Mini kit (cat. no. 52906, QIAGEN, Hilden, Germany) according to manufacturer's instructions, and eluted into 100 µl AVE buffer. To detect SARS-CoV-2 RNA, 5 µl RNA was added to the TaqPath 1-step RT-qPCR mastermix. 3 SARS-CoV-2 genes (ORF1ab, S and N) were amplified using the TaqPath COVID-19 Combo Kit and TaqPath COVID-19 CE-IVD RT-PCR Kit (ThermoFisher Scientific, Massachusetts, United States) in a QuantStudio 7 Flex Real-Time PCR system (ThermoFisher Scientific). Data was analyzed using the Design and Analysis software (ThermoFisher Scientific). For positive samples, Ct values are represented as the average of the Ct values of all three genes. A sample was scored positive where at least two out of the three genes were detected, and inconclusive if only one of the genes was detected.

PBMC isolation and immune phenotyping by flow cytometry

PBMC were isolated by density gradient centrifugation using Histopaque 1077 (Sigma-Aldrich, St. Louis, Missouri, United States) and SepMate separation tubes (STEMCELL Technologies, Vancouver, Canada). For T cell and NK cell phenotyping, 10^6 fresh PBMCs were surface stained in 50 microliter antibody mix with the following antibodies from BD Biosciences (Franklin Lakes, NJ, USA): anti-CD45 Hv500 (1:100 dilution, clone HI30, cat. 560777); anti-CD8 BV395 (1:50 dilution, clone RPA-T8, cat. 563795); anti-CD4 BV496 (1:25 dilution, clone SK3, cat. 564651); anti-PD1 BV421 (1:50 dilution, clone EH12.1, cat. 562516); anti-CXCR3 PE-CF594 (1:25 dilution, clone 1C6/CXCR3, cat. 562451). The following antibodies were from BioLegend (San Diego, CA, USA): anti-CD19 Bv605 (1:100 dilution, clone HIB19, cat. 302244); anti-CD16 Bv650 (1:50 dilution, clone 3G8, cat. 302042); anti-CD56 Bv711 (1:50 dilution, clone HCD56, cat. 318336); anti-CD3 Bv785 (1:25 dilution, clone OKT3, cat. 317330); anti-CXCR5 FITC (1:25 dilution, clone J252D4, cat. 356914); anti-HLA-DR PE (1:50 dilution, clone L243, cat. 307606); anti-CCR7 PerCP-Cy5.5 (1:25 dilution, clone G043H7, cat. 353220); anti-CD38 PE-Cy7 (1:25 dilution, clone HIT2, cat. 303516); anti-ICOS APC (1:25 dilution, clone C398.4A, cat. 313510) and anti-CD45RA AF700 (1:25 dilution, clone HI100, cat. 304120). PBMCs were incubated with antibodies for 20 min at room temperature. For B-cell phenotyping, the following antibodies were used: (all from BioLegend) anti-CD45 APC (1:25 dilution, clone HI30, cat. 304012); anti-CD3 Bv711 (1:50 dilution, clone OKT3, cat. 317328), anti-CD14 Bv711 (1:25 dilution, clone M5E2, cat. 301838); anti-CD19 Bv605 (1:50 dilution, clone HIB19, cat. 302244); anti-CD27 Hv500 (1:50 dilution, clone O323, cat. 302836); anti-CD38 PE-Cy7 (1:25 dilution, clone HIT2, cat. 303516) and anti-CD138 BV785 (1:25 dilution, clone MI15, cat. 356538). Cells were then washed twice in PBS and fixed in 2% paraformaldehyde and stored at 4˚C before acquisition on FACSAria Fusion III flow cytometer (BD) and analyzed with FlowJo software version 9.9.6 (Tree Star). Depending on the volume of blood which was drawn, full phenotyping was only available for participants where sufficient blood was available for the assay.

Statistical analysis

Data is described with the non-parametric measures of median and interquartile range, and significance determined using the non-parametric Mann-Whitney U test for pairwise comparisons, Fisher Exact test for pairwise comparisons of frequencies, and the Kruskal-Wallis test with multiple comparison correction by the Dunn Method for comparisons involved more than two populations. All tests were performed using Graphpad Prism eight or Stata software.

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Additional information

Group author details

COMMIT-KZN Team
Moherndran Archary: Department of Paediatrics and Child Health, University of KwaZulu-Natal, Durban, South Africa; Kaylesh J Dullabh: Department of Cardiothoracic Surgery, University of KwaZulu-Natal, Durban, South Africa; Jennifer Giandhari: KwaZulu-Natal Research Innovation and Sequencing Platform, Durban, South Africa; Philip Goulder: Africa Health Research Institute and Department of Paediatrics, University of Oxford, Oxford, United Kingdom; Guy Harling: Africa Health Research Institute and the Institute for Global Health, University College London, London, United Kingdom; Rohen Harrichandparsad: Department of Neurosurgery, University of KwaZulu-Natal, Durban, South Africa; Kobus Herbst: Africa Health Research Institute and the South African Population Research Infrastructure Network, Durban, South Africa; Prakash Jeena: Department of Paediatrics and Child Health, University of KwaZulu-Natal, Durban, South Africa; Thandeka Khoza: Africa Health Research Institute, Durban, South Africa; Nigel Klein: Africa Health Research Institute and the Institute of Child Health, University College London, London, United Kingdom; Rajhmun Madansein: Department of Cardiothoracic Surgery, University of KwaZulu-Natal, Durban, South Africa; Mohlopheni Marakalala: Africa Health Research Institute and Division of Infection and Immunity, University College London, London, United Kingdom; Mosa Moshabela: College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa; Kogie Naidoo: Centre for the AIDS Programme of Research in South Africa, Durban, South Africa; Zaza Ndhlovu: Africa Health Research Institute and the Ragon Institute of MGH, MIT and Harvard, Cambridge, United States; Kennedy Nyamande: Department of Pulmonology and Critical Care, University of KwaZulu-Natal, Durban, South Africa; Nesri Padayatchi: Centre for the AIDS Programme of Research in South Africa, Durban, South Africa; Vinod Patel: Department of Neurology, University of KwaZulu-Natal, Durban, South Africa; Theresa Smit: Africa Health Research Institute, Durban, South Africa; Adrie Steyn: Africa Health Research Institute and Division of Infectious Diseases, University of Alabama at Birmingham, Birmingham, United States

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Author contributions

Farina Karim, Conceptualization, Resources, Data curation, Formal analysis, Project administration; Inbal Gazy, Formal analysis, Methodology; Sandile Cele, Robert Krause, Mallory Bernstein, Data curation, Formal analysis; Yenzekile Zungu, Daniel Muema, Data curation; Khadija Khan, Yashica Ganga, Dirhona Ramjit, Project administration; Hylton Rodel, Ntombifuthi Mthabela, Investigation; Matilda Mazibuko, Willem Hanekom, Bernadett Gosnell, Tulio de Oliveira, Resources; Thumbi Ndung'u, Methodology; COMMIT-KZN Team, Richard J Lessells, Writing - review and editing; Emily B Wong, Conceptualization; Mahomed-Yunus S Moosa, Resources, Supervision; Gil Lustig, Formal analysis; Alasdair Leslie, Formal analysis, Supervision; Henrik Kløverpris, Formal analysis, Supervision, Investigation; Alex Sigal, Conceptualization, Funding acquisition, Writing - review and editing

Author ORCIDs

Farina Karim https://orcid.org/0000-0001-9698-016X
Robert Krause https://orcid.org/0000-0003-1558-0397
Thumbi Ndung'u http://orcid.org/0000-0003-2962-3992
Richard J Lessells http://orcid.org/0000-0003-0926-710X
Alex Sigal https://orcid.org/0000-0001-8571-2004

Ethics

Human subjects: The study protocol was approved by the University of KwaZulu-Natal Institutional Review Board (approval BREC/00001275/2020). Adult patients (>18 years old) presenting either at King Edward VIII, Inkosi Albert Luthuli Central or Clairwood Hospitals in Durban, South Africa, between June 2020 to May 2021, diagnosed to be SARS-CoV-2 positive as part of their clinical workup and able to provide informed consent were eligible for the study. Written informed consent was obtained for all enrolled participants.

Decision letter and Author response

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Additional files

Supplementary files

- Source data 1. Participant information.
- Supplementary file 1. Summary of case visits.
- Supplementary file 2. Timing of enrollment in PLWH and HIV negative participants.
- Supplementary file 3. ART regimen in PLWH as determined by LC-MS/MS.
- Supplementary file 4. Infection wave 1 COVID-19 disease severity by HIV status.
- Supplementary file 5. Infection wave 2 COVID-19 disease severity by HIV status.
- Supplementary file 6. Comparison between HIV negative participants requiring and not requiring supplemental oxygen.
- Supplementary file 7. Comparison between PLWH requiring and not requiring supplemental oxygen.
- Transparent reporting form

Data availability

All data generated or analysed during this study are included in the manuscript and supporting files.

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CHAPTER 3:

Persistent SARS-CoV-2 infection and intra-host evolution in association with advanced HIV infection

This chapter is a case study to determine the effect of advanced HIV disease mediated immunosuppression on SARS-CoV-2 infection and intra-host evolution. The case study comprised of a severely immunocompromised HIV viremic participant with an enrolment CD4+ T cell count of 6 cells/µL and HIV viral load of 34,151 copies/mL. SARS-CoV-2 was detectable until day 216 post-diagnostic swab. SARS-CoV-2 viruses from the infection were sequenced and a phylogenetic analysis was conducted, and it was found to be consistent with intra-host evolution of mutations which lead to escape from neutralizing antibody immunity. This case study demonstrated the consequences of prolonged SARS-CoV-2 infection in immunocompromised individuals and highlights the potential for the continued emergence of new variants.

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Persistent SARS-CoV-2 infection and intra-host evolution in association with advanced HIV infection

Karim F, PhD[1,2], Moosa MYS, MD, PhD[3], Gosnell BI, MD, PhD[3], Cele S, MSc[1,2], Giandhari J, PhD[4], Pillay S, MSc[4], Tegally H, MSc[4], Wilkinson E, PhD[4], San JE, MSc[4], Msomi N, MD,PhD[5], Mlisana K, MD, PhD[6,7], Khan K, PhD[1], Bernstein M, PhD[1], Manickchund N, MD,PhD[3], Singh L, PhD[1], Ramphal U, MSc[1], COMMIT-KZN Team, Hanekom W, MD, PhD[1], Lessells RJ, MD, PhD[4,7], Sigal A, PhD[1,2,8✉*], de Oliveira T, PhD[4,7,9✉*]

[1] Africa Health Research Institute, Durban, South Africa
[2] School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal, Durban, South Africa
[3] Department of Infectious Diseases, University of KwaZulu-Natal, Durban, South Africa
[4] KwaZulu-Natal Research Innovation & Sequencing Platform, School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal, Durban, South Africa
[5] Discipline of Virology, University of KwaZulu-Natal, School of Laboratory Medicine and Medical Sciences and National Health Laboratory Service, Durban, South Africa
[6] National Health Laboratory Service, Johannesburg, South Africa
[7] Centre for the AIDS Programme of Research in South Africa (CAPRISA), University of KwaZulu-Natal, Durban, South Africa
[8] Max Planck Institute for Infection Biology, Berlin, Germany
[9] Department of Global Health, University of Washington, Seattle, United States

* Prof. Sigal and Prof. de Oliveira contributed equally to this article

✉ Correspondence:
Professor Tulio de Oliveira
KwaZulu-Natal Research Innovation & Sequencing Platform
Nelson R Mandela School of Medicine
1st Floor, K-RITH Tower Builing
719 Umbilo Road
Durban 4001
South Africa
deoliveira@ukzn.ac.za

Professor Alex Sigal
Nelson R Mandela School of Medicine
3rd Floor, K-RITH Tower Builing
719 Umbilo Road
Durban 4001
South Africa
alex.sigal@ahri.org

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Summary (100 words)

While most people effectively clear SARS-CoV-2, there are several reports of prolonged infection in immunosuppressed individuals. Here we present a case of prolonged infection of greater than 6 months with shedding of high titter SARS-CoV-2 in an individual with advanced HIV and antiretroviral treatment failure. Through whole genome sequencing at multiple time-points, we demonstrate the early emergence of the E484K substitution associated with escape from neutralizing antibodies, followed by other escape mutations and the N501Y substitution found in most variants of concern. This provides support to the hypothesis of intra-host evolution as one mechanism for the emergence of SARS-CoV-2 variants with immune evasion properties.

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Introduction

Coronavirus disease 2019 (COVID-19) is an acute respiratory illness caused by the severe acute respiratory syndrome-related coronavirus 2 (SARS-CoV-2). While most people effectively clear SARS-CoV-2 infection[1,2], there are now several reports of prolonged infection associated with underlying immunosuppression[3-14]. In many of these cases, there has been evidence of intra-host evolution, characterised by the emergence of viruses with mutations in the spike glycoprotein. It has been hypothesized that intra-host evolution may be one mechanism for the emergence of SARS-CoV-2 variants[4,15,16]. Here we describe a case of prolonged SARS-CoV-2 infection in a person with advanced Human Immunodeficiency Virus (HIV) and antiretroviral treatment failure without either clinically induced immunosuppression or neutralizing antibody based treatment for SARS-CoV-2 infection, and present analysis of the virus evolution over time.

Case Report

Am HIV-positive female in her late 30s was admitted to hospital in September 2020 with sore throat, cough and dyspnoea. Symptom onset was 12 days prior to admission. She had been on HIV antiretroviral therapy (ART) since 2006, most recently a fixed-dose combination of tenofovir, emtricitabine and efavirenz. Other relevant medical history included a single episode of tuberculosis in 2006, and chronic asthma for which she received inhaled budesonide and salbutamol. On the day of admission (day 0), SARS-CoV-2 reverse-transcriptase polymerase chain reaction (RT-PCR) of a nasopharyngeal swab specimen was positive (Allplex™ 2019-nCoV Assay, Seegene Inc., Seoul, South Korea; mean cycle threshold (Ct) 18.5). Sputum Xpert® MTB/RIF Ultra (Cepheid, Sunnyvale, CA) was negative. Other routine investigations are shown in Table S1. She was managed in a general COVID-19 ward, received oxygen via face mask and a six-day course of dexamethasone, and was discharged home after nine days.

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During the hospital admission, she was enrolled in a prospective cohort study exploring the effect of HIV infection on the natural history and immune response to SARS-CoV-2 infection[17]. She was reviewed at enrolment (day 6 post-admission and day 16 post-symptom onset) when she was placed on non-high flow supplemental oxygen, and in the clinic on day 20 and day 34, when she was asymptomatic. On day 71, she complained of chest tightness. Oxygen saturation (SpO_2) was documented to drop from 96% to 76% on exertion. Chest X-ray showed non-specific perihilar infiltrates. She was treated empirically for *Pneumocystis jiroveci* pneumonia on an ambulatory basis with 21 days of co-trimoxazole and prednisone. She was reviewed on day 106 when she reported only fatigue, and on day 190 when she was asymptomatic. On day 190, antiretroviral therapy was switched to a fixed-dose combination of tenofovir, lamivudine and dolutegravir. On day 206, she achieved HIV viral load suppression (i.e. < 50 cp/ml).

Methods

Ethical considerations

The protocol for the prospective cohort study exploring the effect of HIV and TB on the natural history and immune response to SARS-CoV-2 infection was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (ref. BREC/00001275/2020) and the KwaZulu-Natal Provincial Health Research and Ethics Committee (ref. KZ_202005_003). We obtained written informed consent from all participants. Additionally, we obtained written informed consent for publication from the participant described in this report.

Specimen collection and laboratory testing

We collected nasopharyngeal swabs and blood specimens on day 6 (day of study enrolment), day 20, day 34, day 71, day 106, day 190, day 204, day 216 and day 233. We performed SARS-CoV-2 RT-PCR testing on all swabs using either the Taqpath™ COVID-19 RT-PCR assay (Thermo Fisher Scientific, Waltham, MA) or the Allplex™ 2019-nCoV Assay (Seegene Inc., Seoul, South Korea). Anti-SARS-CoV-2

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antibodies in the blood were measured using the Test-it CoV-2 IgM/IgG test kit (Lifeassay Diagnostics, Cape Town, South Africa), which detects IgM and IgG against the S1 subunit of spike. HIV-1 viral load was measured using the Abbott *m*2000 RealTi*m*e assay (Abbott Laboratories, Abbott Park, IL) and CD4+ count using the BD FACSCanto™ (BD Biosciences, San Jose, CA). We performed antiretroviral drug level testing using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Sample analysis was performed using an Agilent High Pressure Liquid Chromatography (HPLC) system (Agilent Technologies, Santa Clara, CA) coupled to the AB Sciex 5500 (AB Sciex, Framingham, MA), triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) TurboIonSpray source. The LC-MS/MS method was developed and optimised for the quantitation of tenofovir, emtricitabine, efavirenz, lopinavir, ritonavir, nevirapine, zidovudine, lamivudine, abacavir, atazanvir and dolutegravir in the same sample. We performed HIV-1 sequencing on all specimens with viral load >1000 copies/mL using the Illumina MiSeq instrument (Illumina, Inc., San Diego, CA) and used the Stanford HIV drug resistance database (version 9.0) for genotypic resistance interpretation (Supplementary Appendix).

SARS-CoV-2 whole genome sequencing

Six specimens collected as part of the cohort study (day 6 – day 190) and the residual nasopharyngeal swab specimen collected for routine diagnosis on day 0 were all processed at the KwaZulu-Natal Research Innovation & Sequencing Platform laboratory (KRISP). We extracted SARS-CoV-2 ribonucleic acid (RNA) using the Viral NA/gDNA Kit on the automated chemagic™ 360 system (PerkinElmer, Inc., Waltham, MA). We converted RNA to cDNA using the Superscript IV First Strand synthesis system (Life Technologies, Carlsbad, CA) and random hexamer primers, using the ARTIC V3 protocol[18]. We performed SARS-CoV-2 whole genome amplification by multiplex PCR using primers designed on Primal Scheme (http://primal.zibraproject.org/), generating 400bp amplicons with an overlap of 70bp to cover the 30kb SARS-CoV-2 genome. We cleaned up PCR products using AmpureXP purification beads (Beckman Coulter, High Wycombe, UK) and quantified the products

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using the Qubit dsDNA High Sensitivity assay on the Qubit 4.0 instrument (Life Technologies, Carlsbad, CA). We then used the Illumina® Nextera Flex DNA Library Prep kit (Illimuna, Inc., San Diego, CA) to prepare indexed paired end libraries of genomic DNA. We normalized sequencing libraries to 4nM, then pooled and denatured with 0.2N sodium acetate. We spiked the 12pM sample library with 1% PhiX (PhiX Control v3 adapter-ligated library used as a control). We sequenced libraries on a 500-cycle v2 MiSeq Reagent Kit on the Illumina MiSeq instrument (Illumina, Inc., San Diego, CA). Full details of our amplification and sequencing protocol have been previously published[19,20]. We assembled paired-end fastq reads using Genome Detective 1.132 (https://www.genomedetective.com) and the Coronavirus Typing Tool[21]. Low-quality mutations were filtered out of the initial assembly generated from Genome Detective using bcftools 1.7-2 mpileup method. All mutations were confirmed visually with BAM files using Geneious software (Biomatters, Auckland, New Zealand). For analysis of low frequency mutations, we used LoFreq v.2.1.5[22] to call intrahost variants relative to the Wuhan-Hu-1 NC_045512.2 reference. We employed a false discovery rate (FDR) cut-off of 1%. Additionally, LoFreq automatically applies default filters including minimum sequencing depth of x10, dynamic Bonferroni correction for variant quality and strand bias filtering. We annotated the variants using snpEff v4.5[23] and extracted them for additional processing and visualizing using SnpSift v.4.3t[23].

Genomes in this study were analyzed against a global reference dataset of 3566 genomes, including 486 genomes from South Africa, using a custom build of the SARS-CoV-2 NextStrain (https://github.com/nextstrain/ncov). The workflow performs alignment of genomes, phylogenetic tree inference, tree dating and ancestral state construction and annotation. The phylogenetic tree was visualized using ggplot and ggtree. In addition, resulting time scaled phylogeny can be viewed interactively and has been shared publicly on the NGS-SA NextStrain page at: https://nextstrain.org/groups/ngs-sa/COVID19-Alex-2021.04.29. Downstream analysis and visualization was performed using custom scripts in R[24].

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Results

SARS-CoV-2 RT-PCR was positive at all time-points between day 0 and day 216, with mean Ct ranging from 16.4 to 31.6 (Table 1 & Fig. 1). Anti-S1 IgG and IgM were not detected at any time-point using a commercial rapid test kit. At study enrolment (day 6), HIV-1 RNA was 34,151 copies/mL and CD4+ cell count was 6 cells/µL. HIV-1 RNA remained elevated at all time points up to day 204, then decreased to <50 copies/mL two weeks following the switch to TLD. Until viral suppression, CD4+ cell count was persistently below 20 cells/µL. The antiretroviral drugs tenofovir, emtricitabine and efavirenz were detectable in plasma specimens only on day 71 and day 106. At enrolment, there were HIV-1 reverse transcriptase mutations associated with high-level resistance to efavirenz (V106M, K103R + V179D) and low-level resistance to tenofovir (K70Q). At the later-time points when antiretrovirals were detectable, the M184V mutation associated with high-level resistance to emtricitabine was also detected (Table S2).

We generated high quality whole SARS-CoV-2 genomes from samples at all seven time-points. Phylogenetic analysis was consistent with persistent infection and accelerated intra-host evolution (Fig. 1). All genomes fell within PANGO lineage B.1.1.273[25], a South African lineage that was previously part of B.1.1.56, one of the most prevalent lineages in the first wave of the epidemic in the province of KwaZulu-Natal in South Africa, the location of the study[26]. The sequence at day 0 had the D614G mutation in addition to two additional spike mutations (G142V and D796Y). At day 6, the E484K substitution in the receptor-binding domain (RBD) emerged, along with a substitution in the S2 subunit (A1078V) (Fig. 2). The E484K mutation remained in the consensus sequence until day 34, but then persisted at low frequency up to day 190 (Suppl. Fig. 1, Table S3). At the later time points, there was a significant shift in the virus population, with the emergence at day 71 of the K417T and F490S mutations in the spike RBD, followed by the emergence of L455F and F456L at day 106. At day 190, there was the emergence of D427Y and N501Y.

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Figure 1. Phylogenetic analysis of seven SARS-CoV-2 whole genome sequences from a patient with advanced HIV. Shown in Panel A is a maximum-likelihood phylogenetic tree with patient sequences (orange) at seven time-points in relation to 486 representative South African and 3080 other global sequences. Panel B zooms in to show the relationship between the six patient-derived sequences from day 0, day 6, day 20, day 34, day 71, day 106 and day 190. Panel C shows the HIV-1 viral load in log scale (blue, left y-axis) and mean SARS-CoV-2 RT-PCR cycle threshold (Ct) value (orange, right y-axis) at each sampling time point .

S

	9	142	144	190	417	427	455	456	484	490	501	614	796	1078
	P	G	Y	R	K	D	L	F	E	F	N	D	D	A
D0		V										G	Y	
D6									K			G		V
D20									K			G		
D34			-						K			G		
D71	L		-	K	T					S		G	Y	
D106			-				F	L		S		G		
D190			-	K		Y				S	Y	G	Y	

N

	145	203	204	205
	H	R	G	T
D0		K	R	
D6		K	R	
D20		K	R	
D34		K	R	
D71		K	R	
D106		N/A	N/A	
D190	Y			I

ORF1a

	1233	1267	1352	1474	1500	2201	2267	2796	2797	3674	3675	3676	3677
	K	S	A	T	H	N	R	M	S	L	S	G	F
D0			V	I			G						
D6			V	I			G						
D20			V	I			G						
D34			V	I			G			F			
D71	N		V	I			G				-	-	-
D106		A	V	I		Y	G	X	F				
D190	N		V	I	Y		G				-	-	-

ORF1a

	176	314	657	1028	1161	1289	1936
	A	P	M	D	P	T	P
D0	V	L			L		
D6	V	L				H	
D20	V	L			L		
D34	V	L			L		
D71	V	L		A			
D106		L					
D190	V	L	T	A			S

Figure 2. Locations of SARS-CoV-2 amino acid substitutions and deletions over time. Shown are the amino acid substitutions (marked by the substituted amino acid) and deletions (marked by a dash at the amino acid location) in the consensus sequences at each of the seven time-points (D0, D6, D20, D34, D71, D106 and D190). S spike, N nucleocapsid, ORF open reading frame**.**

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Discussion

We present a case of persistent SARS-CoV-2 infection with accelerated intra-host evolution in a patient with advanced HIV and antiretroviral treatment failure. Despite a short clinical illness of moderate severity, SARS-CoV-2 PCR positivity persisted up to 216 days. We demonstrate significant shifts in the virus population over that time, involving multiple mutations at key neutralizing antibody epitopes in the spike RBD and N terminal domain (NTD). Unlike many of the other reported cases, virus evolution was not driven by the receipt of immune-based therapies (convalescent plasma or monoclonal antibodies)[3-7,11]. Most other cases of persistent infection have been described in people with haematological malignancies or people receiving immunosuppressive therapies for solid organ transplants or other chronic medical conditions[3-10,12]. There is one other documented case of prolonged infection in a person with HIV - in that case the person was ART-naïve with profound immunosuppression and genome sequencing revealed only a single emergent mutation in spike (T719I) at day 53[12]. Although awaiting further viral and immunological analysis, the genomic and clinical data suggest that virus evolution may have been driven by selective pressure from an impaired neutralizing antibody response.

The E484K mutation in the spike RBD emerged very early in the course of infection, six days post-diagnosis and about 19 days post-symptom onset. This mutation is associated with resistance to class 2 neutralizing antibodies (NAbs)[27-33]. E484K has been observed in other cases of persistent infection, although in these cases it has emerged at a later stage in the course of infection[3,6,11]. At day 34, the Y144 deletion emerged and persisted up to day 106. This is in one of the recurrent deletion regions (RDR) in NTD that are associated with NAb escape[34], and deletions in that specific region have been observed frequently in persistent infection[3,5-7,9-11,14]. As infection persisted, there were significant shifts in the virus population and the E484K mutation was replaced by mutations at other sites in the RBD associated with resistance to class 1, 2 and 3 NAbs (K417T and F490S at day 71 and then additionally L455F and F456L at day 106)[30]. The pattern of mutations is notable for RBD

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mutations at E484, K417 and N501, three of the signature mutations of the 501Y.V2 (B.1.351) variant of concern[15]. We believe this provides further evidence to support accelerated intra-host evolution as a plausible mechanism for the emergence of 501Y.V2 in South Africa.

There is some evidence that HIV is associated with an increased risk of more severe disease and death from COVID-19[17], although in most studies the association is modest[36-39]. Two studies from South Africa have demonstrated that HIV is associated with suboptimal CD4+ T-cell and humoral immune responses to SARS-CoV-2, particularly in the absence of suppressive ART[17,40]; and one study from the United States suggested that HIV is associated with lower neutralizing antibody titres following natural infection[41]. In the case described here, based on the very low CD4+ count there was putative immunosuppression as a result of antiretroviral treatment failure and HIV drug resistance. The impairment of both cellular and humoral adaptive immunity from HIV was obviously profound enough to delay clearance of SARS-CoV-2[42]. Importantly, clearance of SARS-CoV-2 closely followed more effective suppression of HIV (Fig. 1C).

South Africa, has the largest HIV treatment programme in the world, with an estimated 5.2 million people on ART. Despite this, there remains a considerable burden of advanced HIV disease[43,44]. Disruptions to HIV programmes as a result of COVID-19 could potentially increase the burden of advanced HIV[45]. These findings highlight the importance of maintaining essential health services in high HIV prevalence settings, and accelerating progress towards 90-90-90 targets[46]. Preliminary results in people with well-controlled HIV suggest immune responses to COVID-19 vaccines are equivalent to those in HIV-negative people[47,48], but more work is needed to understand immunogenicity and effectiveness in PLHIV and to design optimal dosing strategies, particularly for those with advanced HIV. If persistent infection does occur more frequently in the context of HIV, it may provide justification for prioritising people living with HIV for COVID-19 vaccination.

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Table 1 Results of SARS-CoV-2 PCR assays, antibody assays, HIV viral load and CD4+ count

Time point	SARS-CoV-2 PCR	Mean Ct*	SARS-CoV-2 IgG	SARS-CoV-2 IgM	HIV-1 RNA (copies/mL)	CD4+ count (cells/µL)
Day 0	Positive	18.5	-	-	-	-
Day 6	Positive†	16.4†	Negative	Negative	34 151	6
Day 20	Positive†	19.2†	Negative	Negative	6394	6
Day 34	Positive	21.6	Negative	Negative	5477	5
Day 71	Positive	19.2	Negative	Negative	4309	7
Day 106	Positive	26.9	Negative	Negative	22 905	15
Day 190	Positive	21.3	Negative	Negative	14 964	6
Day 204	Positive	29.1	Negative	Negative	<50	19
Day 216	Positive	31.6	Negative	Negative	-	64
Day 233	Negative	>40	Negative	Negative	<50	46

* Mean Ct value calculated as mean of the Ct values for each of the three gene targets

† Specimens from day 6 and day 20 tested with the Taqpath™ COVID-19 RT-PCR assay (Thermo Fisher Scientific, Waltham, MA) targeting ORF1ab, N gene, and S gene; specimens from all other time points tested with the Allplex™ 2019-nCoV Assay (Seegene Inc., Seoul, South Korea) targeting E gene, N gene, and RdRp gene

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CHAPTER 4:

Variable SARS-CoV-2 evolution and vaccine response in individuals with advanced HIV disease

This paper examined the SARS-CoV-2 evolution and response to Pfizer BNT162b2 mRNA vaccination of immunosuppressed individuals with advanced HIV disease. Participants had SARS-CoV-2 infection lasting between 130 to 293 days and 3 of 5 had extensive SARS-CoV-2 evolution. The remaining two participants had few substitutions and deletions in the SARS-CoV-2 genome but were re-infected with another variant soon after their original infection. The participants with advanced HIV disease who were HIV viremic at vaccination demonstrated a poor neutralisation response post-vaccination. These findings indicate that HIV infection needs to be controlled by ART to facilitate an effective SARS-CoV-2 neutralizing antibody response by vaccination.

Variable SARS-CoV-2 evolution and vaccine response in individuals with advanced HIV disease

Farina Karim[1,2], Mallory Bernstein[1],..., Janine-Lee Upton[1], ..., Khadija Khan[1,2],... Kajal Reedoy[1], Zesuliwe Jule[1],... Matilda Mazibuko[1], Yashica Ganga[1], Katya Govender[1], Nokuthula Ngcobo[1], Willem Hanekom[1,3], Ron Milo[4], Anne von Gottberg[5,6], Nombulelo Magula[7], COMMIT-KZN Team[§], Nithendra Manickchund[8], Bernadett I. Gosnell[8], Penny L. Moore [5,6,9,10], Richard J. Lessells[9,11], Tulio de Oliveira[9,11,12,13], Mahomed-Yunus S. Moosa[8], Alex Sigal[1,2,9*]

[1]Africa Health Research Institute, Durban, South Africa. [2]School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal, Durban, South Africa. [3]Division of Infection and Immunity, University College London, London, UK. [4]Department of Plant and Environmental Sciences, Weizmann Institute of Science, Rehovot, Israel. [5]National Institute for Communicable Diseases of the National Health Laboratory Service, Johannesburg, South Africa. [6]SAMRC Antibody Immunity Research Unit, School of Pathology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa. [7]Department of Internal Medicine, Nelson R. Mandela School of Medicine. University of Kwa-Zulu Natal. [8]Department of Infectious Diseases, Nelson R. Mandela School of Clinical Medicine, University of KwaZulu-Natal, Durban, South Africa. [9]Centre for the AIDS Programme of Research in South Africa, Durban, South Africa. [10]Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Cape Town, South Africa. [11]KwaZulu-Natal Research Innovation and Sequencing Platform, Durban, South Africa. [12]Centre for Epidemic Response and Innovation, School of Data Science and Computational Thinking, Stellenbosch University, Stellenbosch, South Africa. [13]Department of Global Health, University of Washington, Seattle, USA.

Rapid emergence of new variants through SARS-CoV-2 evolution compromises the effectiveness of current vaccines. Evolution of SARS-CoV-2 can happen during long-term infection in immunosuppressed individuals with advanced HIV disease, where uncontrolled HIV viremia and depletion of CD4 T cells leads to immune compromise. It is unclear how prevalent SARS-CoV-2 evolution is in this group and whether vaccination would effectively induce immunity. Here we tracked five mostly non-hospitalized individuals with advanced HIV disease and uncontrolled viremia at enrollment who were infected with different variants of SARS-CoV-2. All had long-term SARS-CoV-2 infection. The SARS-CoV-2 mutation rate was highly variable and two of the individuals showed strong evidence for re-infection. The neutralizing antibody response after mRNA vaccination was also variable and contrasted with the homogenous response in individuals who were HIV negative or with suppressed HIV and higher CD4 T cell counts, indicating that primary vaccination alone may not offer protection to all individuals with advanced HIV disease. This study reinforces the importance of effective antiretroviral therapy to reduce SARS-CoV-2 evolution and increase vaccine response.

Introduction

Evolution of new variants compromises vaccine induced immunity from being able to prevent symptomatic infection, although protection from more severe disease may be maintained because it requires lower levels of neutralizing antibodies[1] and because there is less variant escape from the T cell response[2]. Emergence of a new variant requires extensive evolution of SARS-CoV-2. The Omicron BA.1 variant, initially detected in Botswana and South Africa in late 2021, did not circulate in intermediate forms prior to its emergence. One possibility of how an extensively evolved variant can abruptly emerge is reverse zoonosis[3-12], the infection of an animal reservoir where the virus mutates to adapt to the new host, and then re-infects a human host. A second possibility is evolution in long-term infection in an immunosuppressed individual[13-25] or possibly a small group of such individuals. Such evolution in immunocompromised hosts is not unique to SARS-CoV-2 and has been shown in influenza and salmonella infections[26,27]. A third possibility, that evolution may have occurred in a graded manner at a location without genomic surveillance and spread from there, is currently not well supported.

Case studies of SARS-CoV-2 infections in immunosuppressed individuals show prolonged infection and the evolution of genomic changes in the SARS-CoV-2 spike protein associated with escape from neutralizing antibodies[22], although mutations outside of spike are also common[28]. SARS-CoV-2 long-term infection and evolution happens in some people who are immunosuppressed because of long-term uncontrolled HIV infection, termed advanced HIV disease[13-15,24]. Uncontrolled HIV infection leads to CD4 T cell depletion, and advanced HIV disease is defined as a CD4 T cell count < 200 cells/microliter. About 1 in 10 people living with HIV (PLWH) in South Africa has advanced HIV disease[29,30]. The number of PLWH in South Africa is about 8 million as of 2021 according to UNAIDS[31]. Therefore, the number of people immunosuppressed because of advanced HIV disease is ~800,000 in South Africa alone.

We have previously reported on the evolution of SARS-CoV-2 from ancestral virus infection in an individual who was immunosuppressed because of advanced HIV disease (participant 27 in this study)[13,14]. The virus which evolved over 6 months gained immune escape from neutralizing antibodies elicited by ancestral SARS-CoV-2, Delta variant infection, Pfizer BNT162b2 vaccination, and to a lesser extent Beta variant infection. In addition, the virus evolved an increased propensity for cell-to-cell fusion, possibly as a second mechanism to escape antibody neutralization[32].

Vaccination may be one possible strategy to reduce long-term SARS-CoV-2 infection and evolution in people with advanced HIV disease. PLWH have been shown to have moderately worse outcomes with SARS-CoV-2[33-40]. We and others have found decreased neutralizing antibody responses to SARS-CoV-2 infection in PLWH[40,41] which was more pronounced when detectable HIV viremia was present[41]. However, we did not observe a difference between PLWH and HIV negative individuals in neutralizing antibody activity after the Johnson and Johnson Ad26.CoV2.S vaccine alone or in hybrid immunity elicited by infection followed by vaccination[41]. This was similar to the results observed in antibody immunity elicited by ChAdOx and mRNA vaccines[42-46] which also did not substantially differ between the HIV negative and PLWH groups. However, in the subset of PLWH with CD4 counts of <200 cells/microliter, the vaccines were reported to be less effective at eliciting a neutralizing response[40,47,48].

It is unclear if extensive SARS-CoV-2 evolution is a typical outcome in advanced HIV disease or whether there is heterogeneity between individuals in the evolution rate. It is also unclear if vaccination could prevent such evolution by eliciting an effective neutralizing antibody response. Here we tracked SARS-CoV-2 infection in five participants with advanced HIV

disease. We determined that there was extensive variability in both the rate of acquisition of SARS-CoV-2 mutations and in the response to the Pfizer BNT162b2 mRNA vaccine.

Results

Five participants with advanced HIV disease and HIV viremia were enrolled in our study tracking SARS-CoV-2 infection in South Africa. These participants were between 20 and 42 years of age and with a Covid-19 diagnosis date ranging between September 2020 and December 2021 (Table S1). Participants were outpatients for 82% of study visits. During each study visit, a combined nasopharyngeal and oropharyngeal swab was taken for sequencing. When practicable, the virus was isolated by outgrowth. To avoid possible erroneous sequencing due to contamination of samples with low levels of SARS-CoV-2 template, only sequences derived from samples with a qPCR cycle threshold (Ct) below 30 or sequences from outgrown viruses were analyzed.

Sequencing covered a time span between 28 days for participant 255 to 190 days for participant 27, but the duration of infection was substantially longer, with time to first undetectable Ct ranging from 130 days for participant 96 to 293 days for participant 255 (Table S1). For all participants except 255, diagnosis was closely followed by study enrollment. Participant 255 was enrolled in the study in December 2021 during the Omicron infection wave. However, a record of a positive qPCR result for SARS-CoV-2 was present for this participant from September 2021, corresponding to the period when the Delta variant was dominant in South Africa. Therefore, the original diagnosis date was taken as September 2021 (Figure 1A). Among the other participants, participant 27 was infected in the ancestral infection wave in 2020. Participants 96 and 127 were infected in early 2021, corresponding to the time the Beta variant was dominant in South Africa, and participant 209 was infected in December 2021 during the Omicron subvariant BA.1 infection wave.

Alignment of participant sequences confirmed that participant 27 was infected with ancestral virus and participant 96 was infected with the Beta variant. Participant 127 was also infected with a Beta variant. However, the last sequence was a Delta variant, consistent with a re-infection as this occurred during the Delta infection wave in South Africa. Participant 255 was infected with the Delta variant (Figure 1B), consistent with a continuous infection from the first positive diagnostic test in September 2021. Participant 209 was infected with Omicron BA.1, but the sequence from the last timepoint was an Omicron BA.5 subvariant. As the Omicron BA.5 subvariant was circulating at this time, re-infection is likely (Figure 1B).

We next analyzed all non-synonymous changes across the SARS-CoV-2 viral genomes for each participant. We detected multiple substitutions and deletions relative to the infecting strain or variant. Participant 27 showed changes in multiple genes (Figure 2A) including at the first available sample taken 6 days post-diagnosis and 20 days post-self-reported symptom onset. Mutations included K417T and F490S in the spike receptor binding domain (RBD) which are predicted to mediate escape from neutralizing antibodies[49-51]. The virus added 19 substitutions and deletions in the last available sample relative to the first available sample, which spanned a period of 184 days.

Participant 96, who was infected with the Beta variant, also showed multiple changes relative to the infecting Beta variant, including the T478K substitution in the RBD also present in the Delta variant. This virus gained 13 changes over 109 days (Figure 2B). In contrast, participant 127, also infected with the Beta variant, showed minimal SARS-CoV-2 genomic changes from the first available sequence at day 13 post-diagnosis to day 71 post-diagnosis, a span of 58 days. One of the mutations was at the furin cleavage site but was not the P681R mutation

present in the Delta variant which enhances cleavage of the S1/S2 domains in spike. At the next sequenced timepoint, an abrupt change to the Delta variant sequence occurred.

Participant 255 was only tracked for 28 days, but during this period multiple mutations arose relative to the initially sequenced virus. Mutations included the K417T substitution and a change from E484V to E484A in the RBD, where E484A is also present in Omicron subvariants. Altogether, 11 substitutions and deletions arose within the span of sequencing of 28 days. Unlike participant 255, participant 209 who was infected with Omicron BA.1, showed no changes over a period of 139 days. At the next sequenced timepoint, there was an abrupt change to an Omicron BA.5 sequence consistent with re-infection.

Participants were offered dolutegravir based HIV antiretroviral therapy (ART) and received adherence counselling from the clinical team. All participants showed a decline in HIV viremia to below the threshold of assay detection (40 HIV RNA copies/mL) during the study. Participants 27, 127, and 255 showed temporary resurgence of HIV viremia (Figure 3A, first row). Detection of ART levels in these participants showed poor adherence at the earlier timepoints post-enrollment and that periods of non-adherence preceded the resurgence of HIV (Figure S1). Control of HIV viremia was associated with a recovery of CD4 T cells in all but one participant (Figure 3A, second row). At vaccination, participants 27 and 96 both had suppressed HIV viremia and cleared SARS-CoV-2 (Figure 3A, third row). Participant 127 had suppressed HIV but had a detectable SARS-CoV-2 at vaccination after being SARS-CoV-2 negative by qPCR in the previous timepoint, most likely explained by re-infection. Participants 255 and 209 both had HIV viremia during vaccination. Interestingly, participants 255 and 209 cleared SARS-CoV-2 infection despite HIV viremia. However, this may not be related to vaccination (see below).

We investigated the neutralizing antibody response to vaccination. All participants were vaccinated with two doses of the Pfizer BNT162b2 mRNA vaccine except for participant 127, who had developed synovial inflammation in the wrists and hands 6 days post-first vaccine dose, a rare adverse event associated with Covid-19 mRNA vaccines[52,53]. The interval between doses followed as much as practicable the South African guidelines at the time of vaccination of 6 weeks.

We tested for neutralizing antibodies in the participant blood plasma against ancestral virus and the Beta, Delta and Omicron BA.1 variants using a live virus neutralization assay as well as spike antibody levels. Testing for neutralization capacity and antibody levels was done at baseline and after each dose of the vaccine (Figure 3B). Neutralization capacity was quantified as $FRNT_{50}$, the reciprocal of the plasma dilution which resulted in 50% neutralization of the live virus in a focus forming assay (Materials and methods).

In the three participants with suppressed HIV at vaccination (27, 96, and 127), there was an increase in neutralization capacity for all strains/variants tested after the first, and if administered, second dose. In participant 127, who received only one vaccine dose, this vaccine induced neutralization waned quickly, with neutralization capacity dropping approximately 10-fold in about 6 months. Omicron BA.1 was least neutralized in all participants except 209, who was Omicron BA.1 infected. In participant 27, neutralizing antibodies to Omicron BA.1 showed very rapid waning. In participant 96, neutralization capacity against BA.1 was poorly elicited by the vaccine but was abruptly increased a short period thereafter. Participants 255 and 209, in whom HIV viremia persisted, showed a poor initial response to the vaccine, although participant 255 had anti-spike antibody levels similar to the participants where stronger neutralization was elicited. For participant 209, neutralization capacity increased with time. This participant also likely had Omicron BA.5 re-infection post-vaccine, which may have elicited this increase.

For participants 255 and 209, SARS-CoV-2 was present close to the time of vaccination. Participant 255 cleared SARS-CoV-2 when neutralization of the Delta variant (the variant responsible for the original infection) had an $FRNT_{50}$ of 139. This occurred just before administration of the first vaccine dose (Figure 3B, timepoint marked with a star). Participant 209 cleared SARS-CoV-2 infection between day 103 (the last visit with a SARS-CoV-2 positive qPCR result) and day 133 post-first vaccine dose (Figure 3B, timepoint marked with a star) at which point the plasma neutralized Omicron BA.1 with an $FRNT_{50}$ of 378. The participant was Omicron BA.1 then Omicron BA.5 infected in the post-vaccination period and this, rather than vaccination, may have led to a gradual increase in antibody neutralization titers.

To compare our results to BNT162b2 vaccination in PLWH without advanced HIV disease who were also previously infected, we tested neutralization capacity after each vaccine dose in five participants with either suppressed HIV or who were HIV negative (Figure 3C, see Table S2 for participant details). These participants showed a relatively homogenous response, with a large fold-change post-first dose, then limited waning followed by a smaller fold-increase after second dose.

We next investigated a larger group of participants, who were PLWH (n=10) or HIV negative (n=16). We determined the neutralization capacity about 1 to 2 months post-second dose (Table S3) for each of the tested strains/variants. All participants in this group had a history of previous infection (Table S3). We examined both fold-change from baseline and absolute neutralization level in a 2-dimensional graph (Figure 3D). At peak response, the highest neutralization quantified as $FRNT_{50}$ was against ancestral virus and lowest for Omicron BA.1. Geometric mean titer $FRNT_{50}$ in these participants was 8,450 for ancestral, 5,300 for Delta, 2,501 for Beta, and 711 for Omicron BA.1. Fold-increase in neutralizing capacity was more similar between the variants: 63-fold increase in ancestral virus neutralization, 65-fold in Delta, 42-fold in Beta, and 39-fold in Omicron BA.1 neutralization. When we compared the peak neutralization capacity and fold-increase in the advanced HIV group against this group, we observed that the advanced HIV disease participants with suppressed viremia did not markedly differ from the HIV negative participants and PLWH with suppressed HIV viremia. However, the two advanced HIV disease participants with unsuppressed viremia formed a separate cluster at low fold-change and $FRNT_{50}$ values for each variant (Figure 3F).

Discussion

Here we characterized 5 long-term SARS-CoV-2 infections in individuals with advanced HIV disease who had HIV viremia upon study enrollment. Infections occurred during the ancestral, Beta, Delta, and Omicron infection waves in South Africa. There were extensive changes in the SARS-CoV-2 sequence of three of the participants and little change in two of the participants. The latter two participants showed strong evidence for re-infection with another variant. We observed changes in the SARS-CoV-2 genome and these can be due to the accumulation of mutations in the predominant circulating strain, or competition between multiple circulating strains for dominance in a quasispecies, such that the mutations we saw we the result of a minority strain becoming dominant[54].

The response to the Pfizer BNT162b2 mRNA vaccine was variable and linked to the presence or absence of HIV viremia. Two participants with HIV viremia had an initially weak neutralizing response to the vaccine, while the participants who had suppressed HIV at vaccination had similar responses as those in HIV negative participants and PLWH with suppressed HIV viremia without a low CD4 count. This may indicate that high HIV viremia interferes with the ability of a vaccine to elicit effective neutralizing antibodies. Whether such extensive interference can happen in the absence of a low CD4 count should be examined. Interestingly, in one of the participants with HIV viremia and poor vaccine response post-second vaccine

dose, spike binding antibody levels rose to values similar to participants eliciting a good neutralization response. However, the neutralization capacity did not substantially increase. This may indicate a lack of neutralization potency, which can be associated with poor affinity maturation of neutralizing antibodies[55,56].

Both participants with HIV viremia cleared SARS-CoV-2. At clearance, neutralizing antibody levels were moderate but within the ballpark range where neutralizing activity starts to become effective (plasma dilution for 50% neutralization is at least 100-fold[57]). Whether vaccination contributed to SARS-CoV-2 elimination in the participant with SARS-CoV-2 infection post-vaccination is unclear as the re-infection combined with a declining HIV viral load and recovering CD4 count may also have contributed to clearance.

Limitations of this study include the small number of participants and the partial coverage of the infection interval by sequencing. Also, we studied a subset of individuals with HIV viremia at SARS-CoV-2 infection and a low CD4 count. The results may therefore not apply to individuals who have suppressed HIV viremia but poor recovery of CD4 T cell numbers.

The continued evolution of SARS-CoV-2 is a major barrier to effective control of the infection through vaccination, although the continual presence of potent hybrid immunity from Omicron breakthrough infection may provide a barrier to other variants[58-60] and it is unclear whether a new extensively mutated variant can evolve to dominate infections. However, given the disruption such a variant may cause, investment in an effective global HIV antiretroviral therapy strategy could reduce the probability of such an event occurring and may also reduce the potential evolution of other pathogens.

Methods

Informed consent and ethical statement

All blood samples used for neutralization studies, nasopharyngeal swabs from the advanced HIV disease participants for outgrowth sequencing as well as nasopharyngeal swabs for isolation of the ancestral/D614G, Beta, and Delta virus were obtained after written informed consent from adults with PCR-confirmed SARS-CoV-2 infection enrolled in a prospective cohort of SARS-CoV-2 infected individuals at the Africa Health Research Institute approved by the Biomedical Research Ethics Committee at the University of KwaZulu–Natal (reference BREC/00001275/2020). The Omicron/BA.1 virus was isolated from residual swab used for diagnostic testing (Witwatersrand Human Research Ethics Committee reference M210752).

Reagent availability statement

Viral isolates are available upon reasonable request. Sequences of isolated SARS-CoV-2 used in this study have been deposited in GISAID with accession (MODIFY):

Virus	GISAID	Virus	GISAID
D614G	EPI_ISL_602626.1	**0127-D57**	Submitted
Beta	EPI_ISL_678615	**0127-D71**	Submitted
Delta	EPI_ISL_3118687	**0127-D195**	EPI_ISL_14666773
BA.1	EPI_ISL_7886688	**0127-D205**	EPI_ISL_14666774
0027-D6	EPI_ISL_15541746	**0127-D210**	EPI_ISL_14666775
0027-D20	EPI_ISL_15541747	**0255-D20**	EPI_ISL_14599778
0027-D34	EPI_ISL_15541748	**0255-D23**	EPI_ISL_14599779
0027-D71	EPI_ISL_15541749	**0255-D30**	EPI_ISL_14599780

0027-D106	EPI_ISL_15541750	**0255-D49**	EPI_ISL_13986497
0027-D190	EPI_ISL_2397313	**0209-D5**	EPI_ISL_8578314
0096-D1	EPI_ISL_14666761	**0209-D19**	EPI_ISL_12970431
0096-D15	EPI_ISL_14666763	**0209-D26**	EPI_ISL_12970430
0096-D68	EPI_ISL_14666764	**0209-D103**	EPI_ISL_12268488
0096-D77	EPI_ISL_14666765	**0209-D110**	EPI_ISL_12268487
0096-D110	EPI_ISL_14666766	**0209-D137**	EPI_ISL_12970429
0127-D13	Submitted	**0209-D144**	EPI_ISL_12970433
0127-D27	Submitted	**0209-D159**	EPI_ISL_14666777
0127-D34	Submitted	**0209-D165**	EPI_ISL_13963398
0127-D41	Submitted		

Clinical laboratory testing

SARS-CoV-2 Ct and HIV viral load quantification was performed from a nasopharyngeal swab universal transport medium aliquot and 4 ml EDTA tube of blood respectively at an accredited diagnostic laboratory (Molecular Diagnostic Services, Durban, South Africa). CD4 count, CD8 count, and a full blood count panel were performed by an accredited diagnostic laboratory (Ampath, Durban, South Africa).

Whole-genome sequencing

RNA was extracted on an automated Chemagic 360 instrument, using the CMG-1049 kit (Perkin Elmer, Hamburg, Germany). The RNA was stored at −80◦C prior to use. Libraries for whole genome sequencing were prepared using either the Oxford Nanopore Midnight protocol with Rapid Barcoding or the Illumina COVIDseq Assay. For the Illumina COVIDseq assay, the libraries were prepared according to the manufacturer's protocol. Briefly, amplicons were tagmented, followed by indexing using the Nextera UD Indexes Set A. Sequencing libraries were pooled, normalized to 4 nM and denatured with 0.2 N sodium acetate. An 8 pM sample library was spiked with 1% PhiX (PhiX Control v3 adaptor-ligated library used as a control). We sequenced libraries on a 500-cycle v2 MiSeq Reagent Kit on the Illumina MiSeq instrument (Illumina). On the Illumina NextSeq 550 instrument, sequencing was performed using the Illumina COVIDSeq protocol (Illumina Inc, USA), an amplicon-based next-generation sequencing approach. The first strand synthesis was carried using random hexamers primers from Illumina and the synthesized cDNA underwent two separate multiplex PCR reactions. The pooled PCR amplified products were processed for tagmentation and adapter ligation using IDT for Illumina Nextera UD Indexes. Further enrichment and cleanup was performed as per protocols provided by the manufacturer (Illumina Inc). Pooled samples were quantified using Qubit 3.0 or 4.0 fluorometer (Invitrogen Inc.) using the Qubit dsDNA High Sensitivity assay according to manufacturer's instructions. The fragment sizes were analyzed using TapeStation 4200 (Invitrogen). The pooled libraries were further normalized to 4nM concentration and 25 µL of each normalized pool containing unique index adapter sets were combined in a new tube. The final library pool was denatured and neutralized with 0.2N sodium hydroxide and 200 mM Tris-HCL (pH7), respectively. 1.5 pM sample library was spiked with 2% PhiX. Libraries were loaded onto a 300-cycle NextSeq 500/550 HighOutput Kit v2 and run on the Illumina NextSeq 550 instrument (Illumina, San Diego, CA, USA). For Oxford Nanopore sequencing, the Midnight primer kit was used as described by Freed and Silander55. cDNA synthesis was performed on the extracted RNA using LunaScript RT mastermix (New England BioLabs) followed by gene-specific multiplex PCR using the Midnight Primer pools which produce 1200bp amplicons which overlap to cover the 30-kb SARS-CoV-2 genome. Amplicons from each pool were pooled and used neat for barcoding with the Oxford Nanopore

Rapid Barcoding kit as per the manufacturer's protocol. Barcoded samples were pooled and bead-purified. After the bead clean-up, the library was loaded on a prepared R9.4.1 flow-cell. A GridION X5 or MinION sequencing run was initiated using MinKNOW software with the base-call setting switched off. We assembled paired-end and nanopore.fastq reads using Genome Detective 1.132 (https://www.genomedetective.com) which was updated for the accurate assembly and variant calling of tiled primer amplicon Illumina or Oxford Nanopore reads, and the Coronavirus Typing Tool. For Illumina assembly, GATK HaploTypeCaller --min-pruning 0 argument was added to increase mutation calling sensitivity near sequencing gaps. For Nanopore, low coverage regions with poor alignment quality (<85% variant homogeneity) near sequencing/amplicon ends were masked to be robust against primer drop-out experienced in the Spike gene, and the sensitivity for detecting short inserts using a region-local global alignment of reads, was increased. In addition, we also used the wf_artic (ARTIC SARS-CoV-2) pipeline as built using the nextflow workflow framework. In some instances, mutations were confirmed visually with .bam files using Geneious software V2020.1.2 (Biomatters). The reference genome used throughout the assembly process was NC_045512.2 (numbering equivalent to MN908947.3).

Phylogenetic analysis

Sequences were loaded into and aligned by Nextclade version 2.9.1 (https://clades.nextstrain.org/). The json file output from the Nextclade analysis was loaded into Auspice (https://auspice.us/). Visualization was filtered to include reference sequences from clades 20A, 20B, 20H (Beta), 21J (Delta), 21M, 21K, 21L and 22B (Omicron), and the input sequences (new nodes), for a combined 1408 genomes. The tree was then filtered to show new nodes only. Tip labels were removed and SVG downloaded for final processing using Microsoft Powerpoint software.

Analysis of substitutions and deletions

RL to fill

Cells

The H1299-E3 (H1299-ACE2, clone E3) cell line used in the live virus infections was derived from H1299 (CRL-5803) as described in previous work [61,62] and propagated in growth medium consisting of complete Roswell Park Memorial Institute (RPMI) 1640 with 10% fetal bovine serum containing 10mM of HEPES, 1mM sodium pyruvate, 2mM L-glutamine and 0.1mM nonessential amino acids.

Live virus neutralization assay

H1299-E3 cells were plated in a 96-well plate (Corning) at 30,000 cells per well 1 day pre-infection. Plasma was separated from EDTA-anticoagulated blood by centrifugation at 500 rcf for 10 min and stored at −80 °C. Aliquots of plasma samples were heat-inactivated at 56 °C for 30 min and clarified by centrifugation at 10,000 rcf for 5 min. Virus stocks were used at approximately 50-100 focus-forming units per microwell and added to diluted plasma. Antibody–virus mixtures were incubated for 1 h at 37 °C, 5% CO_2. Cells were infected with 100 μL of the virus–antibody mixtures for 1 h, then 100 μL of a 1X RPMI 1640 (Sigma-Aldrich, R6504), 1.5% carboxymethylcellulose (Sigma-Aldrich, C4888) overlay was added without removing the inoculum. Cells were fixed 18 h post-infection using 4% PFA (Sigma-Aldrich) for 20 min. Foci were stained with a rabbit anti-spike monoclonal antibody (BS-R2B12, GenScript A02058) at 0.5 μg/mL in a permeabilization buffer containing 0.1% saponin (Sigma-Aldrich), 0.1% BSA (Sigma-Aldrich) and 0.05% Tween-20 (Sigma-Aldrich) in PBS. Plates were

incubated with primary antibody overnight at 4 °C, then washed with wash buffer containing 0.05% Tween-20 in PBS. Secondary goat anti-rabbit HRP conjugated antibody (Abcam ab205718) was added at 1 μg/mL and incubated for 2 h at room temperature with shaking. TrueBlue peroxidase substrate (SeraCare 5510-0030) was then added at 50 μL per well and incubated for 20 min at room temperature. Plates were imaged in an ImmunoSpot Ultra-V S6-02-6140 Analyzer ELISPOT instrument with BioSpot Professional built-in image analysis (C.T.L).

Statistics and fitting

All statistics and fitting were performed using custom code in MATLAB v.2019b. Neutralization data were fit to:

$Tx=1/1+(D/ID_{50})$. (1)

Here Tx is the number of foci at plasma dilution D normalized to the number of foci in the absence of plasma on the same plate. ID_{50} is the plasma dilution giving 50% neutralization. $FRNT_{50} = 1/ID_{50}$. Values of $FRNT_{50}$ <1 are set to 1 (undiluted), the lowest measurable value. We note that the most concentrated plasma dilution was 1:25 and therefore $FRNT_{50}$ < 25 were extrapolated.

Detection of ART concentrations in plasma by LC-MS/MS

Sample analysis was performed using an Agilent High Pressure Liquid Chromatography (HPLC) system coupled to the AB Sciex 5500, triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) TurboIonSpray source. The LC-MS/MS method was developed and optimized for the quantitation of tenofovir, lamivudine and dolutegravir in the same sample. A protein precipitation extraction method using acetonitrile was used to process 50 μL plasma samples. 50 μL of water and 50 μL of ISTD solution was added and the sample was briefly mixed. 150 μL of acetonitrile was subsequently added to facilitate protein precipitation, vortex mixed and centrifuged at 16000 g for 10 min at 4°C. 170 μL of the clear supernatant was then transferred to a clean micro-centrifuge tube and dried down using a SpeedVac dryer set at 40°C. The dried samples were then reconstituted in 100 μL of 0.02% sodium deoxycholate (Sigma) in Millipore filtered water, vortex mixed, briefly centrifuged, placed in a small insert vial, capped, placed in the auto sampler compartment (maintained at 4°C) and analyzed using LC-MS/MS. The analytes were separated on an Agilent Zorbax Eclipse Plus C18 HPLC column using gradient elution. The column oven was set at 40°C, a sample volume of 2 μL was injected and the flow rate was set to 0.2 mL/min. Mobile phase A consisted of water with 0.1% formic acid and B consisted of acetonitrile with 0.1% formic acid. The drug analytes were monitored using multiple-reaction monitoring mode for positive ions except for efavirenz which was monitored in the negative ion scan mode. Analyst software, version 1.6.2 was used for quantitative data analysis. Blanked values for EFV, FTC and TFV were in the range of 3 ng/mL and this was set as the detection limit.

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Competing interest statement

AS received an honorarium for a talk given to Pfizer employees.

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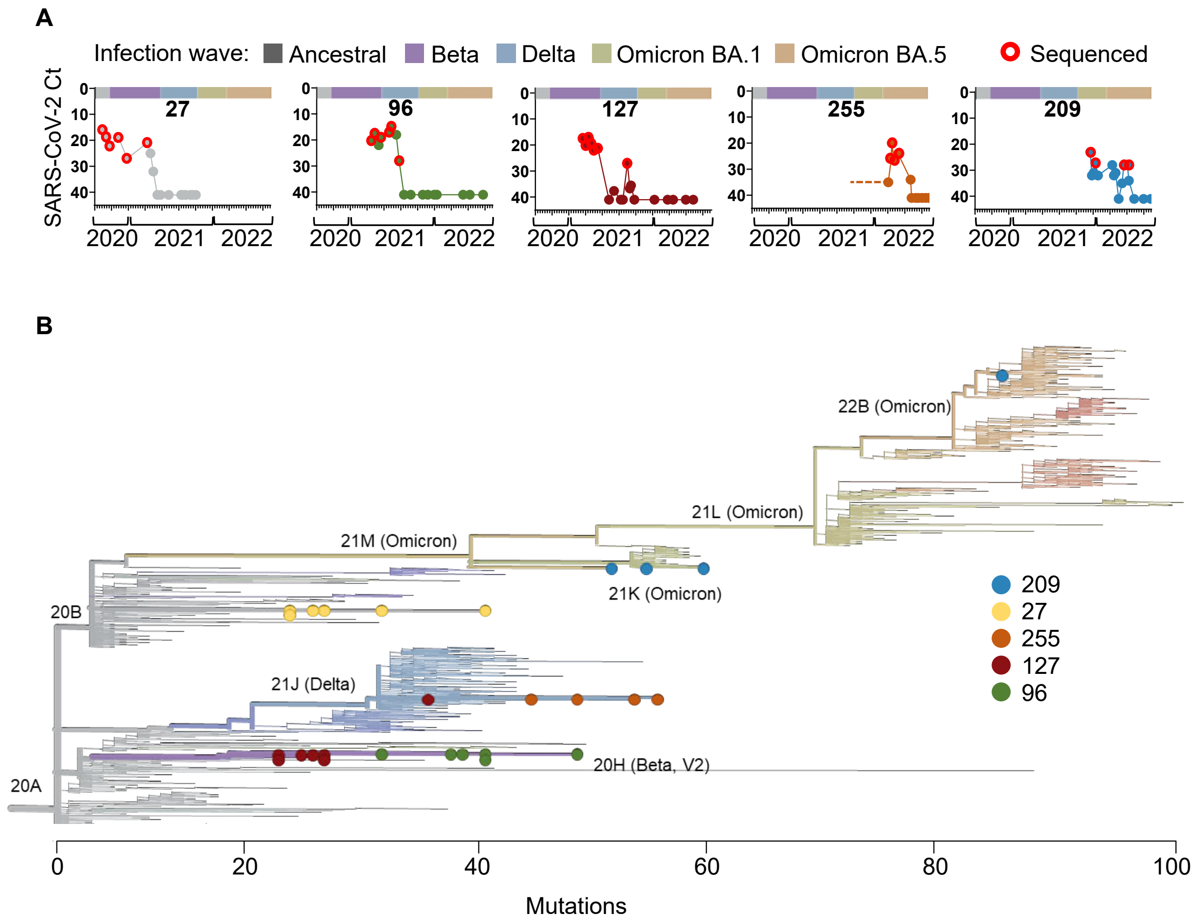

Figure 1: SARS-CoV-2 long term infection and evolution in people with advanced HIV disease. (A) SARS-CoV-2 qPCR cycle threshold (Ct) values over time per infection with sequenced timepoints marked with a red circle. Top bar denotes infection wave and numbers in bold within graphs denote participant. For participant 255, dashed horizontal line denotes delay from SARS-CoV-2 diagnosis to first available study sample at enrollment. For the other participants, the delay between diagnosis to enrollment was minimal. (B) Phylogenetic analysis of the SARS-CoV-2 consensus sequences from the long-term infections. Participant virus sequences are shown as points. Note that for participants 127 and 209, the consensus sequence changes clade.

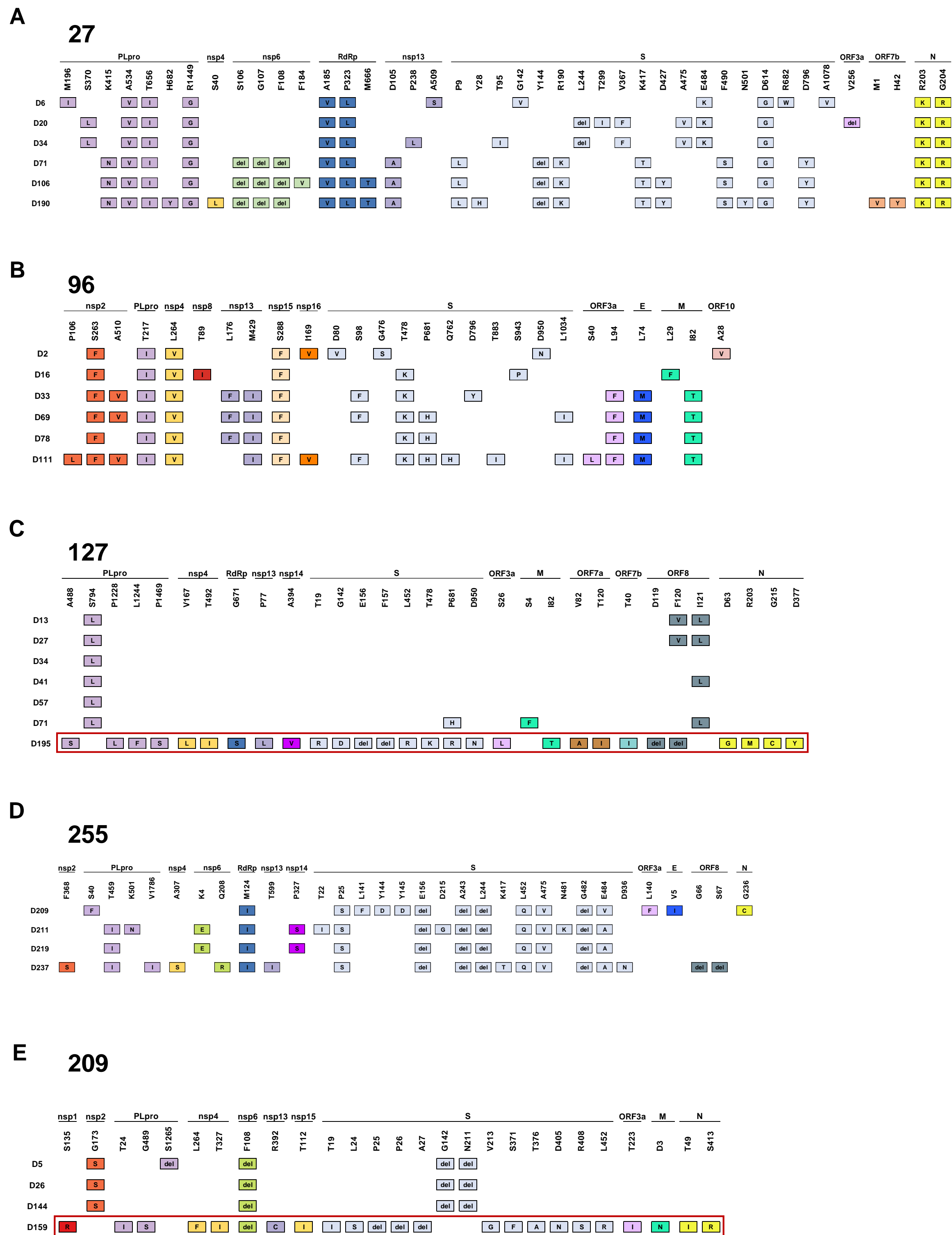

Figure 2: Substitutions and deletions in SARS-CoV-2 isolated from advanced HIV participants over time. Changes are relative to the most likely infecting variant. Numbers on the left denote the day post-diagnosis at which the virus sample was obtained. Probable re-infections are marked with a red rectangle.

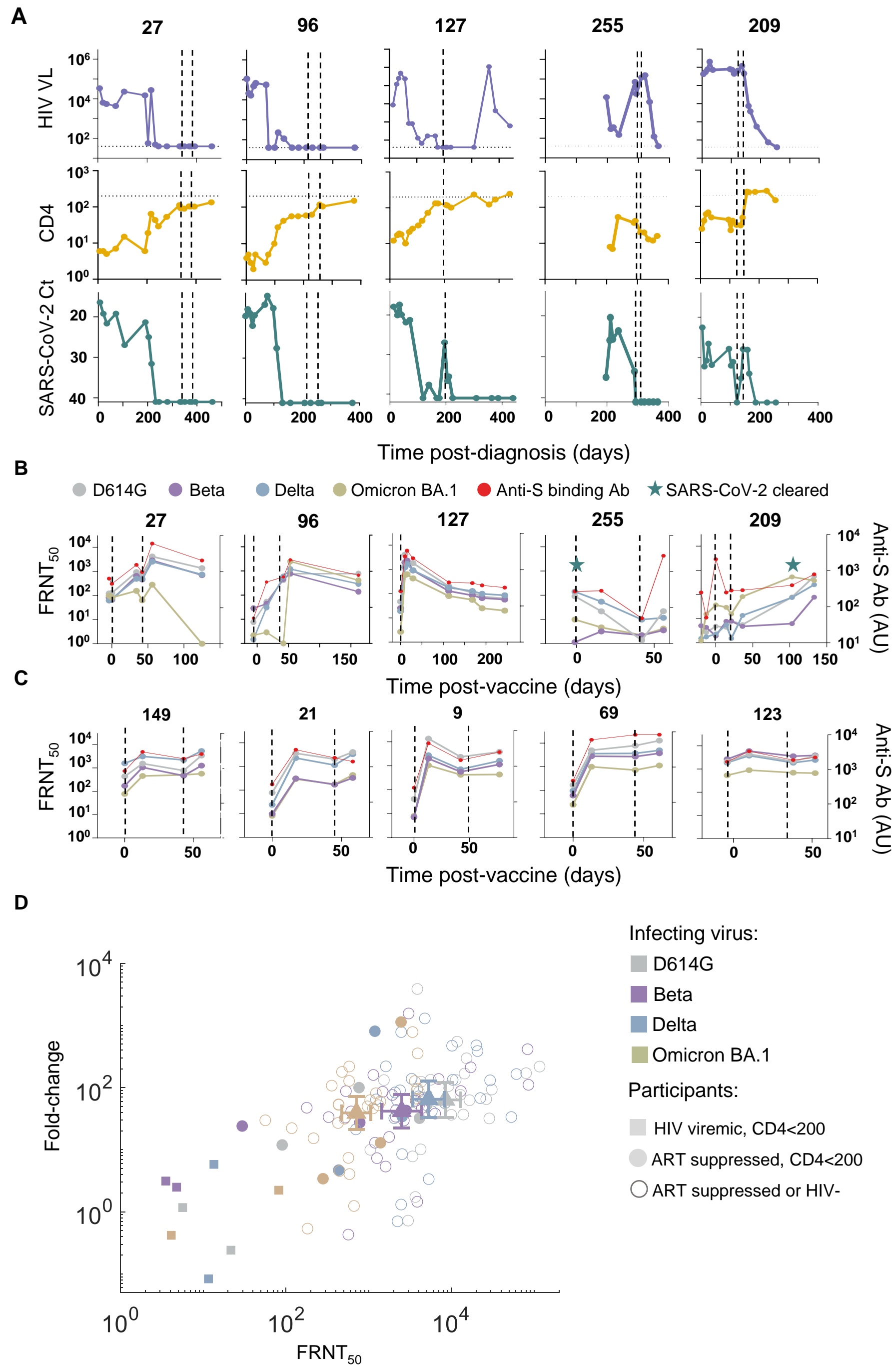

Figure 3: Neutralizing antibody response to the Pfizer BNT162b2 mRNA vaccine. (A) HIV viral load, CD4 count, and SARS-CoV-2 qPCR cycle threshold (Ct) over time. Dashed vertical lines denote Pfizer BNT162b2 vaccine doses (B) Plasma neutralizing antibody response of advanced HIV disease participants against ancestral D614G virus and the Beta, Delta, and Omicron BA.1 variants pre- and post-vaccine doses. X-axis denotes time post-first vaccine dose and Y-axis denotes neutralization capacity as $FRNT_{50}$, the reciprocal plasma dilution required for 50% neutralization. Timing of vaccine doses are denoted by vertical dashed lines. A star denotes time of SARS-CoV-2 clearance for participants with ongoing SARS-CoV-2 infection near the time of vaccination. (C) Plasma neutralizing antibody response of five non-advanced HIV disease participants pre- and post-vaccine doses. (D) Fold-change in neutralization capacity versus $FRNT_{50}$ against D614G virus and the Beta, Delta, and Omicron BA.1 variants post-second vaccine dose in participants with advanced HIV disease and HIV viremia at vaccination (filled squares), advanced HIV participants with a suppressed HIV viral load at vaccination (filled circles), and non-advanced HIV disease (empty circles) participants (n=26, with n=10 PLWH and 16 HIV negative).

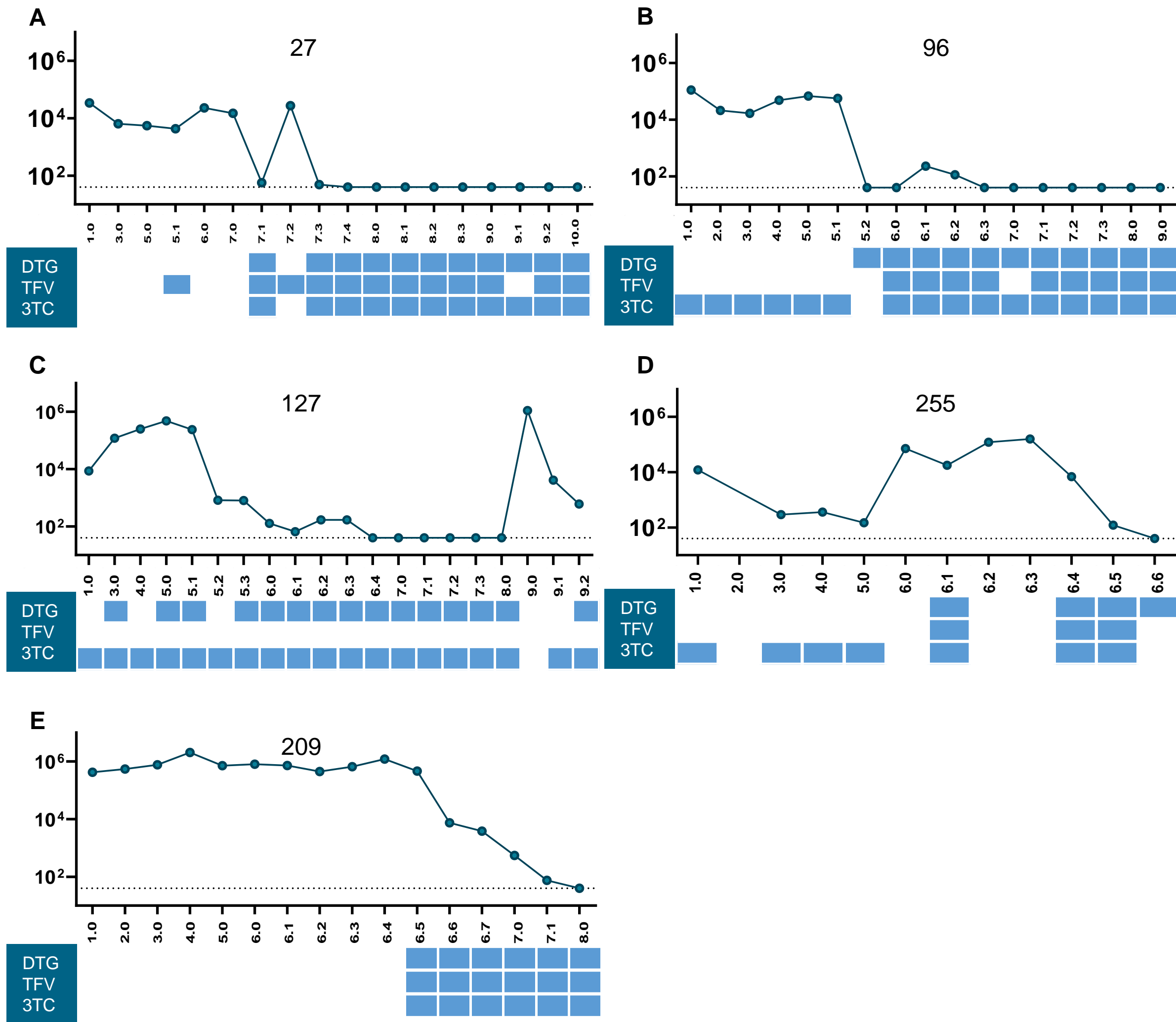

Figure S 1: Antiretroviral drug concentrations in advanced HIV disease participants. Detection of dolutegravir (DTG), tenofovir (TFV), and lamivudine (3TC) in participant plasma at different sampled timepoints, where timepoint 1 is enrollment and 2-5 are at weekly intervals within the first month of enrollment. Later timepoints were at 3-month intervals.

CHAPTER 5:

Discussion

South Africa is a place where there was a strong overlap between two pandemics: HIV and SARS-CoV-2. There are several reasons to study the effects of this overlap on COVID-19. First, it is important to know how HIV modulates SARS-CoV-2 infection as this may have direct health implications for PLWH. Interventions, such as additional vaccine booster doses and prioritization for SARS-CoV-2 antiviral therapies, can then be implemented.

Second, if HIV suppression state and history have an effect on SARS-CoV-2 control and clearance, then the consequences of long-term infection and shedding in terms of viral evolution must be understood to mitigate such evolution, especially if the pattern of evolved mutations is very similar to that found in SARS-CoV-2 variants of concern, to prevent the formation of new variants.

Last, how a new virus adapts to the human host must be understood to prepare ourselves for the next pandemic. HIV, SARS-CoV, and MERS are all examples of viruses that recently jumped to humans from other animals before SARS-CoV-2, yet the determinants of this process are poorly understood. Looking at intra-host evolution is one way to decipher the drivers of viral evolution.

In the work which forms the basis of this thesis, I have led three projects and assisted with multiple other projects which have informed both the effect of HIV/SARS-CoV-2 co-infection on COVID-19 disease and immunity, and the effect of advanced HIV disease on prolonged SARS-CoV-2 shedding and evolution. With a more in-depth study of the functional significance of the mutations which arise in intra-host evolution, the data I generated may also shed light on the changes viruses need to make to better replicate in the human host.

In the first part of the work (presented in Chapter 2), I investigated the effect of SARS-CoV-2 infection on disease severity in PLWH. The primary measure was the requirement for supplemental oxygen during hospitalization. The reason for this choice is that it marks a clear and easily quantifiable separation between a less dangerous

form of the disease where the patient is likely to recover without support to a more dangerous form which may cause disability or death if support in the form of supplemental oxygen is not provided (Young et al., 2020). This latter form of the disease is associated with infection of the lower respiratory tract of pneumocytes expressing ACE2 in conjunction with TMPRSS2, and leads to inflammation and cellular syncytia which are detrimental to oxygen exchange (Delorey et al., 2021; Meng et al., 2022; Mlcochova et al., 2021).

The main observation of the work was higher requirement for supplemental oxygen in PLWH relative to HIV negative study participants in the Beta infected wave. This confirms a previous report from South Africa showing HIV worsens the outcome of SARS-CoV-2 infection, although in that report the tracked outcome was mortality (Ambrosioni et al., 2021; Boulle et al., 2020). This effect was not observed in the first, ancestral SARS-CoV-2 infection wave, but this does not necessarily indicate that there is a fundamental difference in how the ancestral virus and Beta variant infection interact with HIV infection, although this is a possibility. The other possibilities are that the difference stems from the difference in disease severity measured as supplemental oxygen requirement between waves, as the disease severity in the Beta infection wave was higher, thus making differences more pronounced and significant, or that the lower levels of HIV suppression in the second wave contributed to poorer immune control of SARS-CoV-2.

The limitation of this main result of the study was that this was not a sampling of the population but rather a cohort of hospitalized participants. Hence, differences in linkage to care between PLWH and HIV negative participants as well as behavioural, logistical, and other unknown factors which may have changed who accesses hospital care have not been accounted for.

On the level of the immune response, I found that the participants accessing hospital care in the Beta infection wave had a lower CD4+ T cell count and this was especially pronounced in PLWH, where the CD4 count dropped from 420 cells/μL to 172 cells/μL during the active SARS-CoV-2 infection phase, where the virus was detectable by qPCR, below the value of 200 cells/μL usually used as the cut-off for profound immunosuppression (De Maria et al., 2003). This contrasted with HIV negative

participants, where the drop was negligible and T cell counts in both infection waves were above 700. This explains the higher severity of infection of PLWH during the Beta infection wave, as CD4+ T cells are required in the orchestration of both the B cell driven antibody response and the CD8 T cell response, in addition to secreting antiviral factors such as interferon-gamma and IL2, important in T cell survival (Sette & Crotty, 2021). Interestingly, CD4+ T cell counts in PLWH recovered post-clearance of SARS-CoV-2, indicating that the low CD4 count at infection was not a product of HIV infection alone but rather the interaction between the two viruses, as SARS-CoV-2 also causes lymphopenia (Chen et al., 2020).

Another indication of possible immune dysregulation of the SARS-CoV-2 immune response in PLWH which I found in this study was lower expression of CXCR3 on T cells. CXCR3 is a tissue homing marker, and lower levels may indicate either that less T cells are available for trafficking to the site of infection in the lung, or that more such cells have trafficked out of the circulation and into the lung because of more severe infection at this site. Consistent with the second option, I found that there was a higher fraction of PD1+HLA-DR+ T cells, indicating a higher level of immune activation, during active SARS-CoV-2 infection in PLWH. The sum of the data showed evidence that SARS-CoV-2 infection is modulated by HIV and consistent with the notion that PLWH constitute a vulnerable population where SARS-CoV-2 infection can have a worse outcome, and should be prioritized for interventions for this reason.

The third project (presented in chapter 4) I led continued to examine SARS-CoV-2 immunity in PLWH, and specifically in people with advanced HIV disease, defined as those with a CD4+ T cell count of below 200 cells/µL. All participants in this group had very low CD4 counts when enrolled (range was 2 to 24 cells/µL), and all were HIV viremic with high viral loads. Subsequently, all became HIV suppressed with ART during the study. These study participants chose to vaccinate during the study with the Pfizer BNT162b2 mRNA vaccine and we could therefore observe their neutralizing antibody immune response to the vaccine. Antibody neutralization is a correlate of vaccine efficacy (Cromer, Steain, Reynaldi, Schlub, Sasson, et al., 2022; Cromer, Steain, Reynaldi, Schlub, Wheatley, et al., 2022; Khoury et al., 2021) and we used it in the study to understand whether vaccination was effective.

We checked neutralization of four different strains/variants of SARS-CoV-2: ancestral SARS-CoV-2, and the Beta, Delta and Omicron BA.1 variants. We found that 3 out of the 5 advanced HIV disease participants, who had suppressed their HIV viremia in the blood by vaccination, showed neutralizing antibody responses against the different SARS-CoV-2 viruses on par with PLWH with suppressed HIV and higher CD4 counts as well as HIV negative participants. However, the two advanced HIV disease participants whose viremia was not yet suppressed showed poor neutralizing responses. While the lack of response in viremic people with advanced HIV disease may not be surprising and is consistent with our previous studies showing that unsuppressed HIV viremia interferes with the SARS-CoV-2 neutralizing antibody response (Hwa et al., 2023; Khan et al., 2021), the relatively normal response to the vaccine in suppressed people with advanced HIV disease is encouraging and indicates that vaccination may be effective in this group. The main limitation of this conclusion is the low number of participants with advanced HIV disease analysed in the study. This is explained that longitudinal follow up of participants with advanced HIV disease is difficult because of loss to follow-up, and vaccination in this group is rare.

An important focus of the two latter studies (presented in chapter 3 and 4) is understanding what happens to SARS-CoV-2 virus during infection in someone with advanced HIV disease. I started this investigation in the study presented in chapter 3 with a case study of one participant with advanced HIV disease. This study participant had a CD4 count of 6 and an HIV viral load of about 34,000 on the enrolment study visit and was indicated for a dolutegravir based regimen (TLD, coformulation of dolutegravir, lamivudine, and tenofovir-DP) by the study clinician. However, poor adherence prevented rapid HIV suppression.

During a period of approximately 6 months, we observed high SARS-CoV-2 viral titres from the nasopharyngeal swab sample which were culturable using a cell line, indicating the shedding of infectious virus (Case, Bailey, Kim, Chen, & Diamond, 2020). Once HIV viremia was effectively suppressed by ART, SARS-CoV-2 first became unculturable, and then cleared. This indicates the strong relationship between HIV viremia and the inability to clear SARS-CoV-2, and that HIV suppression is the key step in SARS-CoV-2 control. However, this was an observation based on a single

case. In the subsequent expansion of the study presented in chapter 4, we added 4 participants to the analysis, and obtained a more complete picture: in 3 of the 5 participants with advanced HIV disease, suppression of HIV viremia was closely followed by clearance of SARS-CoV-2. In one participant, SARS-CoV-2 was cleared when HIV viremia was partially suppressed and the CD4 count was recovering due to initiation of ART, but neither process was complete. Finally, in one participant, clearance occurred after a partial CD4 count recovery because of ART initiation, yet the HIV viral load was still high. Therefore, the requirement for the suppression of HIV viremia for the clearance of SARS-CoV-2 in advanced HIV disease may not be absolute, but some combination of ART mediated suppression of HIV viremia and CD4+ T cell count recovery is necessary.

We sequenced the SARS-CoV-2 virus from first the case study (chapter 3) and then the additional case series of 4 advanced HIV disease participants (chapter 4). The virus from the case study participant evolved non-synonymous mutations and showed deletions in the spike protein during the course of the prolonged infection. These mutations included the mutations in the ACE2 receptor binding domain (RBD) of spike, including mutations at residues 417 and 484 known to confer escape from neutralizing antibodies elicited during ancestral and Delta variant SARS-CoV-2 infection and vaccination based on ancestral SARS-CoV-2 sequence (Greaney, Eguia, et al., 2022; A. J. Greaney et al., 2021; Allison J. Greaney et al., 2021; Greaney, Starr, et al., 2022; Tyler N. Starr et al., 2020), and which are also present in the Omicron variant (https://covdb.stanford.edu/variants/omicron_ba_1_3/, accessed 14.01.2023). This was the first report, made available on medRxiv in June 2021, showing that immunosuppression because of advanced HIV disease can lead to prolonged SARS-CoV-2 infection and extensive SARS-CoV-2 evolution (Karim et al., 2021).

Our subsequent study showed that this evolved virus could escape neutralizing antibody immunity elicited by ancestral SARS-CoV-2, the Pfizer BNT162b2 mRNA vaccine, and especially the Delta variant (Sandile Cele et al., 2022). Interestingly, mutations at these sites also occur in the Beta variant, and the evolved virus had the lowest escape from neutralizing antibodies elicited by Beta variant infection (Sandile Cele et al., 2022). The timing of SARS-CoV-2 clearance was explained by neutralizing antibody levels against SARS-CoV-2 measured in the blood of the participant with

advanced HIV from whom the virus was derived. The level of these antibodies increased substantially about 6 months after initial infection, and this was tightly associated with both suppression of HIV viremia (which may have led to return of an effective immune response in the participant) and the clearance of SARS-CoV-2 (possibly the result of these antibodies being made).

A more extensive analysis of the mutations was performed in the study examining the five participants with advanced HIV disease. Here I analysed mutations and deletions in spike and also in the other SARS-CoV-2 genes. For each participant, between four to seven nasopharyngeal samples were successfully sequenced and allowed us to detect changes to the viral genomes across time. Participants were infected with ancestral, Beta, Delta and Omicron BA.1 variants and in all cases persistent infection was observed with extensive evolution in 3 out of 5 participants. In the remaining 2 participants, we observed relatively minor differences in non-synonymous mutations. However, both showed strong evidence of re-infection rapidly following (or possibly occurring during) the initial infection. If infection occurred concurrently, recombination between the infecting SARS-CoV-2 variants could occur (Jackson et al., 2021).

Across participants, changes were most pronounced in the spike RBD, and included neutralizing antibody escape mutations such as L452Q, K417T, and E484A in participant 255 on a Delta variant backbone. These mutations, while found individually in different variants, have not been reported in this combination. During this study, we were able to culture many of the sequenced SARS-CoV-2 viruses, indicating the participants were infectious for much or possibly all of the period of their prolonged SARS-CoV-2 infection. The combination of prolonged SARS-CoV-2 infection with probable shedding of infectious virus and extensive evolution and possibly opportunity for recombination of multiple variants may indicate the danger of the emergence of a new variant during this type of infection. There were also many substitutions in other parts of the SARS-CoV-2 genome whose physiological significance is not yet determined, but can potentially be adaptations that enhance features such as viral replication and fusion (Lustig et al., 2022).

The mutations we observed evolving in advanced HIV disease participants may lead to reduced neutralization by monoclonal antibody therapies, convalescent sera, and

post-vaccination sera (Aleem, Akbar Samad, & Slenker, 2022). Resistance mutations to monoclonal antibody (mAb) therapy have been determined in variants. Table 2 lists commonly available antibodies.

Table 2. *Monoclonal antibodies currently used as Covid-19 treatment adapted from (https://covdb.stanford.edu/, accessed on the 27th July 2023)*

Antibody	Company	Short name	References
Bamlanivimab	AbCellera Biologics, Eli Lilly	BAM	(Dougan et al., 2021; Gottlieb et al. 2021)
Casirivimab	Regeneron Pharmaceuticals	CAS	(Bierle, Ganesh, & Razonable, 2021)
Tixagevimab	AstraZeneca	TIX	(Keam, 2022; Ustianowski, 2022)
Romlusevimab	Brii Biosciences	ROM	(Hoy, 2022; Liu, 2022)
C144	The Rockefeller University	C414	(Fenwick et al., 2021)
Etesevimab	Junshi Biosciences, Institute of Microbiology, Chinese Academy of Science	ETE	(Gottlieb et al., 2021)

Table 3 lists how the known antibody escape mutations we observed in advanced HIV disease participants are predicted to affect the function of monoclonal antibodies.

Table 3. *Substitutions in SARS-CoV-2 viral isolates from immunocompromised participants in our cohort*

Spike Mutations	Study Participant	Variants found	BAM	CAS	TIX	ROM	C144	ETE	References
E484K	27, 96, 127	Beta, Gamma	+++	++	++	+	+++	++	(Muecksch et al., 2021)
A475V	27, 255	D614G					+	++	(Muecksch et al., 2021; T. N. Starr et al., 2021)
K417T	27, 255	Beta, Gamma, Omicron		++		+	+++	+++	(Fenwick et al., 2021)
N501Y	27, 127, 209	Alpha, Beta, Gamma, Omicron	+++	++	+		+++	++	(Fenwick et al., 2021)
D405N	209	Omicron BA.2, BA.4/5			+				(Roe et al., 2023)
K417N	96, 127, 209	Beta, Gamma, Omicron	++				++		(Q. Li et al., 2021)

+ *2-5-fold resistance*
++ *5-24-fold resistance*
+++ *>25-fold resistance*

These results show that the mutations found in SARS-CoV-2 in the advanced HIV participants in our cohort are known antibody escape mutations that appear in

variants. In addition, they are expected to mediate escape from existing clinically used monoclonal neutralizing antibodies.

One limitation of this study is that we had analysed the viral sequences from only five participants and therefore whether such prolonged infection and extensive evolution is as common as we observed is difficult to determine because of the small sample. Moreover, we have not yet determined whether in the participants with re-infection, the two different infecting variants were present at the same time in the individual. We are currently determining this using an analysis of the individual viral sequences as opposed to determining the consensus sequence, which would hide minority strains.

Conclusion

In this body of work which constitutes my PhD thesis, I have made several contributions to a better understanding of the interaction between SARS-CoV-2 and HIV infection in the same individual. First, I have determined that COVID-19 disease severity in PLWH is higher than in HIV negative individuals, an effect likely driven by individuals with unsuppressed HIV viremia. This closely associates with low CD4+ T cell count in PLWH upon SARS-CoV-2 infection, which recovers in most individuals upon convalescence, and indicates that the adaptive immune response may be compromised.

Second, I have determined that immunosuppression because of advanced HIV disease leads to prolonged SARS-CoV-2 infection and can lead to extensive SARS-CoV-2 evolution also observed in variants of concern. Possibly, this mechanism is what has led to the emergence of first the Beta and then the Omicron BA.1, BA.2, and BA.5 variants in South Africa. While such evolution in other types of immunosuppression also happens, the approximately 800,000 advanced HIV disease cases in South Africa (Chihana et al., 2019) would increase the probability of a variant to emerge by the mass of numbers.

The implications are that, first, PLWH should be prioritized for interventions that can clear SARS-CoV-2, including therapeutics such as Paxlovid (Extance, 2022). The effect of additional booster doses of vaccine should be investigated in this population.

Most importantly, ART and adherence counselling should be made readily available to all who need it. These approaches will both benefit PLWH and may help reduce the chances that the next variant will come from our region.

CHAPTER 6:

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