The reactivation of Kaposi's Sarcoma-associated Herpesvirus (KSHV) by SARS-CoV-2 in non-hospitalised HIV-infected patients

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Declaration

I, Humaira Lambarey, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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Lesmes-Rodríguez LC, **Lambarey H**, Chetram A, Riou C, Wilkinson RJ, Joyimbana W, Jennings L, Orrell C, Jaramillo-Hernández DA, Schäfer G. Previous exposure to common coronavirus HCoV-NL63 is associated with reduced COVID-19 severity in patients from Cape Town, South Africa. Frontiers in Virology. 2023 Feb 8;3:1125448.

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Abbreviations

ACE2	angiotensin-converting enzyme 2
AIDS	acquired immunodeficiency syndrome
ALT	alanine transaminase
ART	antiretroviral therapy
CI	confidence interval
COVID-19	coronavirus disease 2019
CRP	C-reactive protein
E	Envelope
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EBNA	EBV nuclear antigen
EBV	Epstein-Barr virus
ERV-3	endogenous retrovirus 3
FU	follow-up
HR	hazard ratio
HHV	human herpesvirus
HIV	human immunodeficiency virus
HCMV	human cytomegalovirus
h	hour
HREC	Human Research Ethics Committee
HSV	herpes simplex virus
Ig	immunoglobulin
IL-6	interleukin-6

IQR	interquartile range
kg	kilogram
KICS	KSHV inflammatory cytokine syndrome
KS	Kaposi's sarcoma
KSHV	Kaposi's sarcoma-associated herpesvirus
LANA	latency-associated nuclear antigen
LTFU	lost to follow-up
M	membrane
MCD	Multicentric Castleman disease
min	minute
MSM	men who have sex with men
Mtb	*Mycobacterium tuberculosis*
N	nucleocapsid
NHLS	National Health Laboratory Services
OD	optical density
OR	odds ratio
ORF	open reading frame
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline-tween 20
PCR	polymerase chain reaction
PD-1	anti-programmed cell death protein-1
PEL	primary effusion lymphoma
PLWH	people living with HIV
RBD	receptor-binding domain

RT	room temperature
RTA	replication and transcription activator
S	Spike
SA	South Africa
SD	standard deviation
SSA	Sub-Saharan Africa
SARS-CoV-2	severe acute respiratory syndrome coronavirus-2
TNF	tumour necrosis factor
UCT	University of Cape Town
vFLIP	viral homolog of the Fas-associated death domain-like IL-1-β-converting enzyme inhibitory protein
VL	viral load
WHO	World Health Organization

Abstract

High exposure to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of the COVID-19 pandemic, occurred primarily in densely populated, low-income communities which are additionally burdened by a high prevalence of Human Immunodeficiency Virus (HIV) and Kaposi's Sarcoma Associated Herpes Virus (KSHV). SARS-CoV-2 co-infection with herpesviruses has been suggested to have an impact on acute and/or long-term disease progression by triggering their reactivation from latency. We have previously reported that lytic KSHV infection (assessed by blood viral load (VL)) was associated with morbidity and mortality in critically ill COVID-19 patients. However, the impact of SARS-CoV-2 exposure on HIV/KSHV co-infected non-hospitalised individuals is currently unknown.

We therefore performed a longitudinal observational cross-sectional study (n = 407) on non-hospitalised HIV-infected adult patients attending antiretroviral therapy (ART) services in Gugulethu, South Africa, from October 2020 to April 2023. The start of recruitment for this study coincided with the decline of SARS-CoV-2 infections from the first COVID-19 wave and before nation-wide COVID-19 vaccine roll-out, continuing throughout subsequent waves and vaccine introduction. Exposure to SARS-CoV-2 was very high and increased from an initial quarterly 76.2 % seropositivity (before COVID-19 vaccine roll-out) to 94.9 % by the end of the recruitment; 32.2 % of this cohort was self-reportedly vaccinated against COVID-19. The overall KSHV seroprevalence was 53.5 %, with the quarterly percentage of patients with detectable KSHV VL in the peripheral blood increasing from 3.3 % to 69.2 %. When assessing SARS-CoV-2 seroprevalence and its potential association with KSHV reactivation, we found that KSHV VL presence was significantly associated with SARS-CoV-2 RBD IgG antibody titres in unvaccinated patients, and logistic regression revealed significantly higher odds of KSHV lytic reactivation in unvaccinated patients who were previously exposed to SARS-CoV-2 (adjusted OR 1.28 [95 % CI: 1.05 – 1.55], p = 0.015), compared to vaccinated patients (adjusted OR 0.83 [95 % CI: 0.67 – 1.02], p = 0.080).

In addition, we invited KSHV seropositive patients with or without previous SARS-CoV-2 infection for follow-up (FU) (n = 46) every 6 months over a 12-month period to determine the effect of SARS-CoV-2 infection on lytic reactivation of KSHV. Supporting our observations of the cross-sectional study design, the number of unvaccinated individuals with detectable KSHV VL increased, particularly from the 6- (13.3 %) to 12-month (22.2 %) visit but decreased steadily in the vaccinated patients from initial recruitment (15.8 %) to 12-month FU (0 %). Further analysis using a cox regression model confirmed a higher probability of KSHV

detection (as a measure of KSHV reactivation) over time in unvaccinated compared to vaccinated patients in response to SARS-CoV-2 exposure.

Moreover, we identified one patient with an unusually high KSHV VL early in the recruitment phase who self-reportedly remained unvaccinated against COVID-19 throughout the study period. This patient was invited for FU visits every 6 months for a total of 2 years and exhibited persistent KSHV viremia, together with increased SARS-CoV-2 and KSHV serology. While non-adherence to TB/HIV treatment, his living circumstances and/or malnutrition may be the cause of his uncontrolled KSHV viremia, other underlying infections and specifically (repeated) SARS-CoV-2 infection may have played contributing roles.

Cumulatively, the results of this study indicate a positive association between high SARS-CoV-2 exposure and the risk of KSHV reactivation in unvaccinated HIV-infected patients suggesting that, conversely, COVID-19 vaccination plays a protective role against the downstream effects of SARS-CoV-2 infection that we postulate causes lytic reactivation. As lytic reactivation of KSHV may have long-term consequences, particularly in the context of patients with impaired immune functions, identifying and monitoring patients at risk for KSHV reactivation, prevention of KSHV-associated pathologies and appropriate treatment strategies are therefore important in the post-pandemic era.

1. Introduction

1.1 Navigating the South African infectious disease landscape: An introduction into KSHV infection and co-infections

Kaposi sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus-8 (HHV-8), is an oncogenic member of the gamma-herpesvirus subfamily. Etiologically, KSHV infection is linked to Kaposi's sarcoma (KS), the most common acquired immunodeficiency syndrome (AIDS)-related malignancy [1, 2]. It is also the etiological agent for other malignant pathologies which include primary effusion lymphoma (PEL), multicentric Castleman disease (MCD) and KSHV inflammatory cytokine syndrome (KICS). These diseases, along with KS, primarily occur in individuals infected with human immunodeficiency virus (HIV) (see section 1.4) [3-9].

Even before the onset of the HIV/AIDS epidemic, KSHV was endemic in Sub-Saharan Africa (SSA). In comparison, KS was as common in regions of SSA (such as Uganda) as particular cancers (e.g. colon cancer) are in Europe and the USA [10, 11]. Endemic KS is still prevalent in SSA, despite the high incidence of epidemic KS resulting from the HIV epidemic [11].

KSHV, like other herpesviruses, exhibits a biphasic life cycle which consists of a latent and a lytic phase (see section 1.2). While latency is essential for the establishment of persistent infection, reactivation from latency to lytic replication is required for transmission to occur [12, 13]. Pathogenesis thus requires a balance between the two phases of the virus' life cycle and this process has been found to be controlled by specific stimuli (see section 1.3) [13].

While KSHV infection is a necessary requirement for the emergence of KSHV-related malignancies, it does not possess the capability to initiate tumorigenesis on its own. Other co-factors are required for this process, such as immune suppression. Indeed, co-infection with HIV is known to be one of the most important co-factors in KSHV-related malignancy progression [2, 14-16]. Moreover, individuals that are co-infected with HIV and KSHV may develop more than one KSHV-related pathology, thus emphasising the need for clinicians to carefully consider all possible diagnoses when making decisions on treatment [1].

In addition to HIV, co-infection with *Mycobacterium tuberculosis* (Mtb), *Plasmodium* sp., human cytomegalovirus (HCMV), Hepatitis C Virus (HCV) and, most recently, severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) are common infectious agents in SSA [17] which potentially affect KSHV reactivation and KSHV-associated disease progression [2, 17].

In particular, the high prevalence of tuberculosis (TB) which continues to be the leading cause of mortality in HIV-infected individuals in SSA (resulting in a third of all AIDS-related deaths) [18, 19] has caused overdiagnosis and overtreatment of TB and a delay in the diagnosis of non-TB-related pathologies, such as lymphomas and lung cancer, which can mimic TB symptoms at presentation [18-22]. These symptoms include, but are not limited to, fever, weight loss, chest pains, night sweats and lymphadenopathy [21, 22]. KSHV-associated pathologies may also mimic these symptoms [19]. Even though KSHV usually establishes a state of asymptomatic, life-long latency subsequent to acute infection, it also has the capability of both causing inflammatory symptoms upon lytic reactivation as seen in MCD and KICS (see section 1.4.3 and 1.4.4) and inducing malignancies as seen in KS, PEL and MCD (see sections 1.4.1-1.4.3) in individuals who are infected and who exhibit weakened immune systems [2]. Indeed, previous work from our laboratory assessed and confirmed an association of elevated KSHV viral load (VL) and mortality in critically ill HIV-infected patients with suspected but not confirmed TB [19]. Since elevated KSHV VL has been linked to KSHV-associated malignancies [23], it was concluded that an elevated KSHV VL should be considered an important marker to guide diagnostic and therapeutic evaluation in HIV-infected patients investigated for TB or for those that meet the criteria for KICS (see section 1.4.4 for KICS working definition) [19].

The recent outbreak of the coronavirus disease 2019 (COVID-19) pandemic added an additional burden to already vulnerable populations. SARS-CoV-2 co-infection with HIV and/or herpesviruses has been suggested to have the potential to impact on acute and/or long-term disease progression by triggering lytic reactivation of latent viruses like KSHV, Epstein-Barr Virus (EBV), Herpes Simplex Virus (HSV), human cytomegalovirus (HCMV), HHV-6 and HHV-7 [24, 25]. Indeed, it has been suggested that SARS-CoV-2 encoded proteins have the potential to induce lytic reactivation of KSHV from latently infected cells *in vitro*, through the manipulation of intracellular signalling pathways [26]. In addition, it has previously been reported that systemic herpesvirus (EBV, HCMV and HHV-6) reactivation may occur in critically ill COVID-19 patients which was associated with morbidity and mortality [27]. This is supported by previous work from our laboratory which assessed the contribution of KSHV to COVID-19 severity and outcome in hospitalised South African patients (41.1 % were KSHV seropositive). The results of this study indicated that detectable KSHV VL, which is an indicator of lytic reactivation, was associated with mortality in these patients [28]. However, due to the cross-sectional study design it is still speculative whether the underlying KSHV infection was contributing to COVID-19 severity or if SARS-CoV-2 directly and/or the COVID-19-associated “cytokine storm” (see section 1.6.2) was causing the reactivation of KSHV [28].

Evidently, there are still visible gaps with regards to the COVID-19 pandemic and how it impacts on underlying co-infections. In addition, the long-term effects of COVID-19 on virus-associated cancer morbidity and mortality is still unknown, particularly in populations with high prevalence of oncogenic viruses and HIV. This thesis therefore characterised the impact of SARS-CoV-2 infection on reactivation of KSHV in HIV-infected patients with the long-term aim to inform post-pandemic prevention and/or monitoring strategies of potential KSHV-associated pathologies in high-risk patients with immunodeficiencies.

1.2 KSHV

There are over 100 herpesviruses, with 8 currently known to infect humans. These human herpesviruses have distinct host cell specifications and can be classified into three subfamilies: alpha-, beta- and gamma herpesviruses. The alphaherpesvirus subfamily includes herpes simplex virus type 1 (HSV-1 or HHV-1), herpes simplex virus type 2 (HSV-2 or HHV-2) and varicella-zoster virus (VZV or HHV-3), while HCMV (or HHV-5), HHV-6 and HHV-7 belong to the betaherpesvirus subfamily. EBV (or HHV-4) and KSHV (or HHV-8), the two oncogenic herpesviruses, belong to the gammaherpesvirus subfamily [17, 29].

1.2.1 The structure of KSHV

As a typical herpesvirus, the structure of KSHV is four layered and consists of a core containing the large, double-stranded DNA genome, a nucleocapsid that is surrounded by a lipid bilayer envelope, and a tegument, the layer between the capsid and the bilayer, which is divided into an inner and outer layer [4, 13, 15, 29] (Figure 1.1A).

Herpesviruses are usually equipped with glycoproteins which have various roles in the infection process. At the initial stages, a group of viral glycoproteins are usually implicated in adhering to the surface of the host cell. Subsequently, certain interactions between viral glycoproteins and receptors on the host cell ensue, molecular interactions and triggers occur, which ultimately result in the fusion of the viral envelope with the cell membrane. KSHV glycoproteins consist of those specific to KSHV (such as K8.1A, ORF4, ORF28, ORF45 and ORF68) and those that are homologous to other herpesvirus glycoproteins (such as gB, gH/gL, gM and gN; Figure 1.1A) [30]. While the gH/gL complex together with gB represent the components of the conserved herpesviral core fusion machinery, K8.1, the major antigenic component of the KSHV virion, functions in a highly cell-specific manner during KSHV entry

at the attachment step, playing an important role in the infection of epithelial cells (Figure 1.1B) [31].

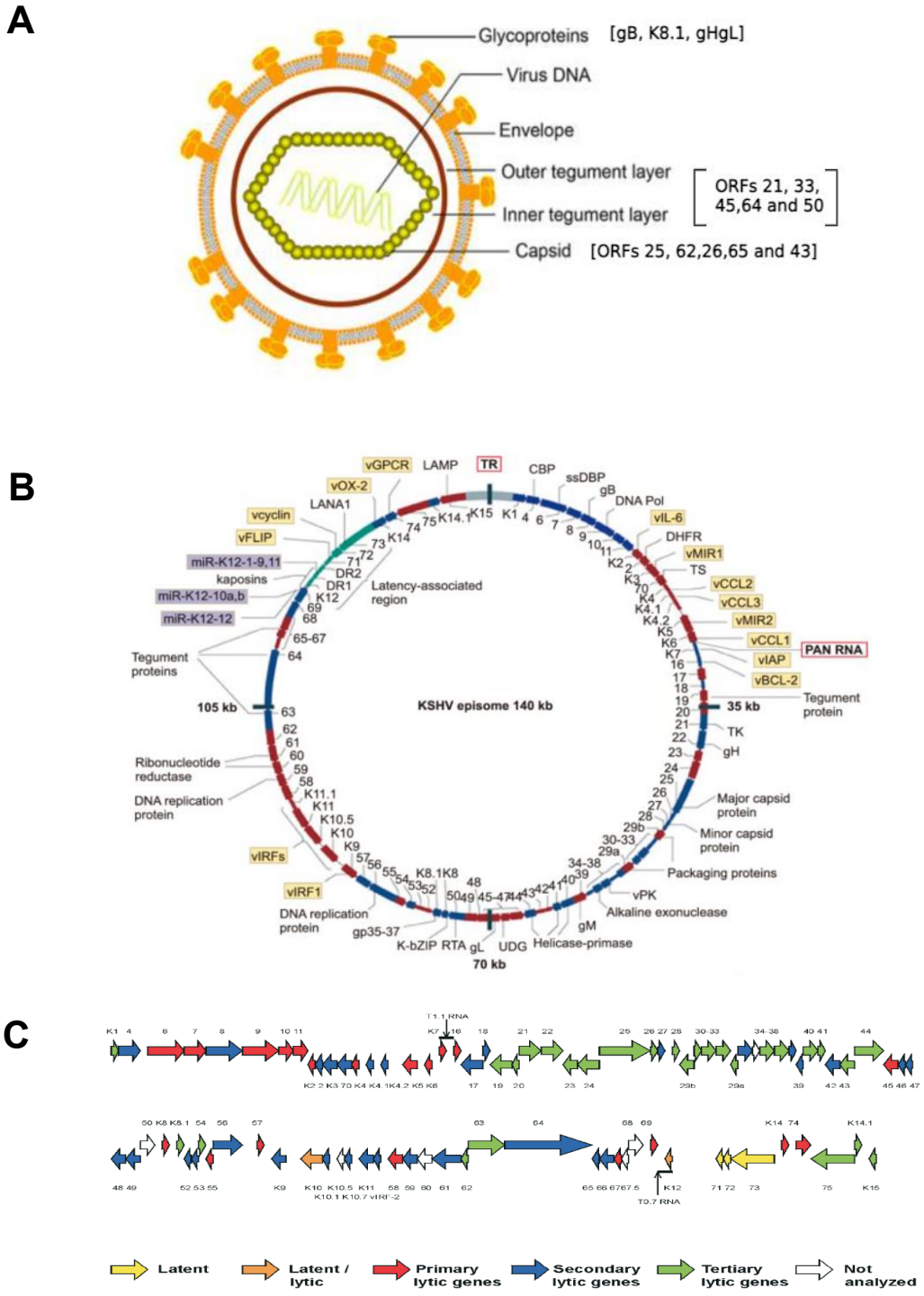

Figure 1.1: KSHV Structure. A) A schematic presentation of the structure of KSHV adapted from Yan *et al*. [32] and modified in BioRender (BioRender.com). **B)** Episome of KSHV with latent genes indicated in green, early lytic genes indicated in red, late lytic genes indicated in blue, miRNAs indicated in purple and cellular orthologues indicated in yellow. Adapted from Mesri *et al*. [16]. **C)** Linear map of the KSHV genome colour coded according to expression pattern. Adapted from Jenner *et al*. [33].

KSHV with an approximately 140 kb genome encodes 87 open reading frames (ORFs) and 17 viral miRNAs (Figure 1.1B and 1.1C) with 14 of these co-expressing as a cluster. The episome of KSHV contains a latency-associated region characterised by genes important in the latent cycle, while genes encoding for lytic transcripts make up the remainder of the episome. Early lytic genes include those that encode viral proteins that are required for DNA replication or viral gene expression, while late lytic genes include those genes that encode viral structural proteins (envelope and capsid proteins) which are required for viral assembly (see section 1.2.4) [16]. In Figure 1C, a linear map of the KSHV genome shows the locations of genes (colour coded) according to expression pattern class. Latent, lytic, primary lytic (coincides with early genes), secondary lytic (coincides with late lytic genes) and tertiary (coincides with the peak stage of lytic replication) genes are indicated (Figure 1C) [33].

1.2.2 KSHV prevalence

The prevalence of KSHV varies geographically and can be divided into three patterns: high-level (seroprevalence between 30 – 70 %), intermediate-level (seroprevalence between 10 – 25 %) and non-endemic (<10 %) [1, 15, 32, 34]. Areas such as the Mediterranean and SSA are endemic areas with the highest prevalence of KSHV found in SSA (40 – 50 % seropositivity), followed by Mediterranean countries (10 – 25 %). KSHV is much less common in the US, Europe and Asia (<10 %) [15, 32, 35, 36]. In addition to geographical regions, the prevalence of KSHV varies in particular population groups [37].

As can be seen in Figure 1.2A, HIV prevalence in SSA is high compared to other regions in the world. This coincides with the high KSHV prevalence in this geographic region (Figure 1.2B). High prevalence of KSHV, however, has been seen in individuals of specific ethnicities regardless of HIV status in this area where KSHV (and KS) was endemic even before the onset of the HIV/AIDS epidemic. These regions include, but are not limited to, Uganda (14 – 95 %), Cameroon (>80 %) and the Ivory Coast (43 – 100 %) [38, 39]. Consequently, KS incidence is highest in SSA (Figure 1.2C) and represents a combination of both endemic and AIDS-related KS (two of the five epidemiological subtypes of KS, see section 1.4.1).

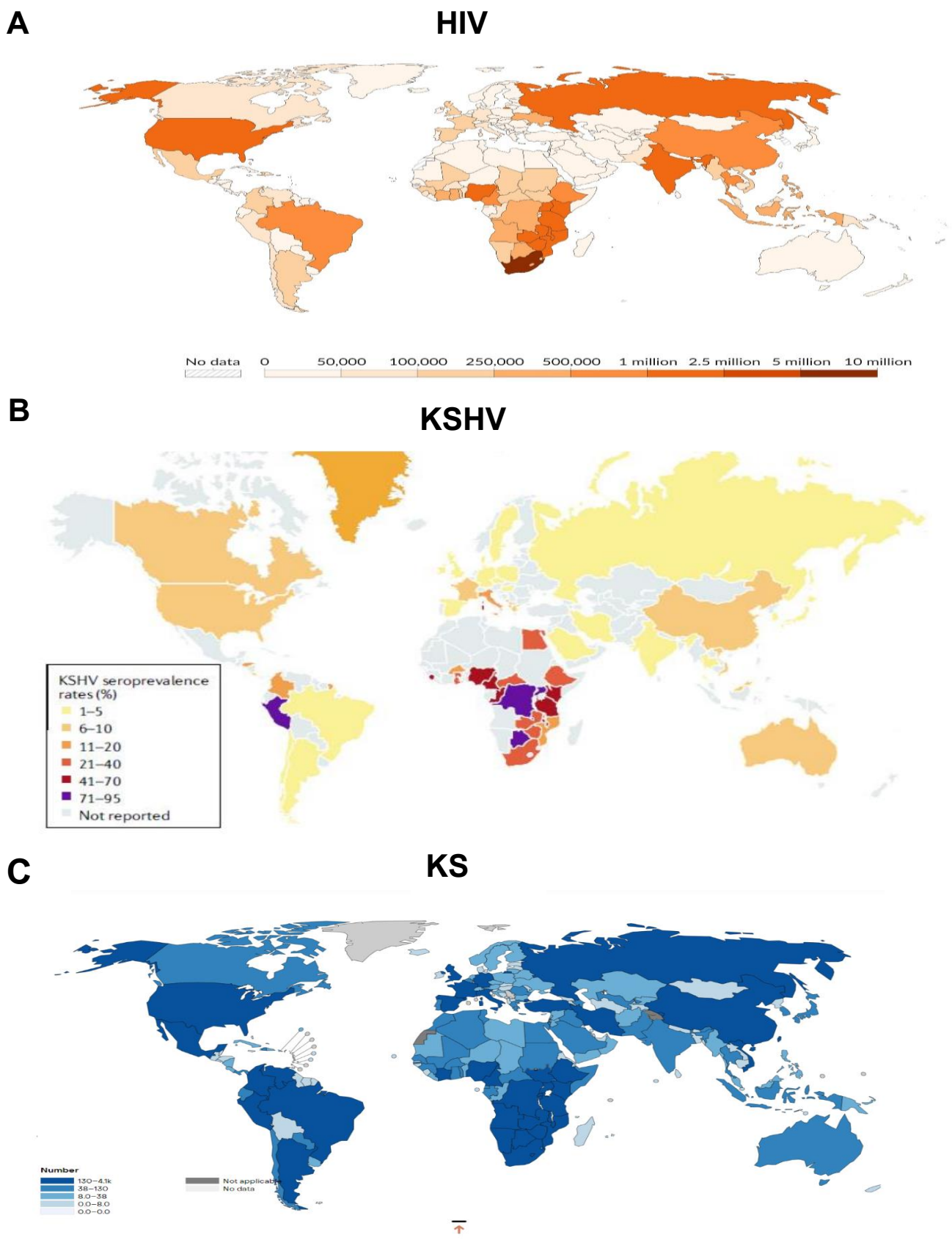

Figure 1.2: Epidemiology of HIV, KSHV and KS. A) Geographic representation of the number of people living with HIV globally in 2019 produced by OurWorldinData [40]. **B)** KSHV seroprevalence rates, adapted from Cesarman *et al*. [36]. **C)** KS incidence as reported in Globocan 2022 [41].

1.2.3 KSHV transmission and symptoms of infection

The transmission of KSHV varies in different geographical populations with the main route of spread propagating through saliva [1, 42]. In SSA, KSHV is usually transmitted during early childhood (from mother to child as well as through siblings with a close age range). For example, Dedicoat *et al*. [43] conducted a study to determine mother to child transmission of KSHV in SA where over 2000 mother-child pairs were recruited from rural clinics in SA. Prevalence of antibodies against both latent and lytic KSHV antigens in the enrolled children increased with increasing antibodies titres of their mothers, supporting mother to child transmission of the virus [43].

KSHV transmission is also possible throughout an individual's life through virus shedding via the saliva and other bodily fluids [1, 12, 44]. For instance, in most western countries, transmission is primarily known to be sexually in HIV-infected homosexual men, although there is little information on the transmission in women [45-47]**.** Transmission is also possible through blood or blood products as well as organ transplantation. Reports on transmission via drug injection (needle sharing) has been controversial where it is reported as uncommon in some groups but prevalent in others [15, 45-48].

There has also been speculation around behavioural practices in SSA which might enhance the risk of KSHV transmission. These include healing and medical practices (e.g. spitting as a form of treatment), the process of initiation or other ritual practices (e.g. certain birth rituals involve using saliva) as well as certain feeding practices which are associated with saliva as means of transmission [12, 49].

KSHV usually manifests as an acute infection (lytic) and subsequently establishes lifelong (often asymptomatic) infection (latency): under specific conditions lytic reactivation may be induced. Most individuals presenting with KSHV infection are asymptomatic; however, when symptoms develop they are usually associated with KSHV-related malignancies [2]. Some individuals may present with mild, non-specific symptoms which include lymphadenopathy, localised rash, fatigue and diarrhoea [50]. Host T-cell responses are likely to control KSHV infection; however, with a decline in T-cell immunity, mostly linked to immunosuppression, KSHV-infected individuals have an increased likelihood of developing KSHV-related malignancies (see section 1.4) [2, 50, 51]. Gene products from both lytic and latent cycles contribute to the development of KSHV-related malignancies (see section 1.4).

1.2.4 KSHV replication and lifecycle

KSHV has a broad cell tropism/spectrum *in vivo*, as it can infect endothelial cells, epithelial cells, monocytes, keratinocytes and B-cells where it can establish a state of latency [52]. The molecular interactions required for infection may vary significantly depending on the cell type. In addition, a major factor contributing to its broad tropism is KSHV's capability to engage with a diverse array of molecules serving as receptors [30].

Like other herpesviruses, KSHV displays a biphasic lifecycle consisting of a persistent latent infection and short lytic replication cycles which have distinct gene expression patterns (Figure 1.3) [32, 53]. Upon infection characterised by short lytic replication, KSHV usually takes up a dormant state and establishes asymptomatic life-long latency, where its genome is maintained as an episome within the host cells' nuclei with no progeny being produced [2, 13, 54]. Lytic replication can be activated by chemical stimulation, by cytokines or through co-infections (see 1.3) [13, 17].

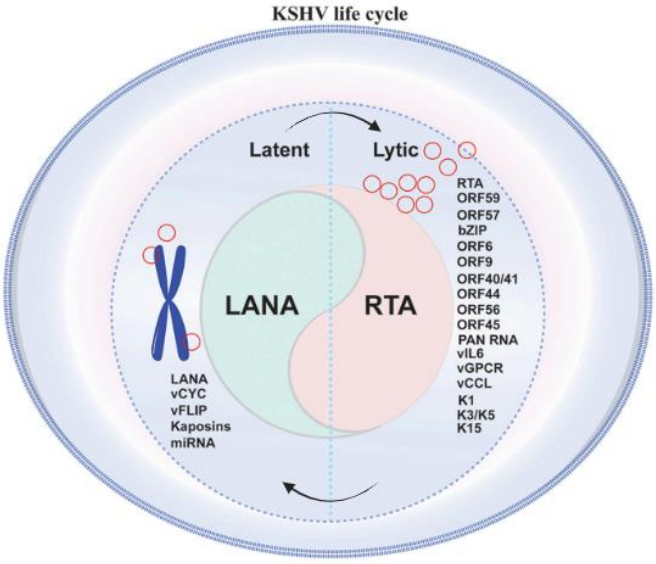

Figure 1.3: **Schematic diagram of the KSHV life-cycle adapted from Purushothaman *et al*. [55] and modified in BioRender (BioRender.com).**

KSHV lytic gene expression occurs in the order of immediate early, early and late transcripts, these genes are also indicated as Primary (early lytic), Secondary (late lytic) and Tertiary (peak lytic) (Figure 1.1C) [13]. During the latency period, only a few genes are expressed, these include genes encoded by ORF71 (viral homolog of the Fas-associated death domain-like IL-1-β-converting enzyme inhibitory protein, vFLIP), ORF72 (vCyclin), ORF73 (LANA), ORFK10.5 (vIRF3), ORFK12 (kaposin) and viral miRNAs [1, 56, 57]. The lytic phase involves

the expression of all genes with the switch to lytic replication mediated by the ORF50 encoded replication and transcription activator (RTA) (Figure 1.1B and C) [1, 58].

The balance between latent and lytic states is controlled by various viral and host gene products, with the lytic gene regulator, RTA, and the latent gene product latency associated nuclear antigen (LANA) playing key roles in this process [13, 54].

1.2.4.1 Latent phase

This phase of the KSHV life cycle can be described as the dormant or non-productive state, with only a few viral genes being expressed and no virus particles being actively produced [53]. During this state the virus establishes persistence in the host avoiding immune recognition and ensuring its survival [2, 59]. Viral persistence occurs through replication of the infected cell and segregation of viral episomes during mitosis to daughter cells [60]. Latent phase gene products have the potential to promote tumorigenesis. These genes include LANA, which is also the most abundantly expressed protein, vCyclin, kaposins, the viral FLICE inhibitory protein as well as miRNAs (Figure 1.1B and 1.3) [59].

Latency relies on controlled gene expression; this is facilitated by epigenetic modifications of the virus genome and its associated histones. After entry into the host cell nucleus, the KSHV genome relies on the epigenetic machinery to convert the genome into a fully chromatinised, latent KSHV episome. The initially open chromatin causes low-level RTA expression which contributes to the activation of H3K4 trimethylation and H3K27 acetylation (these contribute to the initial activation of the KSHV genome). After 24 – 72 h, the KSHV genome shifts to repressing H3K27 trimethylation and H2AK119 ubiquitylation marks which contribute to setting up KSHV for latency [60].

Of the latency associated genes, LANA plays an important role in both initial KSHV latency establishment and maintenance of the latent stage. Many of LANA's functions depend on its ability to recruit host-specific DNA modifying enzyme complexes to the viral genome. The recruitment of these host machinery complexes is to ensure DNA replication, persistence of its episome and to maintain latency which is important for survival and pathogenesis as the virus does not have its own cellular machinery [61].

Once latency has been established, LANA maintains this state with the cooperation of the other latent gene products such as vFLIP, vCyclin and kaposins. LANA and vFLIP stimulate the upregulation of enhancers (such as the H3K27 methyltransferase zeste homolog 2) that are important in solidifying a repressive chromatin state [55, 60]. vCyclin phosphorylates a

histone chaperone, nucleophosmin, that has a role in transcriptional control and the organisation of chromatin, thereby enabling LANA binding, promoting latency [60]. Kaposins are proteins encoded by ORF K12, consisting of kaposin A, B and C. Of interest is kaposin B, which has been reported to increase pro-inflammatory cytokine expression by inhibiting cytokine mRNA degradation, resulting in a tumour-friendly KS microenvironment, typical for KSHV-associated malignancies [16, 55, 62]. Kaposin B activates the p38/MAPK-associated protein kinase 2 pathway (MK2), which, together with the elevated kaposin B-mediated cytokine release, may contribute to an autocrine/paracrine amplification loop that further enhances p38 activity. The activation of the p38/MK2 pathway has been suggested as a link between KSHV infection and the increased cytokine production. Moreover, viral miRNAs are also involved in maintaining KSHV latency through suppression of viral lytic genes as well as inducing host survival pathways through hijacking the NF-κB pathway [55, 63-65]. All KSHV miRNAs target RTA and inhibit viral lytic gene promoter activation [55].

When KSHV latent cells are reactivated to a lytic state by various stimuli (see section 1.3), the virus episome's compact chromatin structure begins to relax leading to viral gene expression and infectious virion particle production [55].

1.2.4.2 Lytic phase

The lytic phase of infection is inducible by external factors leading to the production of infectious virions [32]. This phase therefore allows for virus propagation and the spread to a new host and is characterised by viral gene expression that follows a meticulous order. It allows efficient replication of the viral DNA as well as efficient packaging into new virions [2, 32]. Once activated, several viral proteins and cellular factors are involved to regulate this process [32]. A key factor regulating the latent to lytic infection switch is the immediate early gene, ORF50/RTA. It is the first lytic gene to be expressed in B lymphocytes during replication. This gene product then triggers expression of early and then late genes [55].

Importantly, the transition between latency and lytic reactivation is controlled by the LANA/RTA interaction in KSHV-infected cells [66]. Once RTA has accumulated to a sufficient level it can outcompete LANA allowing RTA to target distinct sites in the KSHV genome and transactivate 34 lytic genes [67] in the order immediate early – early – late, as mentioned in section 1.2.4. The immediate early genes mediate the transition from latent to lytic phase and include RTA/ORF50 (i.e. RTA autoregulates its own transcription), ORF70, ORF29b, ORF48, K3, K4.1, K4, K8α and K8.2 and are involved in transcription of genes and cell modifications required for viral replication. RTA also transactivates the early genes which include ORF6,

ORF21, ORF57, ORF74, ORF37, ORF59, ORF65, K1, K8, K5, K2, K12, K3, K9, K1, K8.1A, viral interleukin-6 (IL-6), and viral interferon regulatory factor 1 (vIRF1; Figure 1.1B). These genes encode viral proteins that are necessary for DNA replication and gene expression. The late genes which consist of K8.1 and gB are viral structural and membrane glycoproteins (Figure 1.3). They also encode the small viral capsid antigen ORF65 which is required for assembly and maturation of virions [55, 66, 68]. As mentioned above, the RTA promoter transactivates an autoregulatory feed-forward loop, thereby further promoting lytic gene expression. Certain lytic gene products, like K8 and ORF57, maintain this loop, ensuring KSHV reactivation sustains itself through lytic protein contributions to the lytic cycle [55].

Lytic reactivation is important for the dissemination of virus particles within the host. While the complete reversion back to latency may be complex (due to the interplay between both viral and host factors), dampening lytic reactivation to re-establish latency is an intriguing area of research and provides scope for potential therapeutic interventions [60].

1.3 Triggers of KSHV lytic reactivation

Both internal and external stimuli have been described that can potentially trigger KSHV lytic reactivation from latency. These include inflammation, oxidative stress, immune suppression, hypoxia and cytokines and various infectious agents [17, 69], which can transmit the signal via miRNA, alterations of histone modifications, reactive oxygen species, cellular stress responses and modifications of host cell transcription factors binding to viral promoters [70].

Co-infections of KSHV with HIV, HSV-1, HSV-2, HHV-6, HHV-7, HCMV, Mtb, *Plasmodium* sp. and most recently SARS-CoV-2 may also cause KSHV reactivation [17, 71]. Viral co-infections may trigger the secretion of inflammatory cytokines like oncostatin M and interferon γ, which may in turn trigger lytic KSHV reactivation [2, 72, 73]. Indeed, HIV was found to reactivate KSHV both directly or through enhanced cytokine production [2]. In addition, the decrease or lower CD4+ count (and expansion of CD14++CD16+ monocytes) in HIV-positive individuals, causes an unbalanced population of CD4+ cells and the associated inflammation triggers KSHV lytic reactivation [71]. Reactivation of KSHV can also be influenced by HIV proteins, specifically trans-activator of transcription (tat), which has the potential to activate the immediate early gene RTA. HIV can additionally induce lytic replication through KSHV RTA in a tat-independent manner [17, 74].

HCMV (also endemic to SSA) is usually asymptomatic in healthy individuals, with reactivation especially occurring in immunocompromised individuals who are also at an increased risk of

KSHV-associated malignancies. Moreover, HCMV triggers KSHV reactivation by stimulating RTA through its UL112-113 region. The region encodes four nuclear phosphoproteins via alternative splicing which plays a crucial role in HCMV DNA replication [2, 75].

As mentioned previously, the high prevalence of Mtb in SSA has led to an overdiagnosis and overtreatment of TB, resulting in underdiagnosis of KSHV-related pathologies that mimic these symptoms [17, 19]. KSHV VL and antibodies in patients with underlying Mtb infection indicated higher titres in co-infected patients than in controls [76]. In addition, secreted virulence factors were found to induce reactivation *in vitro*. This was confirmed by the expression of the lytic gene RTA and other genes in endothelial and PEL cell lines [77].

Plasmodium sp. has also been indicated as a risk factor for KSHV reactivation [2]. Nalwoga *et al*. [78], showed an association between exposure to the malaria parasite and KSHV seropositivity [2, 78]. *Plasmodium* sp. can cause anaemia (following the blood stage of infection), and this may cause hypoxia in the affected individuals, potentially triggering KSHV reactivation. Repeated infections can lead to reactivation due to B- and T-cell immunity impairment. Furthermore, the drugs used to treat malarial infection may cause immune suppression, ultimately leading to lytic replication of KSHV [2, 78, 79]. Additionally, *Plasmodium* sp. triggers a T helper type 2 immune response, characterised by the production of cytokines such as IL-4, IL-5 and IL-13. It has been shown that IL-4 is involved in the reactivation of KSHV *in vitro*, which was found to be associated with an increase in the expression of immediate early (RTA, ORF45 and ORF57) and late (ORF19) lytic transcripts [17, 80].

Both HSV-1 and HSV-2 are infectious co-factors with the potential to reactivate KSHV. Indeed, infection with HSV-1 was shown to activate RTA through downregulation of two cellular miRNAs (miR-498 and MiR-320d) which directly target RTA and inhibit its expression [17, 81]. Similarly, infection with HSV-2 was reported to lead to RTA activation which resulted in expression of viral proteins, production of infectious viral particles and lytic phase mRNA transcripts in BCBL-1 cells [17, 82].

HHV-6 co-infection can occur in KSHV infected cells, especially those with a macrophage/ monocyte lineage, influencing gene expression through direct interactions (e.g. HHV-6 infection was noted in monocytes infiltrating KS lesions). Infection with HHV-6 may cause a deregulation of the expression of certain cytokines such as interleukin 1β, tumour necrosis factor (TNF) α and interferons [2].

Most recently, emerging evidence indicates that co-infection with SARS-CoV-2 may induce KSHV lytic reactivation (see section 1.7).

1.4 KSHV-associated pathologies

1.4.1 Kaposi's sarcoma

KS is a multicentric hyperproliferative disease presenting with lesions, which appear as spindle-shaped tumour cells of endothelial origin, that are usually accompanied by fibrosis, vascular slits and inflammatory infiltrates [1]. KS is classified by five epidemiological subtypes, namely: classic KS (found in Mediterranean and Eastern European regions), endemic KS (found in Africa), transplant-associated or iatrogenic KS, KS involving men who have sex with men (MSM), and AIDS-associated KS, the latter being the most common AIDS-related malignancy worldwide [1, 32, 37]. KS involving MSM is the most recent classification to be described, i.e. HIV negative MSM with an increased risk of KS. Here, KS presents in a classic KS manner in a younger cohort of males [37, 83]. A new classification system for KS has very recently been proposed, dividing patients into immunocompromised and non-immunocompromised groups [37]. This new classification may be more relevant in guiding clinical and therapeutic approaches compared to the traditional KS classification.

Although the introduction of ART and combination ARTs has reduced the incidence of KS worldwide, the disease is still common in SSA compared to other geographical regions. In some countries in SSA, such as in Uganda, KS is the most common tumour found in men [1, 84].

Besides HIV-related immunosuppression facilitating carcinogenesis generally, the interplay between HIV and KSHV specifically contributes to the development of KS. Both HIV and KSHV lytic infection promote the production of cytokines, which may initiate both tumour development and progression [85]. Additionally, KSHV promotes HIV viral transport, where KSHV stimulated dendritic cells capture more HIV viral particles and increase HIV transport to CD4+ T cells [86]. The HIV-1 encoded tat stimulates growth, migration, invasion and adhesion of endothelial cells and KS tumour cells. Tat is able to trigger KSHV reactivation from latency, and additionally accelerates tumour progression. Tumour progression is induced by KSHV-encoded proteins such as viral G-protein coupled receptor (vGPCR) (which can initiate production of vascular endothelial growth factor (VEGF), IL-6, IL-8 and TNF α, kaposin A and vIL-6. Additionally, the KSHV oncogene K1 and HIV-1 negative factor (nef) together promote angiogenesis in KS lesions by inducing the miRNA, miR-718, to regulate the PTEN/AKT/mTOR signalling pathway. This interaction results in neovascularisation, a characteristic seen in KS patients [87]. In addition to the increased incidence of KS in HIV-infected individuals, KS occurs frequently in transplant patients compared to the general population. This increased risk of KS development is associated with or may be a

consequence of immunosuppressive therapy which differs to HIV-related KS which is a consequence of immunosuppression through HIV co-infection [88].

Other genes central in KS pathogenesis include LANA, vFLIP and vCyclin. As mentioned previously, LANA is the key latent gene involved in viral genome maintenance, while vFLIP inhibits apoptosis and promotes survival of the cells. vCyclin is able to dysregulate the cell cycle. These genes are involved in various cell proliferation and tumorigenesis pathways (such as NF-κB, MAPK, JAK/STAT and PI3K/AKT/mTOR, Notch, RB) to maintain latent infection. Additionally, vCyclin (transcribed from the same promoter element as LANA) in these pathways promotes progression of the cell cycle and might contribute to latency by promoting cell proliferation by phosphorylating proteins (e.g. pRb), histones (e.g. H1), CDK inhibitor and p27 [55]. LANA directly deregulates the MAPK, JAK/STAT, MEK/ERK, PI3K/AKT and Notch pathways in order to establish latency; it is involved in the MAPK pathway through interaction with a mediator complex [55, 89]. In the JAK/STAT pathway the regulation by LANA ultimately results in the release of angiogenic factors; it associates directly with STAT3 and enhances its transcriptional activity [55, 90]. In the Notch signalling pathway, LANA targets the Notch signalling effector which drives latently infected cells towards angiogenesis [55, 91]. Taken together, while the above-described proteins all contribute to KS carcinogenesis, LANA, vFLIP, vCyclin, vIL-6 and vGPCR are crucial oncoproteins in this process [16].

1.4.2 Primary effusion lymphoma

PEL develops from KSHV-infected B-cells and arises in body cavities such as the pleural, pericardial and the peritoneal cavity, without detectable tumour mass [1, 93]. It is considered a rare malignancy and a late occurrence in HIV infection [94]. PEL can occur concurrently with KS and MCD (see section 1.4.3)

PEL cells express latent KSHV genes, such as vCyclin, LANA and the vFLIP gene which activates the NF-κB pathway, a key survival pathway, in latently infected PEL cells [55, 95, 96]. This enhances cell proliferation and survival during latency by means of inhibiting lytic replication [55, 94].

1.4.3 Multicentric Castleman disease

MCD is a rare, likely underreported, lymphoproliferative disorder representing with plasmablasts with the disease often presenting concurrent with KS [1, 19, 97]. Like PEL, MCD

usually occurs in HIV-positive individuals. It is associated with KSHV lytic activation, i.e. patients have elevated KSHV VL in the peripheral blood and present with inflammatory syndromes (involving human IL-6 and -10) [9, 19, 98]. Additionally, vIL-6 is particularly highly expressed in MCD and is the most characteristic protein. The viral processivity factor ORF59, and vIRF1, viral protein kinase (encoded by ORF36) and thymidine kinase (encoded by ORF21) can also be detected in MCD [99]. In addition, patients with MCD present with symptoms that include night sweats, fever, weight loss, fatigue and lymphadenopathy, cytopenia and splenomegaly [100].

The underreporting of MCD in Africa is primarily due to the lack of the invasive diagnostic procedures needed for conclusive diagnosis which include specific histopathological staining of lymph node biopsies for LANA [100-102]. In addition, MCD histology may overlap that of HIV infection features. Therefore, staining for KSHV is necessary to correctly diagnose MCD; however, the procedure for and interpretation of this is technically difficult and requires specialised training which may contribute to the general underreporting of MCD in low-resource settings [101].

1.4.4 KSHV inflammatory cytokine syndrome (KICS)

KICS is the most recently described KSHV-associated pathology which resembles MCD clinically but without prominent lymphadenopathy nor MCD-characteristic histology [9, 98]. KICS patients, often in the context of concurrent KS as well as HIV co-infection, present with inflammatory symptoms such as fever, sweats, fatigue, wasting, cytopenia, as well as elevated vIL-6, IL-10, and IL-6. Importantly, KICS patients have elevated KSHV VL in the peripheral blood indicating lytic reactivation of KSHV. To distinguish KICS from MCD a KICS working case definition for investigation has been developed which requires that a patient has at least two clinical manifestations drawn from at least two of the categories (symptoms, laboratory abnormalities and/or radiographic abnormalities); elevated KSHV VL, evidence of systemic inflammation (elevated C-reactive protein, CRP) and no evidence of MCD [8, 98]. To date, there are not many published reports on KICS, likely due to the difficulty in diagnosing this KSHV-related disease; KICS therefore, requires further investigation to better understand its prevalence and pathology [98].

1.5 Management of KSHV infection, therapeutic strategies, and treatments

Management of KSHV infection involves the treatment of the associated clinical conditions as well as managing an individual's overall health. In SSA, where AIDS-related KS is the most common KSHV-associated pathology, ART is used to control the HIV infection which in turn also manages KS. Indeed, the introduction of ART in SSA which saw 46 % (or 11.8 million) of people living with HIV (PLWH) receiving treatment by 2017 [103], has improved the clinical outcomes of individuals with KS significantly, with 20 – 80 % ART compliant patients reportedly being in complete remission [103, 104]. A Nigerian study showed a decrease in KS incidence from 2.53 to 1.58 per 1000 person with the reduction most likely being driven by earlier HIV care enrolment and ART initiation [105]. Studies from South Africa (SA), Kenya and Uganda indicated effectiveness in reducing KS incidence by 70 – 80 % in SA [106], 78 % in Uganda and 50 % in Kenya [107], respectively. ARTs also play a crucial role in managing PEL and MCD in addition to other therapeutic approaches [108].

Additionally, anti-programmed cell death protein 1 (PD-1) therapy (i.e. treatment with the anti-PD1 antibodies nivolumab or pembrolizumab) has indicated positively for KS patients, counteracting programmed death ligand 1 (PDL-1) which is increased in KSHV-infected monocytes, possibly contributing to immune evasion [67]. For MCD and PEL, rituximab, an anti-CD20 antibody, has shown some benefit in the clinical setting. This treatment decreases the levels of IL-6 and induces B-cell death after binding [67]. Individuals with PEL are treated with a cyclophosphamide, doxorubicin, vincristine and prednisone chemotherapy regimen [108]. Furthermore, in addition to rituximab, a high dose of zidovudine with valganciclovir (AZT/VGC) or rituximab with liposomal doxorubicin are options for treatment for MCD, and paclitaxel and doxorubicin are also used for the treatment of KS [109]. In terms of KICS, individuals may be treated with approaches similar to MCD [98]. However, there are significant disparities between high- and low-income countries regarding access to these treatments primarily due to the high costs involved [110].

As mentioned earlier, there is a high possibility that many cases of KSHV-associated pathologies may be underdiagnosed/underreported in low-resource settings. Due to the unspecific inflammatory symptoms of those pathologies caused by reactivated KSHV (such as MCD and KICS) mimicking common infectious diseases (such as TB), misdiagnosis of KSHV-associated pathologies is not unlikely. Determining KSHV VL in the blood would be a potential solution in those patients presenting with unspecific symptoms at routine clinical visits. This is especially important in areas such as SSA where prevalence of KSHV is high.

1.6 SARS-CoV-2 and COVID-19

SARS-CoV-2 has had a significant impact in SA since it was first detected in March 2020. The timeline of the pandemic can be described by distinct waves, with the first wave peaking in July 2020. This was followed by a second wave (peaking in January 2021), a third wave (peaking in July 2021), a fourth wave (peaking in January 2022) and a fifth wave (peaking in April 2022) (Figure 1.4). A widespread transmission of the virus occurred early on in the pandemic especially in low-income communities where social distancing was limited due to overcrowding. These low-income communities were therefore at an increased risk of SARS-CoV-2 exposure, particularly during the first COVID-19 wave in SA. Interestingly, these communities were somewhat protected from infection and/or COVID-19 disease during the second wave, suggesting some level of attained immunity [111, 112].

Although COVID-19-related mortality and morbidity was substantially lower in SSA compared to Asia, Europe and the Americas, SA reported the highest mortality and morbidity compared to other African countries with the risk factor of age outweighing any other factor (e.g. hypertension, diabetes, cardiovascular disease) as also seen globally [111-114]. The comparably lower reported SARS-CoV-2-related morbidity and mortality in SSA might be due to the generally younger age structure of the population, the lack of long-term care facilities, previous coronavirus infection, limited SARS-CoV-2 testing, genetic factors as well as an effective and swift response from governments [113].

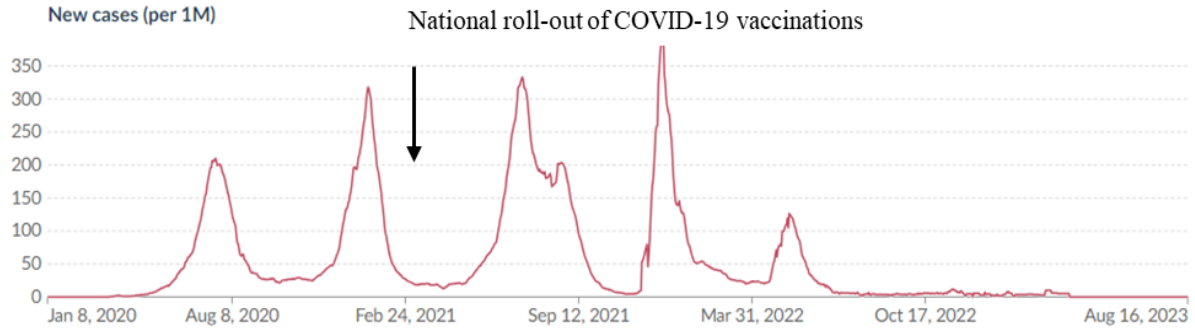

Figure 1.4: Outline of SARS-CoV-2 infection patterns in SA. The 7-day rolling average of daily new confirmed COVID-19 cases per million people in SA is shown as reported on OurWorldInData.org [115]. The start of vaccinations in SA is indicated by the arrow.

The occurrence of the COVID-19 pandemic unexpectedly spread rapidly around the world with initial focus being on controlling infection and treating of presenting symptoms. SSA experienced the COVID-19 pandemic in addition to a long standing and enduring HIV epidemic. At the onset of the COVID-19 pandemic in 2020, there were an estimated 7.8 million PLWH in SA alone, of which 73 % were on ART [116]. In addition, SA is one of the countries

with one of the highest TB burdens in the world (often in the context of concurrent HIV infection) with the addition of the COVID-19 pandemic posing an increased burden on the health system. Interestingly, co-infection with HIV only slightly exacerbated COVID-19 outcome with a large SA study reporting adjusted Odds Ratios (OR) of 1.34 for HIV infection, 1.26 for past TB and 1.42 for current TB in association with hospitalised COVID-19 mortality [92, 114]. However, the measures put in place in SA to handle the COVID-19 pandemic have hampered the containment of TB and HIV by causing disruptions to the treatment of individuals with HIV/TB and a widened socioeconomic gap. This has led to individuals living with HIV facing a higher risk of developing severe COVID-19. As we move on from the COVID-19 pandemic, the impact and influence of the pandemic on both the HIV and TB epidemics will likely become more evident [117].

1.6.1 Structure and classification of SARS-CoV-2

Sequencing of SARS-CoV-2 has shown that it is genetically similar to the previously known SARS-CoV and was thus categorised in the *Coronaviridae* family [118]. Coronaviruses are highly prevalent and have a wide range of hosts ranging from birds (avian hosts) to various mammals (camels, mice, dogs, cats) and humans [119]. They are classified into four genera, namely: Alpha (including the human common cold coronaviruses 229E and NL63), Beta (including the human common cold coronaviruses HKU1 and OC43 as well as the more pathogenic SARS-CoV, MERS and SARS-CoV-2), Gamma (including infectious bronchitis, Beluga whale coronavirus SW1) and Delta (including Bulbul coronavirus HKU11 and Porcine coronavirus HKU157) [119, 120].

SARS-CoV-2 is a single stranded RNA virus with a spherical structure (Figure 1.5A) consisting of a lipid bilayer that envelopes a helical nucleocapsid containing the RNA genome. On the surface of the bilayer are crown-like spikes [119, 121] which incorporate a polybasic cleavage site that is known to increase transmissibility and pathogenicity. In addition to the spike (S) protein, the SARS-CoV-2 virion is made up of the structural proteins envelope (E) and membrane (M) which make up the viral envelope, and nucleocapsid (N). The SARS-CoV-2 genome consists of 12 ORFs: at the 5’ end of the virus genome (overlapping ORFs 1a and 1b) are genes encoding RNA polymerase and other non-structural proteins, occupying almost two-thirds of the genome, while genes encoding structural proteins occupy the last third of the genome. This is in addition to several genes that encode for non-structural proteins and accessory proteins (Figure 1.5B) [118].

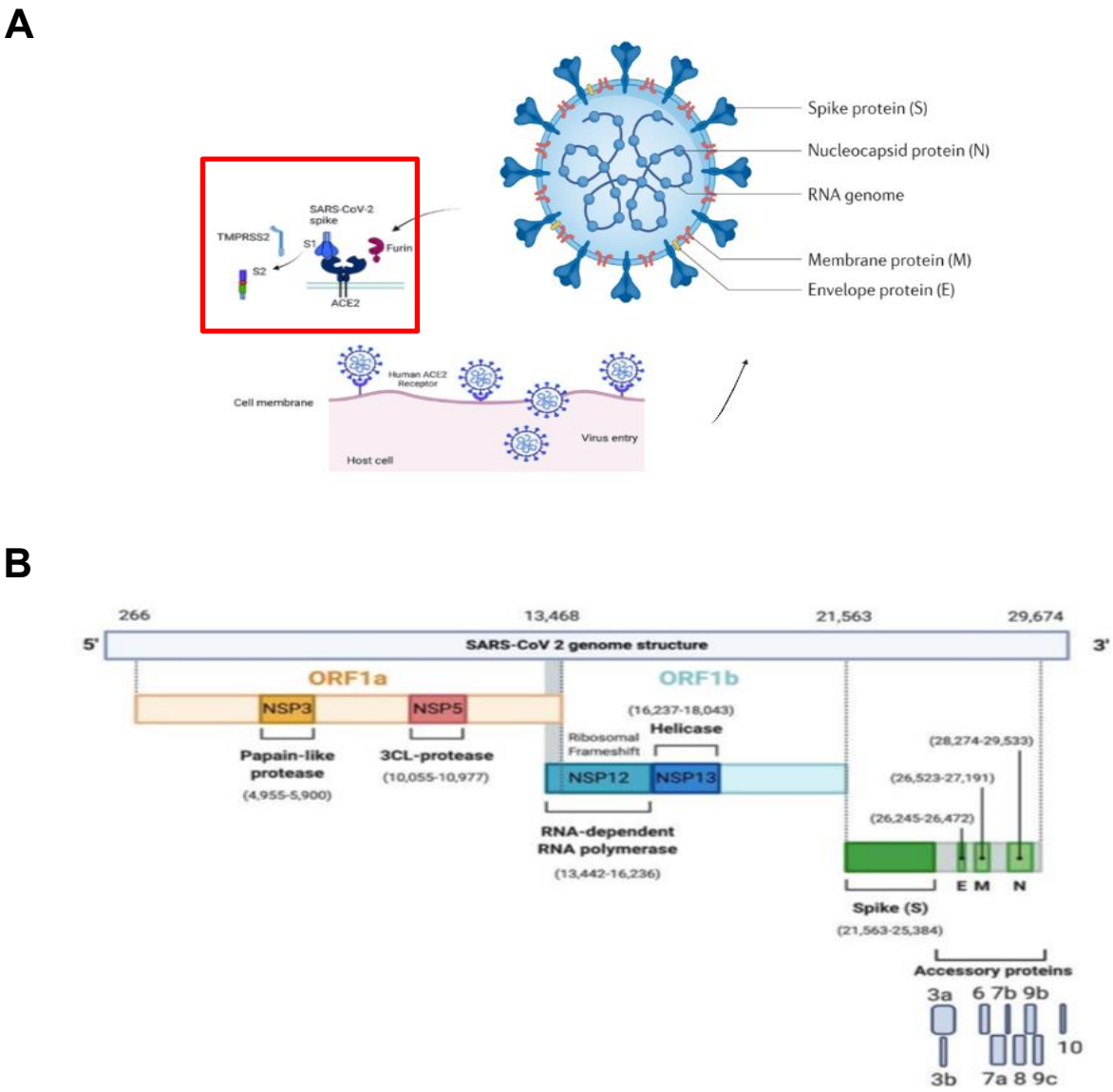

Figure 1.5: A) Schematic presentation of the structure of SARS-CoV-2, the spike protein, and the ACE2 receptor, adapted from Lamers *et al*. [122] and Artik *et al*. [123], modified in BioRender (BioRender.com). **B) Genomic organisation of SARS-CoV-2 showing the large ORF 1a (yellow) and ORF 1b (blue), the regions containing S, and accessory proteins.** Adapted from Alangreh *et al*. [124] and modified in BioRender (BioRender.com).

During infection, the role of S is to facilitate virus adherence to the host epithelial cells situated in the nasal cavity as well as to merge the virus-host cell membranes [119, 121, 125]. The spiky-shaped S protein initially binds to the host receptor Angiotensin-converting enzyme 2 receptor (ACE2) via the receptor binding domain (RBD) situated in the S1 subunit and thereafter leading to fusion of the viral and host membranes via the S2 subunit [126, 127].

1.6.2 SARS-CoV-2 transmission, symptoms and interventions against infection

The spread of SARS-CoV-2 from human to human was quite rapid, primarily causing respiratory illnesses by targeting respiratory epithelial cells [128]. Virus transmission primarily occurred via saliva droplets or any inanimate object that had been exposed to the infectious agent upon close contact (such as hands on door handles that have been exposed). Both symptomatic and asymptomatic SARS-CoV-2 infected individuals were found to be able to transmit the virus, with airborne transmission via aerosols being the dominant mode [121, 129, 130]. Indeed, with an estimated 40 – 45 % of SARS-CoV-2 positive individuals being asymptomatic but infectious [131], undetectable spread of the virus was very common. Therefore, understanding the role of asymptomatic patients and the spread of SARS-CoV-2 is important in developing effective interventions. There have also been studies suggesting transmission via the faecal-oral route through infection of the gastrointestinal tract [128, 132].

Symptoms of SARS-CoV-2 infection can occur at three different levels: mild, severe and critical. The majority of infected individuals experience mild to moderate symptoms, and infections are characterised by viral replication being confined to the upper respiratory tract.

Infection with SARS-CoV-2 usually present with “flu”-like symptoms that may progress to pneumonia and sometimes death. The most common symptoms include, but are not limited to, cough, fever, dyspnea, sore throat and headaches [122, 125]. Some patients, however, experience further disease progression which affect the lower respiratory tract and may lead to an unregulated immune response which in turn leads to an uncontrolled cytokine production (also known as the “cytokine storm”). Cytokines are part of the immune response to various infections; however, a “cytokine storm” indicates an over production of cytokines making it possible to cause multi-organ damage potentially leading to death [133]. In the context of COVID-19, the impact of SARS-CoV-2 is attributed to interferon suppression while at the same time inducing the production of cytokines – this imbalanced reaction is proposed to lead to the “cytokine storm” [134]. In some cases, disease progression like this may advance to fatal pneumonia [17, 122, 125, 135]. A meta-analysis evaluating clinical statistics of almost 9000 patients with varying severity of COVID-19 symptoms indicated the following data: lymphopenia (47.6 %), elevated CRP (65.9 %) and elevated cardiac enzymes (49.4 %) amongst others [136]. A systematic review on 212 published studies from 11 countries/regions reported that COVID-19 was associated with severe disease in 23 % of infected individuals, and a 6 % mortality rate [137].

Certain interventions to reduce SARS-CoV-2 transmission were effective, but this often varied based on adherence by the public, an individual’s behavioural practices as well as the

emergence of new variants amongst others. The introduction of vaccination remains the most powerful tool in terms of disease severity through reducing severe illness; however, it does not prevent infection.

In middle and low-income regions, vaccination rates are usually low and most of an individual's immunity arises from natural infections. In the context of the COVID-19 pandemic, a high rate of natural infections was observed in densely populated areas where social distancing was limited [138] which explains why these communities were relatively protected from SARS-CoV-2 infection and COVID-19 severity in the second wave compared to the first wave [112].

1.6.3 SARS-CoV-2 lifecycle

The SARS-CoV-2 life cycle includes virus entry, translation of viral replication machinery, replication, translation of viral structure proteins, virion assembly and finally the release of the virus. The S protein of SARS-CoV-2 facilities both binding and entry of the virus into the host epithelial cells in the respiratory tract. As mentioned previously (section 1.6.1), the S protein includes two subunits, S1 and S2. S1 encompasses the RBD which interacts with the host cell receptor, ACE2. S2 undergoes conformational changes resulting in fusion of virions and cell membranes [139, 140].

Initial replication is thought to occur in the upper respiratory tract, while later replication occurs in the lower respiratory tract [120]. After successful binding of the virus to the host, fusion and endocytosis, the viral RNA is released into the cytoplasm of the host. Here, the viral RNA is recognised by the host translation machinery as mRNA and is translated into viral proteins which include S, E, M, N and other accessory proteins. In addition, RNA-dependent RNA polymerase replicates viral genomic RNA. The creation of new viral genomes as well as mRNA for the production of viral proteins is required for viral assembly to follow [120, 141].

The nucleocapsids are assembled in the cytoplasm, which is then followed by budding into the lumen of the endoplasmic reticulum to the Golgi apparatus and then to the cell surface. Exocytosis of new virions then follows. The virus then spreads to other regions (cells and organs) that are uninfected [120].

1.6.4 The immune response to SARS-CoV-2 infection

The immune response triggered by SARS-CoV-2 consist of two-phases that include a viral response and an inflammatory phase [140, 142]. The first phase is an incubation phase which is based on defence and protection linked to the innate immune response, as well as non-severe stages where the adaptive immune response will be active. During this first phase, the immune system will try to expel the unwanted virus and prevent the progression to severe disease. The second phase is driven by inflammation, here the virus will spread and infect tissues thereby impairing the immune response [120].

An effective innate immune response results in the induction of a specific adaptive immune response which involves T- and B-cells, linking cell-mediated and humoral responses. During the innate immune response, SARS-CoV-2 is recognised by antigen presenting cells (macrophages, dendritic cells) which, after phagocytosis, present viral peptides to CD4+ T cells. In the host, viral peptides are introduced to CD8+ cytotoxic T cells which divide and result in the lysis of infected cells [120]. The humoral response to SARS-CoV-2 infections involves the production of immunoglobulin (Ig) G, IgM and IgA antibodies [118]. The production of these neutralising antibodies limits infection at a later stage and also prevents re-infection to some extent [120].

B-cells elicit an early response against the SARS-CoV-2 N protein at the onset of SARS-CoV-2 infection; after the appearance of initial symptoms, antibodies against the S protein (the most immunogenic of the SARS-CoV-2 proteins), more specifically the RBD portion of the S protein can be detected [118, 143-145]. The presence of specific IgG, IgM and IgA antibodies usually appear in individuals almost concurrently between 14-23 days post symptom onset [146]. IgM and IgA antibody levels are short lived whereas IgG antibody levels are persistent and can be detected for a longer period of time (Figure 1.6) [118, 147]. The virus-specific antibody levels, namely IgG, IgM and IgA are of importance in predicting population immunity against disease. In addition, it is also important to determine cross-reactivity with other coronaviruses [148].

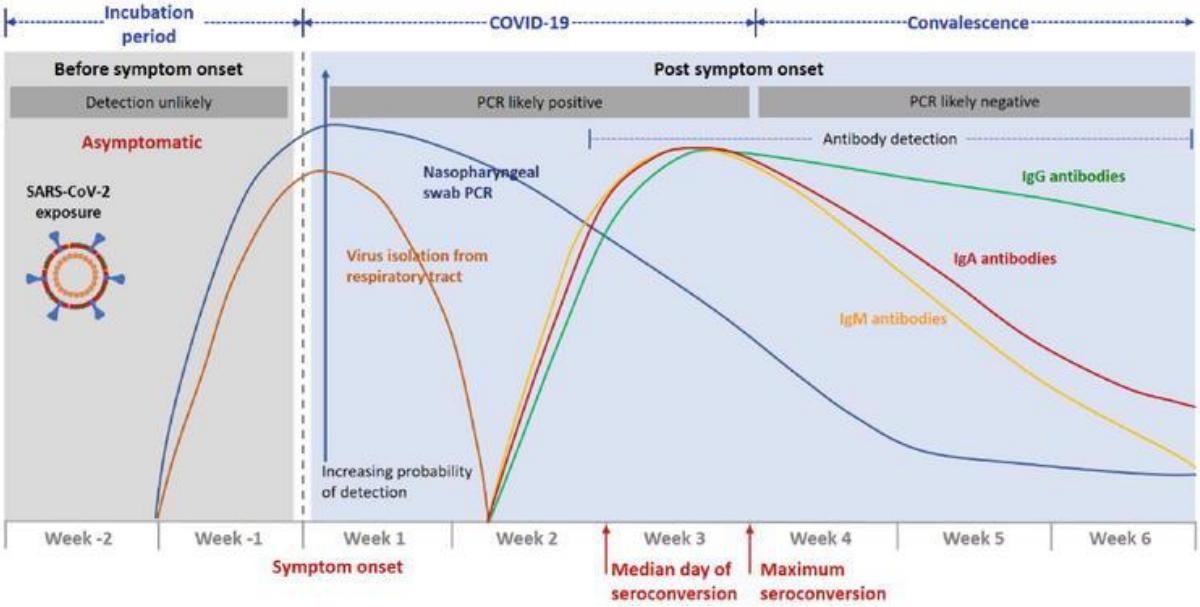

Figure 1.6: The host immune response to SARS-CoV-2 infection. A depiction of the time course of SARS-CoV-2 infection and the appearance of virus specific IgG, IgM and IgA antibodies. Adapted from Vashist *et al*. [146].

1.6.5 The impact of SARS-CoV-2 on HIV infection

The interplay between HIV and SARS-CoV-2 is complex, and a complete understanding of the immune response to SARS-CoV-2 infection in HIV-infected individuals remains elusive. It was initially assumed that unfavourable outcomes would occur in individuals with HIV compared to the overall population [149]. A number of studies have examined susceptibility to SARS-CoV-2 infections and/or progression into COVID-19 disease in PLWH to determine whether PLWH are at a higher risk than non-HIV infected cohorts; however, the results of these studies were conflicting possibly due to the variability of cohorts that were investigated [150]. Mondi *et al*. [149] did not observe an elevated susceptibility to SARS-CoV-2 infection or a more severe progression of COVID-19 in PLWH compared to the general population. It was also observed that patients with more severe immunosuppression experienced a milder disease, suggesting that the impaired immune reactivity of advanced HIV infection contributed to avoiding immune-pathogenetic processes [149]. Additionally, no increased risk of developing severe COVID-19 in HIV-positive compared to HIV-negative individuals has been reported which could possibly be due to the use of ARTs [151]. Moreover, HIV-positive individuals on ART had a lower risk of COVID-19-related hospitalisation compared to those not on ART [151]. Overall, several studies showed that the risk of SARS-CoV-2 infection was found to be comparable between groups that were HIV-negative versus HIV-positive [149-151].

1.7 SARS-CoV-2 infection and the potential to reactivate KSHV

As mentioned in section 1.3, various stimuli have the potential to trigger the transition from latency to lytic reactivation of KSHV. An increasing amount of evidence points to the interplay of the aforementioned pathogens with KSHV; additionally, SARS-CoV-2 may impact on herpesvirus reactivation, particularly in individuals with severe COVID-19 disease [28, 152-155]. Most clinical studies have focussed on herpesviruses other than KSHV [25]. For example, a retrospective study on 100 immunocompromised patients reported reactivation of HSV (12 %), EBV (58 %) and HCMV (19 %) 10 days post-SARS-CoV-2 infection [152]. A clinical investigation of 67 COVID-19 patients from Wuhan (China) found a correlation between EBV reactivation and infection with SARS-CoV-2; here, 55.2 % of patients with a history of infection with EBV tested positive for the EBV viral capsid antigen (IgM) which is an indicator for EBV reactivation [154]. In addition, *in vitro* data reported on SARS-CoV-2 proteins causing KSHV lytic reactivation [26]. In a recent case report, a female patient with a history of KS without the presence of active skin lesions showed a recurrence of KS after being hospitalised for COVID-19. The authors speculated that SARS-CoV-2 caused hyperinflammation contributing to reactivation of KSHV and eventually progression to KS [24, 156]. Additionally, case reports of HIV-patients with a low CD4 count demonstrated a potential interplay between KSHV reactivation and severe COVID-19 [157, 158]. However, the exact mechanism of how SARS-CoV-2 may trigger herpesvirus reactivation is far from understood. Dysregulation of the immune response during SARS-CoV-2 could also influence reactivation, by disabling the host's type 1 interferon response by autoantibodies resulting in decreased control of latent pathogens [70, 159]. There is a growing body of literature linking the persistence/reactivation of latent viruses to post-acute sequalae of SARS-CoV-2 infection. It has been reported that antigens of SARS-CoV-2 may persist in certain organs; the precise mechanism by which this occurs or how it is linked to immune responses still remains unclear [70]. Additionally, the "cytokine storm" may also play a role in the reactivation of KSHV, especially in critically ill COVID-19 patients. One or more of these cytokines, which include IL-6, could result in reactivation [24].

During the first COVID-19 wave in SA, our laboratory conducted an observational study at the Groote Schuur Hospital, Cape Town, between June and August 2020, suggesting that SARS-CoV-2 infection and KSHV co-infection could interact to influence disease severity and outcome [28]. A total of 104 hospitalised COVID-19 cases were included with 29.8 % HIV positive and 41.1 % KSHV seropositive, 30 patients (28.8 % of the cohort) died. In COVID-19 patients, detectable KSHV VL was associated with death after adjusting for potentially confounding factors such as age, sex and HIV status. In addition, data indicated that KSHV

VL may be related to COVID-19 disease severity in HIV-negative individuals (in addition to death) [28].

These findings indicate an interplay between KSHV and SARS-CoV-2 and a potential impact on patient outcomes, especially in critically ill hospitalised patients. However, the interaction between these viruses is complex and warrants further investigation to fully understand the interactions. It is still unclear whether KSHV infection contributes to COVID-19 outcome or whether KSHV reactivation occurs because of SARS-CoV-2 infection and/or its associated "cytokine storm". Moreover, it is of importance that reactivation of KSHV in the post-pandemic era is better understood, especially in populations like SA where the prevalence of KSHV and other infectious diseases is high. It is also important to distinguish between individuals with mild and severe COVID-19 and individuals that are asymptomatic.

In addition to the direct impact of SARS-CoV-2 and/or the COVID-19-related "cytokine storm" for KSHV reactivation, the COVID-19 pandemic might also have indirect implications. As reported by Chen *et al*. [26], anti-COVID-19 drugs were capable of inducing lytic KSHV reactivation through intracellular signalling pathway manipulation. These data suggests that individuals with KSHV, especially those in endemic areas who are exposed to SARS-CoV-2 or are undergoing treatment for COVID-19 may have an increased risk of developing virus associated cancers, even after complete recovery from SARS-CoV-2 infection [26].

1.8 Concluding remarks

With the high KSHV seroprevalence in SSA, particularly in HIV-infected populations [44], it is important to assess the impact of SARS-CoV-2 infection on KSHV reactivation in individuals living in areas where exposure to SARS-CoV-2 infection is very high. As mentioned previously, symptoms of lytic KSHV infection are non-specific and can easily be misdiagnosed with TB and/or other prevalent infections causing inflammatory symptoms. In addition, the occurrence of COVID-19 might have a significant impact on KSHV and/or associated diseases especially in the long-term and this should be investigated further. This knowledge would allow for earlier diagnosis as well as the development of intervention strategies which should be implemented in vulnerable populations to prevent and treat KSHV-associated diseases in the post-pandemic era.

1.9 Rationale and Aims

While previous literature has focused on co-infections in hospitalised COVID-19 patients, it is currently unknown what the impact of exposure to SARS-CoV-2 is on HIV/KSHV co-infected non-hospitalised individuals. We therefore conducted an observational study at the Gugulethu Community Health Centre Antiretroviral clinic (Desmond Tutu HIV Centre, University of Cape Town (UCT)), situated in the Western Province of SA, which provides HIV health care service to communities in the region. The cohort in this study thus represent patients from a low-income area who were most likely unprotected and largely exposed to SARS-CoV-2 from early on in the pandemic because of limited implementation of social distancing measures and non-pharmaceutical interventions.

In this study, we assessed the risk of KSHV reactivation in the context of SARS-CoV-2 exposure on a clinical level. We hypothesised that HIV/KSHV co-infection with SARS-CoV-2 may increase KSHV reactivation in non-hospitalised HIV-infected patients with high exposure to SARS-CoV-2. We addressed this through the following aims:

1. To assess the impact of SARS-CoV-2 infection on KSHV reactivation in non-hospitalised HIV-infected patients (n = 407) enrolled at the Gugulethu Community Health Centre Antiretroviral clinic (Desmond Tutu HIV Centre, UCT) outside Cape Town, SA, using a cross-sectional study design.
2. To determine the effect of SARS-CoV-2 on KSHV VL and KSHV immune responses by following up on KSHV-seropositive patients with or without previous SARS-CoV-2 infection every 6 months up to one year.
3. To report on the case of a selected patient of interest with unusually high KSHV viremia, who was monitored every 6 months for a 2-year period.

2. Materials and Methods

2.1 Ethics approval and funding

Ethics approval for this study was obtained from the Human Research Ethics Committee (HREC), Health Sciences Faculty, UCT, for the enrolment of patients from the Gugulethu Community Health Centre Antiretroviral clinic (Desmond Tutu HIV Centre, UCT) and the collection of blood samples from the respective recruited patients (HREC134/2020). All participants recruited to the study provided written informed consent. This study was conducted according to the declaration of Helsinki and conformed to South African Good Clinical Practice guidelines.

The research was funded by the EDCTP2 programme (Training and Mobility Action TMA2018SF-2446).

2.2 Patient recruitment

2.2.1 Study cohort and study design

A cohort of 407 non-hospitalised adult (>18 years old) HIV-infected patients presenting for routine HIV treatment at the Gugulethu Community Health Centre Antiretroviral clinic (Desmond Tutu HIV Centre, UCT), SA, were enrolled into this study from October 2020 to April 2023. The start of the patient recruitment process coincided with the decline of SARS-CoV-2 infections from the first COVID-19 wave in SA and before COVID-19 vaccinations were available.

Both male and female patients were selected if their latest CD4 count was <350 cells/µl, according to the most recently updated clinical files.

Patients with a positive KSHV serology status as determined by enzyme-linked immunosorbent assay (ELISA, see section 2.6) were invited for a 6- and 12- month follow-up (FU). One particular patient, recorded as GUG116, was invited for further FU after 18 and 24 months.

2.2.2 Clinical and demographic data

At the time of enrolment clinical and demographic details were collected, including any self-reported symptoms and COVID-19 vaccination status at presentation. Peripheral blood was analysed by the National Health Laboratory Services (NHLS) on the day of enrolment for absolute CD4 count using the Aquios PLG panel (CD45-FITC/CD4 PE monoclonal antibodies) together with an Aquios CL Flow cytometer (Beckman Coulter) as well as HIV VL using the ALINITY mHIV-1 ASSAY (Abbott Molecular Inc.), following standard operating procedures. Additional tests to determine the concentrations of sodium, creatinine, albumin, alanine transaminase (ALT), CRP and haemoglobin, as well as full blood count and differential cell count were also performed by the NHLS. Normal thresholds and/or ranges for laboratory results are defined by the NHLS. Information on ART was obtained from pharmacy records.

In-house tests for KSHV and EBV VL and serology, SARS-CoV-2 serology as well as IL-6 levels were conducted as described in sections 2.5, 2.6, 2.7 and 2.8, respectively.

All clinical, demographic and experimental data collected for this study were recorded and stored on an electronic REDCap database [160], hosted by the University of Cape Town. The demographic and clinical characteristics of the patients are presented in Table 3.1 (chapter 3).

2.3 Sample collection, processing, and storage

A trained nurse at the Gugulethu Community Health Centre Antiretroviral clinic (Desmond Tutu HIV Centre, UCT) drew 5x 5 ml tubes of peripheral blood from the arm of each recruited patient during their respective visits to the clinic. Four of the tubes drawn were sent to the NHLS to test for HIV VL (1x Plasma preparation tube (PPT)), CD4 count (1x Ethylenediaminetetraacetic acid (EDTA) tube), full blood and differential count (1x EDTA tube) and sodium, creatinine, albumin, ALT and CRP (1x clotted tube). Samples for in-house analyses were collected in EDTA tubes. All samples were transported at room temperature (RT) to the laboratory and NHLS for processing (see section 2.3.2) on the same day.

Blood samples received for in-house tests were processed in a Biosafety level 2 safety cabinet as soon as possible through centrifugation at 2000x g for 10 minutes (min) at RT (Eppendorf 5810R) to separate the plasma from the blood cells. The plasma was transferred into a new tube. Both the plasma samples and the remaining blood cells in EDTA tubes were stored at -20^{o}C until further analysis.

2.4 Blood DNA extraction

After sample processing, the leukocyte enriched buffy coat and erythrocyte fraction of the patient samples were used for genomic DNA extraction. This process was conducted using the QIAamp DNA Blood Midi kit (Qiagen) according to the manufacturer's instructions. Samples were eluted in buffer AE as supplied in the kit.

Upon completion of the isolation of DNA, samples were quality- and quantity-assessed using a nanodrop (Thermo Fisher Scientific). Thereafter, the processed samples were stored at -20^{o}C until further processing.

2.5 KSHV, EBV and ERV-3 VL assays

To perform VL assays, DNA extracted from whole blood (see section 2.4) was used. KSHV DNA was quantified in all patient samples by TaqMan polymerase chain reaction (PCR) that targeted the KSHV K6 gene [161], while EBV VL was only determined in selected samples as indicated by targeting the EBV polymerase gene [162]. All results were normalised by the expression of human endogenous retrovirus 3 (ERV-3) gene [163]. The samples' DNA concentration was adjusted to 25 ng/µl, with 10 µl of DNA at this concentration used in a PCR reaction of a 50 µl total volume. KSHV, EBV and ERV-3 DNA were detected using 100 pmole of the forward and reverse primers and 50 pmole FAM/TAMRA labelled probe (Inqaba Biosystems, Table 2.1), together with the 2x TaqMan Universal Master Mix (Thermo Fisher Scientific) and run on a Roche 480II LightCycler. KSHV, EBV and ERV-3 DNA was quantified against standard curves that were produced using a K6 plasmid [164], EBV plasmid [165] and an ERV-3 plasmid [166], respectively, provided by Dr Denise Whitby, (National Institute of Health (NIH)). The cycling conditions for all genes were as follows: 50^{o}C for 2 min, 95^{o}C for 8 min, followed by 45 cycles 95^{o}C for 15 sec and 60^{o}C for 1 min.

KSHV and EBV DNA was normalised to the number of cellular equivalents in the given patient samples, using ERV-3 which occurs at two copies per human cell. A given assay was determined to be positive by following a guide of internal/quality controls as seen below in Table 2.2. All samples for this assay were conducted in triplicate, averaged, and then reported as viral DNA copies per million cells. In samples where only one or two technical repeats were detected, or where detectable viral DNA was below the limit of detection (3 copies / reaction), samples were classified as qualitatively positive and assigned the value of 1 copy/10^6 cells [166].

Table 2.1: Primers and probes used in quantitative Taqman PCR to determine KSHV, ERV-3 and EBV VL.

Primer or Probe	Sequence 5'–3'	Fragment length
K6 forward primer	CGCCTAATAGCTGCTGCTACGG	**176bp**
K6 reverse primer	TGCATCAGCTGCCTAACCCAG	
K6 probe	FAM-CACCCACCGCCCGTCCAAATTC-TAMRA	
ERV-3 forward primer	CATGGGAAGCAAGGGAACTAATG	**135bp**
ERV-3 reverse primer	CCCAGCGAGCAATACAGAATTT	
ERV-3 probe	FAM-TCTTCCCTCGAACCTGCACCATCAAGTCA-TAMRA	
EBV forward primer	AGTCCTTCTTGGCTAGTCTGTTGAC	**91bp**
EBV reverse primer	CTTTGGCGCGGATCCTC	
EBV probe	FAM-CATCAAGAAGCTGCTGGCGGCC	

Table 2.2: Quality control for the VL assays.

Control	ERV-1	K6	EBV
No template Control	No amplification	No amplification	No amplification
Negative Control	No amplification	No amplification	No amplification
Positive Control (range- mean copy number)	10-50000	10-100000	10-100000
Standard curve slope (range)	-3.2 – -3.7	-3.2 – -3.7	-3.2 – -3.7
Standard curve R^2 value	>0.97	>0.97	>0.97
Standard curve Y-intercept (range)	37-43	37-43	37-43
Standard curve Ct values (range)	**10^6:** 19.3 – 20.5	**10^6:** 18.3 – 19.5	**10^6:** 19.2 – 20.3
	10^5: 22.5 – 24.0	**10^5:** 21.5 – 23.3	**10^5:** 22.6 – 23.9
	10^4: 26.3 – 27.6	**10^4:** 25.4 – 27.0	**10^4:** 26.2 – 27.4
	10^3: 29.4 – 31.0	**10^3:** 28.6 – 30.4	**10^3:** 29.9 – 30.6
	10^2: 33.3 – 36.0	**10^2:** 32.0 – 34.2	**10^2:** 33.5 – 34.2
	10^1: 37.0 – 39.7	**10^1:** 37.0 – 39.0	**10^1:** 38.1 – 38.8
	10^0: 38.0 – 45.0	**10^0:** 39.1 – 45.0	**10^0:** 42.6 – 43.0

2.6 KSHV and EBV serology

KSHV serology assays were performed for all patients by ELISA for antibodies against the lytic structural glycoprotein K8.1 and the key latency protein LANA, following an established protocol [167]. Plasma samples stored at -20°C were tested using ELISA plates provided by Dr Denise Whitby (NIH), coated with K8.1 or LANA recombinant peptide, respectively. In brief, coated plates (K8.1 and LANA) stored at -80°C were thawed at 37°C. The thawed plates were washed 3x with wash buffer (see Appendix), plates were then inverted and patted dry on paper towel. Plasma samples, previously prepared, were thawed, mixed, and diluted 1:10 with assay buffer (see Appendix). The prepared samples were added to the K8.1 and LANA plates at a final dilution of 1:20 and 1:100, respectively. Assay buffer served as a background control while pooled KS samples from a previous study in our laboratory were used as positive control [168], and fetal bovine serum was used as negative control.

After the addition of all samples and respective controls, plates were sealed and incubated at 37°C for 90 min, thereafter washed 3x with wash buffer to remove any unbound plasma. Plates were inverted and patted dry on paper towel. The phosphatase labelled antibody (ReserveAP Goat anti-Human IgG (H+L)) was diluted (1:5000) in assay buffer and 100 µl was added to each well. The plates were sealed and incubated at 37°C for 30 min and then washed 3x with wash buffer, inverted and patted dry on paper towel. Thereafter, 100 µl 1-step p-nitrophenyl phosphate (PNPP) substrate (Thermo Fisher Scientific) was added to the wells of each plate which were then incubated at RT in the dark for 20 min (K8.1) or 30 min (LANA), respectively. The reaction was stopped with 50 µl of stop solution (see Appendix). The plates were read on a GloMax plate reader (Promega) at a wavelength of 405 nm. To interpret the results of the assay, raw values were adjusted according to the average optical density (OD) of the background controls. Cut-off OD values were calculated using an equation determined by [167] as follows:

For K8.1, cut off values were calculated as –

$$\text{OD of K8.1} = \text{mean of negative controls} + 0{,}95$$

For LANA, cut off values were calculated as –

$$\text{OD of LANA} = \text{mean of negative controls} + 0{,}35$$

Patient samples were considered KSHV seropositive if antibodies to either antigen were detected. Quality control specifications for the blank/background, negative and positive controls can be seen in the Table 2.3 below. Results were only used if the plate passed these specifications.

Table 2.3: Quality control for K8.1/LANA ELISAs.

Control	K8.1 ELISA	LANA ELISA
Blank (raw value)	<0.20	<0.10
Negative control (blank adjusted)	0 – 0.30 Mean x 0.5 - mean x 1.5	0 – 0.20 Mean x 0.5 - mean x 1.5
Positive control (blank adjusted)	>0.30	>1.0

While KSHV serology was measured in the whole cohort, EBV serology was only determined in selected samples as indicated. EBV serology assays were performed by ELISA for antibodies against the EBV nuclear antigen (EBNA) using the Human Anti-Epstein Barr virus IgG ELISA kit (Abcam-ab108731) as per the manufacturer's instructions.

2.7 SARS-CoV-2 serology

The ELISA protocol used to determine SARS-CoV-2 serology was adapted from Makatsa *et al*. [169]. To coat 96-well plates (Nunc, Thermo Fisher Scientific), the SARS-CoV-2 RBD and S protein (Cape Bio Pharms) were diluted in 1x phosphate buffered saline (PBS). Plates were coated by adding 50 µl of diluted antigen to each well at a concentration of 2 µg/ml. Plates were then incubated at 4°C overnight. The next day, plates were washed 5x with 1 % PBS-Tween20 (PBS-T). Thereafter, 200 µl of blocking solution (1 % casein (Sigma) in PBS-T) were added to each plate and incubated for 1 h at RT. Plasma samples and pre-pandemic controls [169] were diluted 1:50 in 0.5 % casein in PBS-T. After plates were blocked, blocking buffer was removed and 100 µl of samples, controls and blanks were added to the respective plates and then incubated for 2 h at RT. Thereafter, the plates were manually washed 5x with PBS-T. The diluted secondary antibody (1:5000 anti-human IgG HRP-labeled in PBS-T containing 0.5 % casein) was added to the wells at a volume of 100 µl and incubated for 1 h at RT. The plates were then washed 5x with PBS-T as previously mentioned.

For development of the plates, 100 µl O-phenylenediamine dihydrochloride (Sigma), was added for 12 min at RT, and the reaction was thereafter stopped with 50 µl 3 M hydrochloric acid (HCl, Sigma). The plates were read immediately at 490 nm using a GloMax plate reader. To interpret results, a cut-off for positivity was set at 2 standard deviations (SD) above the mean OD of the 30 pre-pandemic samples for each plate. The adjusted OD values were then normalised to the cut-off which was set as 1.

2.8 IL-6 ELISA

To measure the amount of IL-6 present in plasma samples, the Human IL-6 ELISA kit (Abcam-ab48478) was used as per the manufacturer's instructions. Samples were quantified against a standard curve of IL-6 recombinant protein which was reconstituted with standard dilution buffer (see Appendix). The dynamic range of the assay was between 6.25 – 200 pg/ml IL-6. Briefly, high-binding 96-well plates (Nunc, Thermo Fisher Scientific) were coated in 100 µl IL-6 Capture Antibody, covered with a sealant and incubated overnight at 4°C. The next day, plates were washed 2x with wash buffer (see Appendix) and blotted against paper towel to remove any excess liquid, thereafter 250 µl of blocking buffer (see Appendix) was added to the plates which were incubated at RT for 2 h. After blocking, the plates were washed 3x with wash buffer and blotted against paper towel. Standards and undiluted plasma were added to the appropriate wells at a volume of 100 µl and 50 µl, respectively, with the blank standard only containing sample dilution buffer.

Detection antibody (biotinylated anti-IL-6) at a volume of 50 µl was added into each well and incubated at RT for 1 h. After incubation, the plates were washed 2x with wash buffer, after which 100 µl of Streptavidin-HRP was added to each well and incubated at RT for 30 min. The plates were washed 2x and blotted against paper towel, thereafter 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate was added to each well and incubated in the dark at RT for 10 min. Next, 100 µl of stop reagent (1 M sulfuric acid) was added to each well and the absorbance was read immediately on a GloMax plate reader at a wavelength of 450 nm. The average for the absorbance of the blank controls were subtracted from all absorbance values. A standard curve was constructed with these calculated values against known concentrations. Sample concentrations were thus calculated by interpolating the absorbance values onto the plotted standard curve. The reference value for elevated IL-6 was defined as 1.80 pg/ml (IQR 1.20 – 2.89) as determined by Borges *et al* [170] .

2.9 Statistical analysis of study data

Statistical analyses were performed using SPSS version 28 (IBM Corp, New York, NY, USA). The analyses on the results shown in sections 3.1 and 3.2 were performed using two different approaches which will be explained below.

In section 3.1, for univariate analyses involving categorical variables, Chi square tests were used for cell counts that were >5, while Fisher's exact test for small contingency tables (2x2) or Fisher-Freeman-Halton tests for larger contingency tables were used. For univariate

analyses involving quantitative variables, Shapiro-Wilk tests were used to assess normality. Levene's test was used to assess homogeneity of variance for normally distributed variables before independent T-tests were used, while Mann-Whitney U test was used for non-parametric variables. Trendlines were fitted as straight lines by linear regression and compared by F test.

Binomial logistic regression was used to assess independent associations of SARS-CoV-2 seropositivity on detection of KSHV VL (categorical variable) while controlling for the relevant variables (demographic and clinical). The study cohort was divided according to COVID-19 vaccination status, this was to account for collinearity of vaccination with OD values for RBD utilising an independent T-test. Participants with missing information were excluded listwise, *p*-values were two-tailed and considered significant if <0.05.

In section 3.2, for univariate analyses involving categorical variables, Chi square tests were used and for analyses involving continuous variables, Kruskal-Wallis tests (>2 comparisons) were used for non-parametric variables. Dunn's test adjusted with Bonferroni correction for multiple comparison was used for any further analysis (post-hoc). Participants with missing information were excluded listwise, *p*-values were two-tailed and considered significant if <0.05.

Over the FU period, KSHV detection was assessed using cox regression. Cox regression was used to plot "times to significant events" and hazard ratios were used to test for significant differences in distributions between vaccinated and unvaccinated groups.

All graphical representations were performed using GraphPad Prism (version 10; GraphPad Software Inc, San Diego, CA, USA) and SPSS version 28 (IBM Corp, New York, NY, USA) where required.

3. Results

During the initial stages of the COVID-19 pandemic, SARS-CoV-2 infection and inflammation caused significant morbidity and mortality; however, the consequences of the long-term effects of exposure to SARS-CoV-2, especially on the reactivation of latent oncogenic herpesviruses like KSHV, is currently unknown. The patient cohort reported on in this thesis represents a unique set of non-hospitalised HIV-infected patients that presented at the Gugulethu Community Health Centre Antiretroviral clinic (Desmond Tutu HIV Centre, UCT) for their routine HIV treatment between October 2020 and April 2023. This cohort therefore includes individuals from a low-income community, largely unprotected by vaccination and highly exposed to SARS-CoV-2 from early on and throughout the pandemic. Indeed, the recruitment process coincided with the decline of SARS-CoV-2 infections from the first COVID-19 wave in SA and continued into subsequent waves as well as the national roll-out of vaccinations (Figure 3.1.1). The recruited individuals from these poorer communities were faced with dense living conditions with limited to no social distancing and non-pharmaceutical interventions. Additionally, the lack of knowledge and awareness around SARS-CoV-2 as well as other diseases have further burdened these communities.

Given the high seroprevalence of KSHV in SSA, particularly in populations infected with HIV, we assessed the potential impact of high exposure to SARS-CoV-2 infection on reactivation of KSHV, in both a cross-sectional study design (section 3.1) and a FU study design (section 3.2). The results presented in section 3.1 have recently been published [92]. Thirdly, one patient of interest, recorded as GUG116 in our REDCap database [160], was monitored over a 2-year period, presenting for FU visits every 6 months, and will be reported as a case study (section 3.3).

3.1. A cross-sectional analysis of the contribution of SARS-CoV-2 to the reactivation of KSHV in non-hospitalised HIV-infected patients

3.1.1 Patient demographic and clinical characteristics

In order to assess whether high and potentially repeated SARS-CoV-2 infection/exposure had any impact on underlying KSHV infection in a cohort of non-hospitalised adult HIV-infected individuals with CD4 counts of <350 cells/µl, we enrolled eligible patients presenting at the Gugulethu Community Health Centre Antiretroviral clinic (Desmond Tutu HIV Centre, UCT) for ART services. The recruitment process was initiated after the decline in the first COVID-19 wave in October 2020 and continued throughout subsequent waves of the pandemic in SA until April 2023 (Figure 3.1.1).

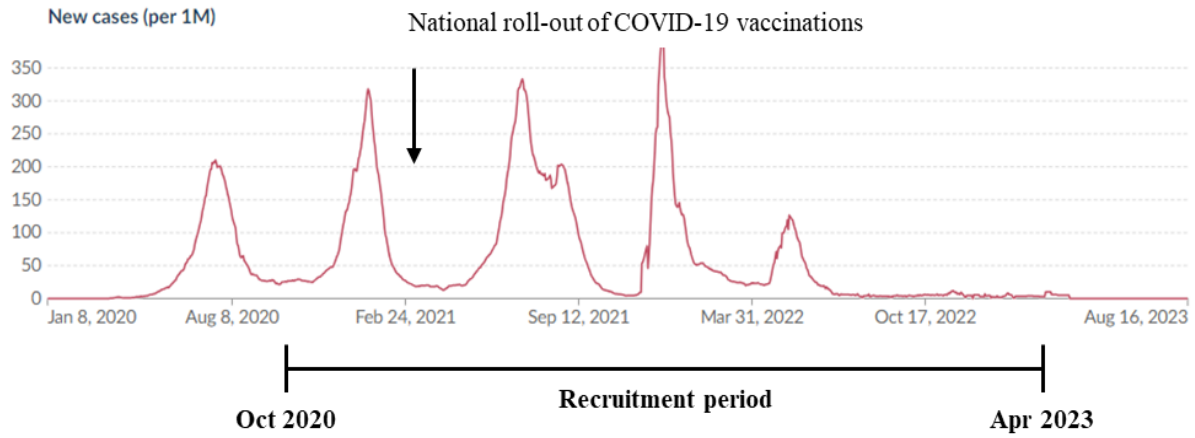

Figure 3.1.1: Outline of SARS-CoV-2 infection patterns in SA in relation to participant enrolment for this study. The 7-day rolling average of daily new confirmed COVID-19 cases per million people in SA is shown as reported on OurWorldInData.org [115]. The timeline of patient recruitment starting in October 2020 at the end of the first wave is shown as well as the start of vaccinations in SA (indicated by the arrow).

At the time of presentation to the clinic, all patients had a confirmed HIV-diagnosis, had a CD4 count of <350 cells/µl, were over the age of 18, and had a negative diagnosis for TB. Symptoms of COVID-19 and vaccination status were self-reported.

Demographic and clinical characteristics obtained at recruitment are summarised in Table 3.1.1. The recruited cohort consisted of 407 individuals of which 274 (67.5 %) were female and 132 (32.5 %) were male with a median age of 39 and a median weight of 70 kilograms (kg). Data collated at recruitment shows that out of the 407, 215 patients (52.8 %) had a suppressed HIV VL (<50 copies/ml), 75 (18.4 %) had low-level viremia (50 – 999 copies/ml) and 107 (26.3 %) had unsuppressed viremia (>1000 copies/ml) with a median HIV VL of 49 copies/ml. Absolute CD4 count was 221 cells/µl. According to pharmacy records, most of the

individuals were receiving ART at the time of recruitment (85.0 %), with 10.8 % having defaulted and 4.2 % of the recruited patients having never been on ARTs.

The World Health Organization (WHO) clinical stage of HIV disease is a criterion which defines HIV disease into clinically relevant stages. These stages guide medical personnel in decision making on treatment for individuals with HIV/AIDS, with stage 1 indicating asymptomatic HIV disease to stage 4 indicating advanced disease which includes severe immune suppression [171]. Most individuals in our cohort had either asymptomatic or minor manifestations of HIV disease. The majority of the cohort was assigned as stage 1 (59.5 %), followed by stage 2 (17.9 %), stage 3 (21.1 %) and stage 4 (1.6 %).

Table 3.1.1: Baseline demographic and clinical characteristics of all study participants (n = 407). Data presented as number and percentage of total (%) or median and interquartile range (IQR) as appropriate.

Characteristic		**N (%) or Median (IQR)**
Sex	Female	274 (67.5 %)
	Male	132 (32.5 %)
Age, years		39 (32 – 45)
Weight, kg		70 (60 – 87)
HIV VL, copies/ml		49 (1 – 1426)
HIV VL	Suppressed (<50 copies/ml)	215 (52.8 %)
	Low-level viremia (50 – 999 copies/ml)	75 (18.4 %)
	Viremia (≥1000 copies/ml)	107 (26.3 %)
Absolute CD4, cells/µl		221 (135 – 332)
Receiving ART	Defaulted	44 (10.8 %)
	Yes	345 (85.0 %)
	No	17 (4.2 %)
Time since ART start, years		0.86 (0.31 – 3.57)
WHO clinical stage of HIV disease	1	226 (59.5 %)
	2	68 (17.9 %)
	3	80 (21.1 %)
	4	6 (1.6 %)
KSHV VL	Not detectable	279 (79.0 %)
	Detectable	74 (21.0 %)
KSHV serology	Negative	187 (46.5 %)
	Positive	215 (53.5 %)
SARS-CoV-2 serology	Negative	71 (17.4 %)
	Positive	336 (82.6 %)
SARS-CoV-2 vaccinated (self-reported)[1]	No	276 (67.8 %)
	Yes	131 (32.2 %)

[1]Data on type of vaccine administered unknown due to self-reported disclosure of vaccination status.

These stages, however, influence when ART is initiated and often cause delay in treatment. SA's ART guidelines have therefore shifted over time to the Universal Test and Treat strategy recommended by the WHO. These guidelines have resulted in ART initiation of all HIV-positive individuals immediately upon diagnosis regardless of clinical indicators (CD4 counts etc.) and/or stages [172, 173].

3.1.2 SARS-CoV-2 prevalence in the recruited cohort

The seroprevalence of SARS-CoV-2 was determined by antibody response to either of the two SARS-CoV-2 S protein fragments, namely RBD and S1. In total, 82.6 % of the recruited cohort were found to be SARS-CoV-2 seropositive (RBD and/or S1) (Table 3.1.1). When the study period was divided quarterly, SARS-CoV-2 seropositivity was 76.2 % at the start of the recruitment period (before the national roll-out of vaccination in SA). SARS-CoV-2 seropositivity increased to 94.9 % at the end of the recruitment period which coincided with the later waves of COVID-19 and an increase in vaccine administration (Figure 3.1.2). These data suggest a very high exposure to SARS-CoV-2 during the first COVID-19 wave, and in the absence of vaccinations. SARS-CoV-2 vaccination was self-reported by patients at the time of recruitment, with an overall 32.2 % of individuals that had received the vaccine by the end of the recruitment process. Vaccine administration in SA began with the Johnson & Johnson vaccine in February 2021, followed by the Pfizer vaccine. However, patients, who self-reported their COVID-19 vaccination status were generally unaware of which vaccine they had received.

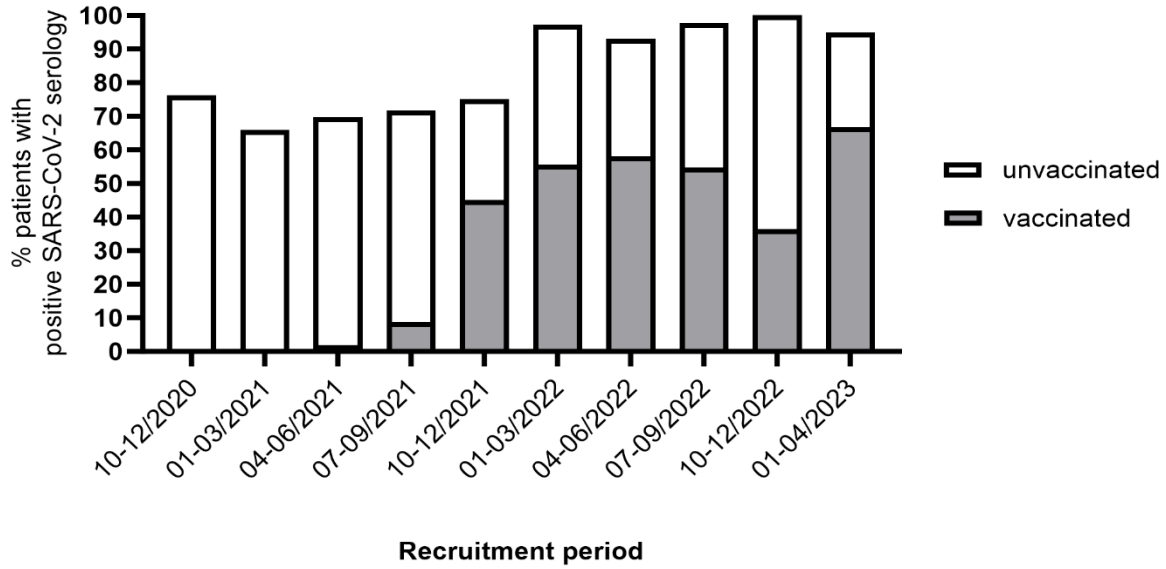

Figure 3.1.2: SARS-CoV-2 seroprevalence over the course of patient recruitment. Quarterly percentage of SARS-CoV-2 seropositive patients over the course of the study period with self-reported vaccination status indicated in white (unvaccinated) and grey (vaccinated).

The data shown here indicate that the steady increase in SARS-CoV-2 seropositivity over the recruitment period was most likely due to both natural infection(s) and the increase in the number of individuals being vaccinated (Figure 3.1.2).

Our study design did not inform on previous SARS-CoV-2 infections, neither with regards to the date of acute SARS-CoV-2 infection nor COVID-19 severity. However, given the high percentage of individuals with positive SARS-CoV-2 serology which steadily increased throughout the recruitment process (even before the roll-out of vaccinations in SA) we can assume that the patients in this cohort were likely exposed to more than one SARS-CoV-2 infection throughout the study.

3.1.3 KSHV prevalence during the recruitment period

KSHV serology was determined by ELISA (see section 2.6), assessing the response of the patient samples to either K8.1, a lytic structural glycoprotein, or LANA, a nuclear antigen associated with latency. Overall, 53.5 % of patients showed a positive response to either K8.1 or LANA. These results were comparable to pre-pandemic HIV-positive samples with an overall seroprevalence (K8.1 and/or LANA) of just under 50 %. KSHV serology remained rather consistent over the recruitment period, with the percentage positivity always being above 50 % (Figure 3.1.3). Taken individually, K8.1 only or LANA only, no obvious trends of changes in serology were observed over the recruitment period. This is not surprising since most KSHV infections in SSA are thought to occur during early childhood [1, 12].

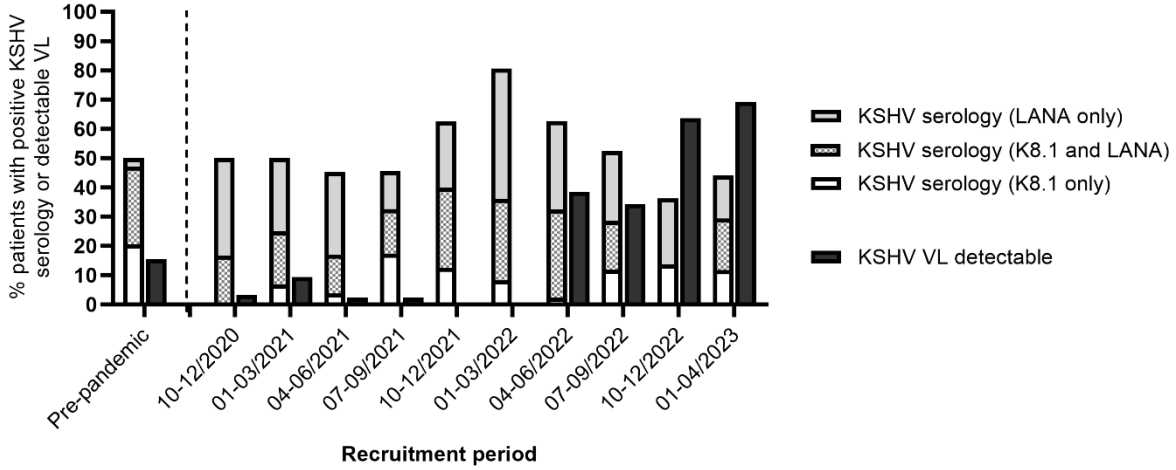

Figure 3.1.3: KSHV seroprevalence and KSHV VL over the course of patient recruitment. Quarterly percentage of KSHV seropositive patients over the course of the study period with single responses to LANA and K8.1 indicated in grey and white, respectively, and dual responses to both LANA and K8.1 indicated with dotted squares. The quarterly percentage of patients with detectable KSHV VL is shown in black bars.

KSHV VL (as a proxy for lytic reactivation) was detectable in a total of 21.0 % of the recruited patients. This percentage was much lower than that of KSHV serology (which is a measure of antibody response to KSHV exposure and indicates seroconversion), indicating that most patients displayed latent KSHV infection without lytic reactivation. Interestingly, when the quarterly percentages of KSHV VL was assessed over the recruitment period, there was initially a very low detectable KSHV VL during the first few months of recruitment until early 2022 (0 - 9.4 %). This result was also comparable to pre-pandemic samples which showed 15.4 % detectable KSHV VL. During the later stages of recruitment, we saw a steady increase in the number of patients with detectable KSHV VL reaching 69.2 % at the end of the recruitment period (Figure 3.1.3).

Of note, detection of KSHV VL was frequently very low and not always quantifiable, as explained in section 2.5. These samples were therefore recorded as detectable or qualitatively positive but not quantifiable and were assigned a value of 1 as reported by Labo *et al*. [166].

It is also important to note that there was a slight discrepancy between the percentage of patients with positive KSHV serology and patients with detectable KSHV VL (Figure 3.1.3) wherein there was a higher quarterly percentage of individuals with detectable KSHV VL towards the end of the recruitment period. This discrepancy could possibly be due to the type of serology assay we used here, where we only detected the presence of two proteins of interest, namely, K8.1 and LANA. Other KSHV-specific proteins not included in our assay could potentially also elicit responses, particularly in individuals that might have KSHV-associated diseases, which would have been missed by our assay. In addition, HIV-infection can also impact on the intensity/strength and diversity of serological responses [174].

3.1.4 The impact of SARS-CoV-2 on the reactivation of KSHV

As mentioned in section 3.1.2, SARS-CoV-2 seropositivity in this cohort of non-hospitalised HIV-infected patients was very high, with 76.2 % already at the beginning of the recruitment process in October 2020, increasing steadily and reaching 94.9 % at the end of the recruitment in April 2023 (Figure 3.1.3). As stated, this increase in SARS-CoV-2 antibodies could possibly be due to multiple SARS-CoV-2 infections as well as the increase in the number of individuals that were vaccinated. As we observed a steady increase in the number of patients with detectable KSHV VL over the recruitment period as described in 3.1.3, we next aimed to determine whether infection with SARS-CoV-2 and/or COVID-19 vaccination had any impact

on reactivation of KSHV. We thus divided our cohort into unvaccinated (n = 276) and vaccinated (n = 131) patients.

Over the course of our recruitment period, we observed an increase in SARS-CoV-2 antibody titre levels (both against RBD and S1) in the unvaccinated patients, following an exponential trend. This observation was not seen in the vaccinated group: antibody levels in the vaccinated group were higher than the levels in the unvaccinated group and remained steady and high for both RBD and S1 throughout the recruitment process, displaying no obvious trends (Figure 3.1.4 and Table 3.1.2).

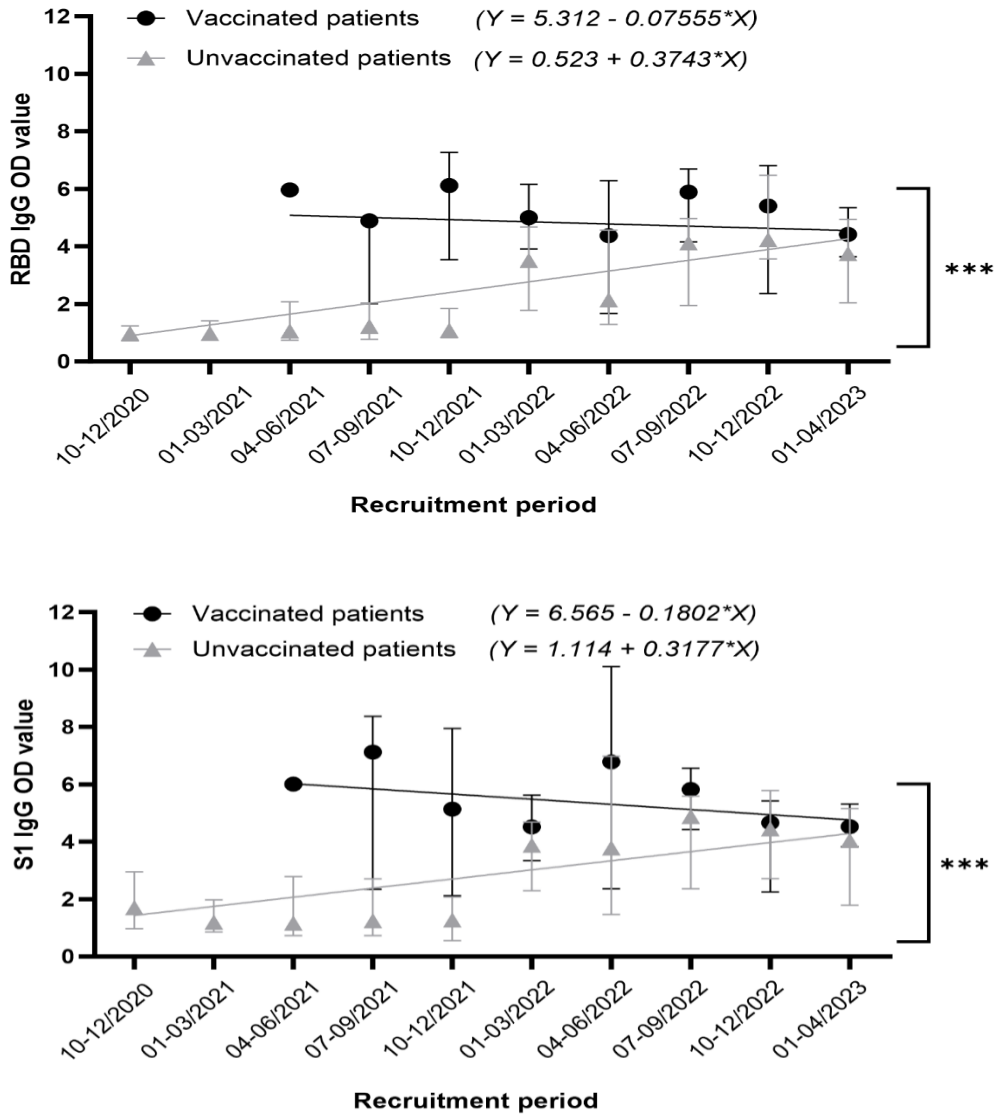

Figure 3.1.4: SARS-CoV-2 antibodies in unvaccinated and vaccinated individuals. Quarterly SARS-CoV-2 RBD IgG titres (top) and S1 IgG titres (bottom) expressed as median IgG OD values of each isotype with IQR. The trendlines over the course of the recruitment period are shown for both COVID-19 vaccinated (black) and unvaccinated (grey) patients and fitted as straight lines by linear regression which were compared by F test.

These results suggest that the increase in SARS-CoV-2 antibody titres in the unvaccinated patients was most likely due to exposure to repeated SARS-CoV-2 infections over the multiple COVID-19 waves experienced in SA [175], and that the higher antibody levels in the vaccinated patient group was due to both vaccination and exposure to SARS-CoV-2 throughout the recruitment process.

To assess any differences between the vaccinated and the unvaccinated patient groups in the context of KSHV reactivation, we analysed our cohort on a univariate level as presented in Table 3.1.2 for demographic and virological parameters and Supplementary Table 1 for clinical parameters. Demographic, virological and clinical variables were analysed with statistical differences seen in a number of variables which include weight, time since ART start, red cell count, mean corpuscular heamoglobin concentration, immature cell count, lymphocyte count, monocyte count, creatinine, SARS-CoV-2-related variables (RBD and S1 IgG OD) and KSHV-related variables (K8.1 OD).

Table 3.1.2: Univariate analysis of demographic and virological variables in COVID-19 unvaccinated (n = 276) and vaccinated (n = 131) patients with undetectable compared to detectable KSHV VL. Data are presented as number and percentage of total or median and IQR where appropriate. For continuous variables, *p*-values are by Mann-Whitney U test. For categorical variables, *p*-values are by Chi-square test, Fisher's Exact test or Fisher-Freeman-Halton test, as appropriate.

		COVID-19 unvaccinated (n = 276)			***COVID-19 vaccinated (n = 131)***		
Variable		**KSHV VL undetectable (n = 194) N (%) or Median (IQR)/Mean (SD)**	**KSHV VL detectable (n = 36) N (%) or Median (IQR)/Mean (SD)**	***p*-value**	**KSHV VL undetectable (n = 85) N (%) or Median (IQR)/Mean (SD)**	**KSHV VL detectable (n = 38) N (%) or Median (IQR)/Mean (SD)**	***p*-value**
Demographic information							
Age, years		39 (32 – 45)	38 (33 – 43.5)	0.99	40 (33 – 45)	41.5 (34 – 46)	0.80
Sex	Female	140 (72.2 %)	21 (58.3 %)	0.096	61 (71.8 %)	26 (68.4 %)	0.71
	Male	54 (27.8 %)	15 (41.7 %)		24 (28.2 %)	12 (31.6 %)	
Weight, kgs		72 (59 – 87)	63.0 (58 – 73.0)	0.031	71 (61 – 97)	64.0 (58.0 – 86.5)	0.079
HIV-related variables							
Time since HIV diagnosis, years[1]		3.62 (0.79 – 9.16)	2.27 (0.18 – 10.25)	0.11	5.62 (1.53 – 13.97)	7.11 (1.46 – 13.25)	0.73
HIV VL, copies/ml		51 (19 – 3283)	50.5 (10 – 2307)	0.92	49 (1 – 1499)	49 (1 – 107)	0.60
HIV VL	Suppressed <50 copies/ml	93 (48.9 %)	18 (50.0 %)	0.54	47 (56.6 %)	24 (63.2 %)	0.23
	Low-level viremia (50 – 999 copies/ml)	35 (18.5 %)	9 (25.0 %)		14 (16.9 %)	9 (23.7 %)	
	Viremia (≥1000 copies/ml)	62 (32.6 %)	9 (25.0 %)		22 (26.5 %)	5 (13.2 %)	
Absolute CD4, cells/µl		218 (126 – 309)	203 (133 – 298)	0.67	273 (151 – 370)	252 (146 – 391)	0.75

		COVID-19 unvaccinated (n = 276)			*COVID-19 vaccinated (n = 131)*		
Variable		**KSHV VL undetectable (n = 194) N (%) or Median (IQR)/Mean (SD)**	**KSHV VL detectable (n = 36) N (%) or Median (IQR)/Mean (SD)**	***p*-value**	**KSHV VL undetectable (n = 85) N (%) or Median (IQR)/Mean (SD)**	**KSHV VL detectable (n = 38) N (%) or Median (IQR)/Mean (SD)**	***p*-value**
Absolute CD4	Within normal range (332 – 1642 cells/µl)[2]	41 (21.7 %)	5 (13.9 %)	0.57	29 (34.5 %)	13 (35.1 %)	1.0
	Low (200 – 331 cells/µl)	63 (33.3 %)	13 (36.1 %)		25 (29.8 %)	11 (29.7 %)	
	Low and immunosuppressed (<200 cells/µl)	85 (45.0 %)	18 (50.0 %)		30 (35.7 %)	13 (35.1 %)	
WHO clinical stage of HIV disease	1	112 (61.5 %)	20 (58.8 %)	0.91	47 (61.0 %)	21 (58.3 %)	0.60
	2	40 (22.0 %)	8 (23.5 %)		9 (11.7 %)	3 (8.3 %)	
	3	29 (15.9 %)	6 (17.6 %)		20 (26.0 %)	10 (27.8 %)	
	4	1 (0.5 %)	0 (0.0 %)		1 (1.3 %)	2 (5.6 %)	
Receiving ART[3]	Yes	169 (87.1 %)	28 (77.8 %)	0.091	66 (78.6 %)	32 (84.2 %)	0.29
	Defaulted	19 (9.8 %)	4 (11.1 %)		12 (14.3 %)	6 (15.8 %)	
	No	6 (3.1 %)	4 (11.1 %)		6 (7.1 %)	0 (0 %)	
Time since ART start[1], years		0.92 (0.30 – 3.58)	0.46 (0.15 – 1.12)	0.025	0.76 (0.31 – 2.95)	0.93 (0.48 – 1.58)	0.56
ART regimen[1]	TDF/3TC/DTG	83 (45.6 %)	21 (67.7 %)	0.17	50 (64.1 %)	21 (56.8 %)	0.32
	TDF/FTC/EFV	65 (35.7 %)	6 (19.4 %)		11 (14.1 %)	4 (10.8 %)	
	AZT/3TC/LPV/r	9 (4.9 %)	0 (0.0 %)		3 (3.8 %)	1 (2.7 %)	
	TDF/FTC/LPV/r	4 (2.2 %)	1 (3.2 %)		5 (6.4 %)	2 (5.4 %)	
	ABC/3TC/LPV/r	0 (0.0 %)	1 (3.2 %)		1 (1.3 %)	0 (0.0 %)	
	ABC/3TC/EFV,	2 (1.1 %)	0 (0.0 %)		0 (0.0 %)	1 (2.7 %)	

		COVID-19 unvaccinated (n = 276)			*COVID-19 vaccinated (n = 131)*		
Variable		**KSHV VL undetectable (n = 194) N (%) or Median (IQR)/Mean (SD)**	**KSHV VL detectable (n = 36) N (%) or Median (IQR)/Mean (SD)**	***p*-value**	**KSHV VL undetectable (n = 85) N (%) or Median (IQR)/Mean (SD)**	**KSHV VL detectable (n = 38) N (%) or Median (IQR)/Mean (SD)**	***p*-value**
	TDF/FTC/ATV/r	3 (1.6 %)	0 (0.0 %)		3 (3.8 %)	3 (8.1 %)	
	ABC/3TC/DTG	5 (2.7 %)	1 (3.2 %)		0 (0.0 %)	1 (2.7 %)	
	AZT/3TC/DTG	2 (1.1 %)	1 (3.2 %)		5 (6.4 %)	2 (5.4 %)	
	ABC/3TC/ATV/r	4 (2.2 %)	0 (0.0 %)		0 (0.0 %)	1 (2.7 %)	
	ABC/3TC/NVP	1 (0.5 %)	0 (0.0 %)		0 (0.0 %)	0 (0.0 %)	
	AZT/3TC/ATV/r	2 (1.1 %)	0 (0.0 %)		0 (0.0 %)	0 (0.0 %)	
	AZT/3TC/NVP	2 (1.1 %)	0 (0.0 %)		0 (0.0 %)	1 (2.7 %)	
SARS-CoV-2-related variables							
SARS-CoV-2[4] serology	Negative	48 (24.7 %)	6 (16.7 %)	0.29	4 (4.7 %)	2 (5.3 %)	1.00
	Positive	146 (75.3 %)	30 (83.3 %)		81 (95.3 %)	36 (94.7 %)	
RBD IgG		1.24 (0.82 – 2.42)	2.83 (1.08 – 4.72)	0.0030	5.13 (4.11 – 6.36)	4.53 (2.90 – 5.92)	0.086
S1 IgG		1.59 (0.81 – 3.28)	2.90 (1.79 – 5.04)	0.0020	5.53 (4.03 – 7.55)	4.61 (3.35 – 5.73)	0.017
KSHV-related variables							
KSHV serology[5]	Negative	90 (46.6 %)	18 (51.4 %)	0.38	33 (38.8 %)	20 (57.1 %)	0.37
	Positive: K8.1 and LANA positive	31 (16.1 %)	6 (17.1 %)		23 (27.1 %)	7 (20.0 %)	
	K8.1 positive only	16 (8.3 %)	5 (14.3 %)		9 (9.4 %)	2 (5.7 %)	
	LANA positive only	56 (29.0 %)	6 (17.1 %)		21 (24.7 %)	6 (17.1 %)	
K8.1 OD of seropositive patients		0.77 (0.36 – 1.45)	1.61 (0.72 – 2.65)	0.012	1.14 (0.60 – 2.06)	1.55 (0.51 – 2.47)	0.67
LANA OD of seropositive patients		1.76 (1.12 – 3.34)	1.84 (0.90 – 5.85)	0.78	2.89 (1.24 – 5.37)	2.25 (1.02 – 4.47)	0.62

	COVID-19 unvaccinated (n = 276)			*COVID-19 vaccinated (n = 131)*		
Variable	**KSHV VL undetectable (n = 194) N (%) or Median (IQR)/Mean (SD)**	**KSHV VL detectable (n = 36) N (%) or Median (IQR)/Mean (SD)**	***p*-value**	**KSHV VL undetectable (n = 85) N (%) or Median (IQR)/Mean (SD)**	**KSHV VL detectable (n = 38) N (%) or Median (IQR)/Mean (SD)**	***p*-value**
Detectable KSHV VL	–	36 (100.0 %)	–	–	38 (100.0 %)	–
Quantifiable KSHV VL	–	14 (38.9 %)	–	–	6 (15.8 %)	–
KSHV VL (copies/10^6 cells)[6]	–	1 (1 – 986.05)	–	–	1 (1 – 1)	–
Quantifiable KSHV VL (copies/10^6 cells)	–	1377.86 (466.88 – 2635.53)	–	–	1941.30 (446.52 – 2914.80)	–

[1]Data collated from pharmacy records. [2]Range as per NHLS definition. [3]Data collated from pharmacy records. "Defaulted" defined as >1 month uncollected ART according to pharmacy records. [4]SARS-CoV-2 antibody positivity detected by ELISA to IgG RBD and S1 ("Negative" indicates that patient sample has an OD value below the assay cut-off for both IgG RBD and S1 ELISAs).[5] KSHV antibody positivity detected by ELISA to K8.1 and LANA ("Negative" indicates that patient sample has an OD value below the assay cut-off for both K8.1 and LANA). [6] KSHV VL includes all detectable (and not quantifiable) samples which were assigned a value of 1.
Abbreviations: FTC, Emtricitabine; EFV, Efavirenz; TDF, Tenofovir; 3TC, Lamivudine; DTG, Dolutegravir, AZT, Zidovudine; LPV/r, Lopinavir/Ritonavir; ABC, Abacavir; ATV/r, Atazanavir/Ritonavir; NVP, Nevirapine; OD, optical density.

While we do see an increase in both quarterly percentage of patients with detectable KSHV VL and SARS-CoV-2 serology over the recruitment period (Figures 3.1.2 – 3.1.4), we did not observe a significant difference in the amount of virus when the quantifiable KSHV VL was plotted against patients that were either unvaccinated or vaccinated (p = 0.9044) (Figure 3.1.5). The median KSHV VL for both the unvaccinated and vaccinated patients was 1 copy/10^6 cells (IQR 1 – 986.05 in unvaccinated patients and IQR 1 – 1 in vaccinated patients) (Table 3.1.2). As previously mentioned, the majority of the KSHV VL was detectable but not quantifiable. KSHV VL was detectable in 36 unvaccinated patients of which 14 patients had quantifiable KSHV VL (38.9 %); whereas in vaccinated patients KSHV VL was detectable in 38 patients of which 6 patients had quantifiable KSHV VL (15.8 %). When we excluded patients with a KSHV VL at the limit of detection from the univariate analysis, we found that the quantifiable KSHV VL was 1377.86 copies/10^6 cells in the unvaccinated patients (IQR 466.80 – 2635.53) and 1941.30 copies/10^6 cells in the vaccinated patients (IQR 446.52 – 2914.80) showing no statistical differences between the two groups (Figure 3.1.5, Table 3.1.2).

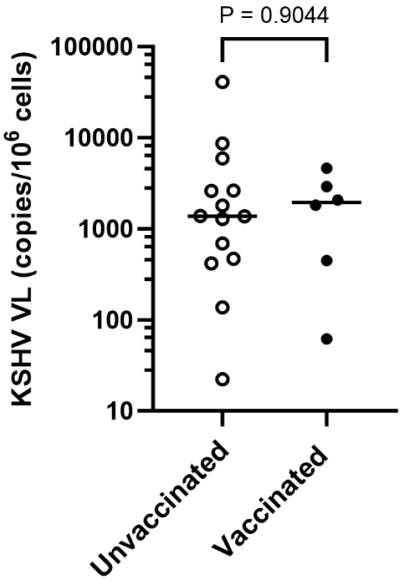

Figure 3.1.5: Quantifiable KSHV VL (expressed as copies/10^6 cells) between COVID-19 unvaccinated and vaccinated individuals. Median values are indicated by the solid line. Groups were compared by Mann-Whitney U test [176].

When analysing SARS-CoV-2-related variables, we found significantly different results for both SARS-CoV-2 RBD and S1 IgG between the unvaccinated and vaccinated patient groups in the context of detectable KSHV VL. Unvaccinated patients with detectable KSHV VL displayed significantly higher titres compared to those with an undetectable VL with a median RBD IgG OD of 2.83 (IQR 1.08 – 4.72) versus 1.24 (IQR 0.82 – 2.42), p = 0.004; and S1 IgG

OD 2.90 (IQR 1.79 – 5.04) versus 1.59 (IQR 0.81 – 3.28), $p = 0.009$. This was not seen in the vaccinated patients with median RBD IgG OD of 4.53 (IQR 2.90 – 5.92) versus 5.13 (IQR 4.11 – 6.36), $p = 0.0849$; and S1 IgG OD 4.61 (IQR 3.35 – 5.73) versus 5.53 (IQR 4.03 – 7.55), $p = 0.0167$ (Table 3.1.2 and Figure 3.1.6). Again, as already shown in Figure 3.1.4, the SARS-CoV-2 OD values for the vaccinated group were substantially higher compared to the unvaccinated group.

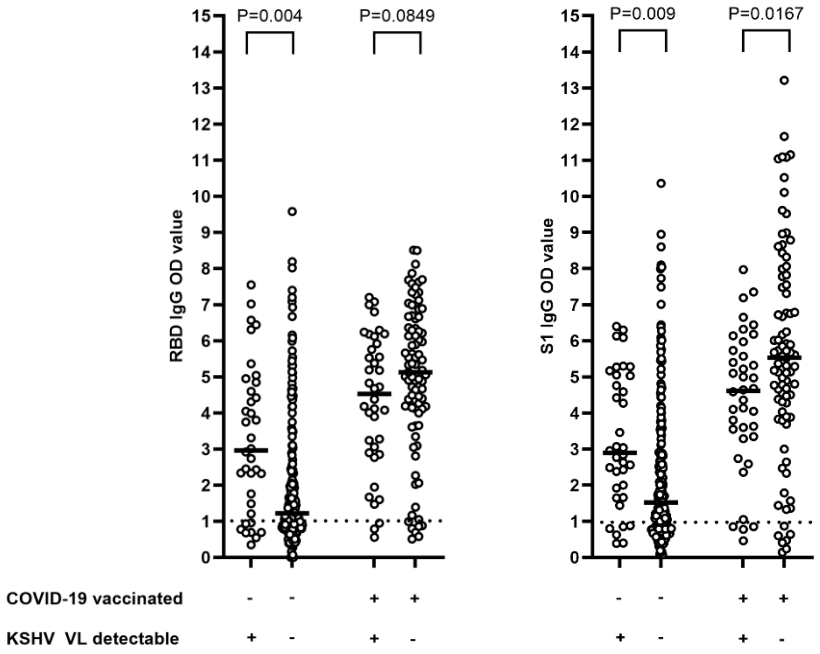

Figure 3.1.6: Detectable KSHV VL is associated with previous SARS-CoV-2 infection. SARS-CoV-2 RBD IgG titres (left) and S1 IgG titres (right) in COVID-19 vaccinated and unvaccinated patients by presence of KSHV VL in the peripheral blood. The cut-offs were determined by the mean OD+2SD of pre-pandemic samples, set as 1 and indicated by the dotted line. Median values are indicated by the solid line. Groups were compared by Mann-Whitney U test [176].

The univariate analysis also revealed that the antibody titres against the lytic KSHV antigen K8.1 were significantly higher in the unvaccinated patient group with detectable KSHV VL compared to undetectable KSHV VL with a median OD of 1.61 (IQR 0.72 – 2.65) versus median OD of 0.77 (IQR 0.36 – 1.45), respectively, $p = 0.012$. This difference was not seen in the vaccinated patient group where patients with detectable KSHV VL had a median K8.1 OD of 1.55 (IQR 0.51 – 2.47) versus a median OD of 1.14 (IQR 0.60 – 2.06) in patients with undetectable KSHV VL, $p = 0.67$ (Table 3.1.2). The antibody titres for the latent KSHV antigen LANA displayed slightly higher OD values in the vaccinated group versus the unvaccinated group which was independent of the presence of detectable KSHV VL. Here, the median OD for the vaccinated group with detectable KSHV VL was 2.25 (IQR 1.02 – 4.47) versus a median OD of 2.89 (IQR 1.24 – 5.37) in patients with undetectable KSHV VL, $p = 0.62$. In unvaccinated

patients, median OD values in the detectable KSHV VL group were 1.84 (IQR 0.90 – 5.85) versus a median OD of 1.76 (IQR 1.12 – 3.34) in patients with undetectable KSHV VL, p = 0.78 (Table 3.1.2).

Interestingly, the univariate analysis did not reveal statistically significant differences in the analysed patient groups with regards to the inflammatory markers CRP and IL-6 (Figure 3.1.7 and Supplementary Table 1). Most individuals in both unvaccinated and vaccinated groups (>70 %) had CRP levels and IL-6 levels that were in the normal range (<10 mg/l and <1.8 pg/l, respectively). Median CRP and IL-6 did not differ between unvaccinated and vaccinated patients with no significant association between CRP or IL-6 levels and COVID-19 vaccination (for CRP p = 0.9357 and 0.9857 for unvaccinated versus vaccinated, respectively, and for IL-6 p = 0.1762 and 0.5000 for the unvaccinated versus the vaccinated groups, respectively). Similarly, neither CRP nor IL-6 levels were associated with KSHV VL detectability (Figure 3.1.7).

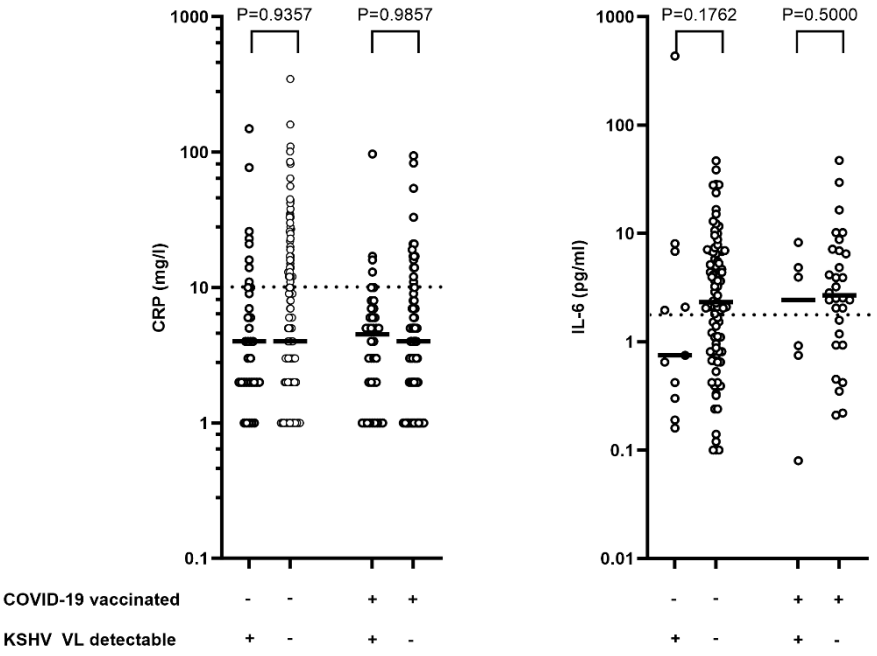

Figure 3.1.7: Detectable KSHV VL is not associated with inflammatory variables. Quantification of inflammatory markers CRP (left) and IL-6 (right) in COVID-19 vaccinated and unvaccinated patients by presence of KSHV VL in the peripheral blood. The cut-off values for elevated CRP (10 mg/l) and IL-6 (1.8 pg/ml) are indicated by the dotted line. Median values are indicated by the solid line. Groups were compared by Mann-Whitney U test [176].

The univariate analysis shown in Table 3.1.2 and Supplementary Table 1 also assessed a broad range of both clinical and virological variables. Besides the interesting observations seen on SARS-CoV-2 and KSHV-related variables that have been mentioned above, we also found a significantly lower weight in unvaccinated versus vaccinated patients (unvaccinated patients

with detectable KSHV VL: median 63 kg (IQR 59 – 73) versus patients with undetectable KSHV VL: median 72 kg (IQR 59 – 87), p = 0.031; vaccinated patients with detectable KSHV VL: median 64 kg (IQR 58 – 86.5) versus patients with undetectable KSHV VL: median 71 kg (IQR 61 – 97); p = 0.079)), shorter time since ART start (unvaccinated patients with detectable KSHV VL: median 0.46 years (IQR 0.15 – 1.12) versus patients with undetectable KSHV VL: median 0.92 years (IQR 0.30 – 3.58), p = 0.025; vaccinated patients with detectable KSHV VL: median 0.9 years (IQR 0.48 – 1.58) versus median 0.76 years (IQR 0.31 – 2.95) in patients with undetectable KSHV VL, p = 0.56) and higher creatinine levels (unvaccinated patients with detectable KSHV VL: median 77 µmol/l (IQR 60.5 – 93) versus median 68 µmol/l (IQR 57.5 – 80) in patients with undetectable KSHV VL, p = 0.040; vaccinated patients with detectable KSHV VL: median 68 µmol/l (IQR 59 – 78) versus patients with undetectable KSHV VL: median 67.5 µmol/l (IQR 59 – 87), p = 0.46). There were also proportionally more individuals with a low lymphocyte count (p = 0.0050) and proportionally more individuals with a high monocyte count (p = 0.013) in the unvaccinated group with detectable KSHV VL compared to the vaccinated group. These significant differences were only seen in the unvaccinated group, but not in the vaccinated group. This could be due to late presentation to the clinic of unvaccinated patients which would explain the shorter time since ART start accompanied by HIV symptoms that present at a later stage. On the other hand, in the vaccinated group, we observed more patients displaying elevated IL-6 without KSHV VL detectable (unvaccinated: p = 0.062; vaccinated: p = 0.030), elevated red cell counts in patients with reactivated KSHV (unvaccinated: p = 0.12; vaccinated: p = 0.031) and higher mean corpuscular haemoglobin concentrations (unvaccinated: p = 0.22; vaccinated: p = 0.026) as well as higher immature cell counts (unvaccinated: p = 0.91; vaccinated: p = 0.048). Other HIV-related variables (such as HIV VL, absolute CD4 count, WHO clinical stage of infection etc); laboratory blood analysis (sodium, albumin, ALT concentrations etc.); and symptoms at presentation (fever, fatigue, edema etc.) were not significantly different between the groups after univariate analysis (Table 3.1.2 and Supplementary Table 1).

Further to our univariate analysis, logistic regression was performed to assess the risk of KSHV reactivation (Table 3.1.3). The logistic regression simultaneously considers multiple variables and combines them to robustly model the probability of an event. Thus, variables with significant associations between patients with undetectable and detectable KSHV VL in the unvaccinated and vaccinated patient groups as identified by univariate analysis (Table 3.1.2 and Supplementary Table 1), based on p-values, were considered for further analysis.

These variables included RBD IgG (OD), age, sex, weight, duration on ART, lymphocyte count, red cell count, creatinine and IL-6 concentrations. The assessment revealed a significantly increased odds of KSHV reactivation in the unvaccinated group of exposed SARS-CoV-2

patients (adjusted odds ratio (OR) 1.28; 95 % confidence interval (CI): 1.05 – 1.55, $p = 0.015$) which was not seen for the vaccinated group (adjusted OR 0.83; 95 % CI: 0.67 – 1.02, $p = 0.080$). A significant association was also detected between low lymphocyte count and KSHV reactivation in the unvaccinated patient group (adjusted OR 2.65; 95 % CI: 1.15 – 6.12, $p = 0.023$) which was not seen in the vaccinated patient group (adjusted OR 0.96; 95 % CI: 0.39 – 2.36, $p = 0.92$).

This logistic regression thus indicates a positive association between the presence of SARS-CoV-2 antibodies (as a proxy for previous SARS-CoV-2 infection) and risk of reactivation of KSHV (as indicated by detectable KSHV VL) in the unvaccinated but not vaccinated group. This suggests that COVID-19 vaccination is protective against KSHV reactivation while in unvaccinated patients previous SARS-CoV-2 infection/exposure and in turn the biological downstream effects of SARS-CoV-2 infection (such as lymphopenia) may cause the reactivation of KSHV. However, this requires further investigation.

Table 3.1.3: Logistic regression for detectable KSHV VL in COVID-19 unvaccinated (n = 276) and vaccinated (n = 131) patients. Data are adjusted for biologically relevant demographic and clinical variables as well as variables associated with detectable KSHV VL on a univariate level (see Table 3.1.2 and Supplementary Table 1) as listed here.

	COVID-19 unvaccinated (n = 276)							***COVID-19 vaccinated (n = 131)***						
Variable	**Unadjusted Odds Ratio**	**95 % CI for Unadjusted Odds Ratio**		**Adjusted odds ratio**	**95 % CI for Adjusted Odds Ratio**		***p*-value**	**Unadjusted Odds Ratio**	**95 % CI for Unadjusted Odds Ratio**		**Adjusted odds ratio**	**95 % CI for Adjusted Odds Ratio**		***p*-value**
		Lower	Upper		Lower	Upper			Lower	Upper		Lower	Upper	
RBD IgG (OD)	1.29	1.10	1.52	1.28	1.05	1.55	0.015*	0.86	0.71	1.04	0.83	0.67	1.02	0.080
Age (years)	1.00	0.96	1.04	0.99	0.94	1.04	0.69	1.00	0.96	1.04	1.00	0.95	1.05	0.98
Sex[1]	1.85	0.89	3.86	2.26	0.94	5.43	0.068	1.17	0.51	2.69	1.28	0.48	3.43	0.62
Weight (kg)	1.00	0.99	1.00	1.00	0.99	1.00	0.29	1.00	0.99	1.00	1.00	1.00	1.00	0.15
Duration on ART (years)	0.92	0.83	1.03	0.96	0.85	1.09	0.53	1.01	0.93	1.10	1.04	0.94	1.14	0.48
Lymphocytes[2]	2.80	1.35	5.80	2.65	1.15	6.12	0.023*	0.95	0.43	2.13	0.96	0.39	2.36	0.92
Red cells[3]	3.36	0.77	14.75	5.58	0.99	31.35	0.051	9.88	1.07	91.65	7.83	0.77	79.22	0.081
Creatinine[4]	4.26	1.27	14.28	4.80	0.98	23.53	0.053	2.42	0.66	8.93	3.18	0.62	16.24	0.17
IL-6[5]	0.40	0.15	1.07	0.31	0.09	1.08	0.065	0.26	0.07	0.94	0.30	0.08	1.15	0.080

[1]Sex is for male compared to female; [2]Lymphocytes is for low count compared to within or above normal range; [3]Red cells is for high concentration compared to below or within normal range; [4]Creatinine is for high concentration compared to below or within normal range; [5]IL-6 is for elevated IL-6 concentration compared to undetectable or less than the reference value.

3.2 A 12-month follow-up analysis on the impact of the COVID-19 pandemic on KSHV-seropositive HIV-infected patients

Given the high seroprevalence of KSHV in SSA, particularly in HIV-infected populations, we assessed the potential effect of high exposure to SARS-CoV-2 infection on reactivation of KSHV in a FU study design. As opposed to the cross-sectional study design shown in section 3.1, this section presents patients who were followed up based on their KSHV-seropositive status at enrolment independent of previous SARS-CoV-2 infection. Eligible patients were invited for FU at 6 months and 12 months post enrolment to determine the effect of SARS-CoV-2 infection on KSHV VL, KSHV immune responses and other clinical markers.

As stated in section 3.1, the initial recruitment process was initiated after the decline of infections of the first COVID-19 wave in October 2020 and continued throughout subsequent waves of the pandemic in SA until April 2023 (Figure 3.1.1 and 3.2.1). These waves were dominated by different SARS-CoV-2 variants which included the wildtype (or ancestral) strain, dominating the first wave, beta (second wave), delta (third wave) and omicron (fourth wave).

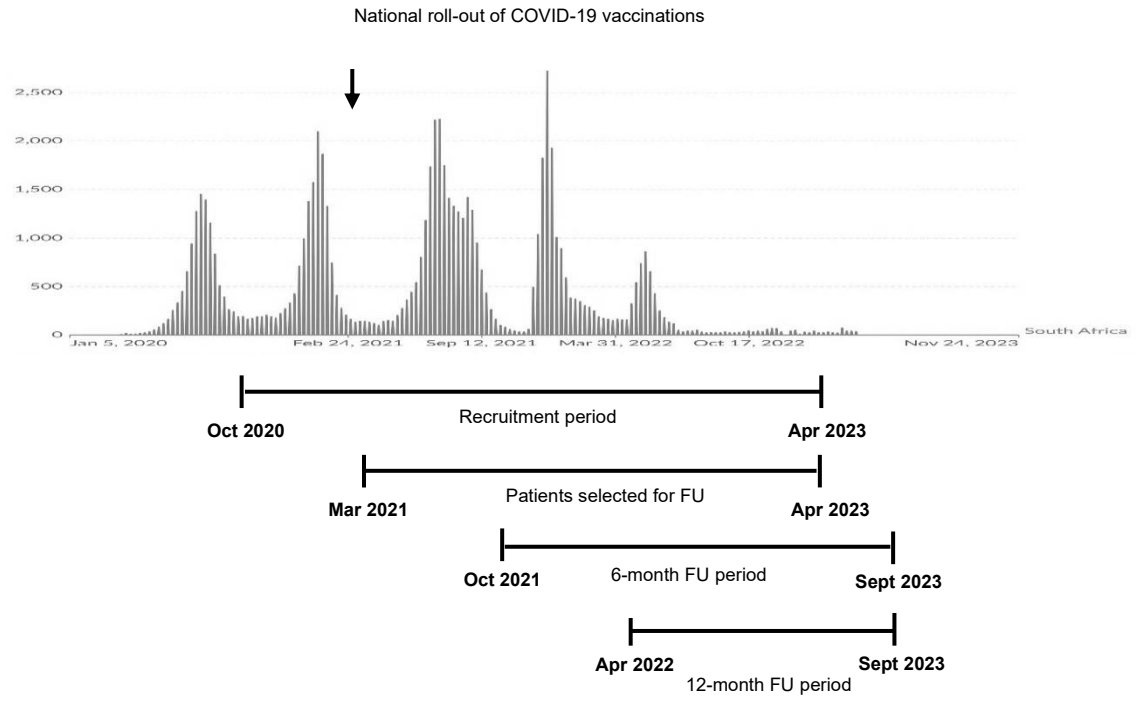

Figure 3.2.1: Outline of SARS-CoV-2 infection patterns in SA in relation to participant enrolment and FU for this study. The 7-day rolling average of daily new confirmed COVID-19 cases per million people in SA is shown as reported on OurWorldInData.org [115]. The timeline represents patient recruitment into the parent study starting in October 2020 at the end of the first COVID-19 wave, patient enrolment into the FU study from March 2021, the start of the 6-month FU period in October 2021, the start of the 12-month FU period in April 2022 as well as the start of national COVID-19 vaccination roll-out in SA in February 2021 (indicated by the arrow).

Although recruitment into the parent study started in October 2020, ethics approval to enrol patients for FU was only obtained in March 2021. The 6-month and 12-month FU periods therefore started in October 2021 and April 2022, respectively, and ran until September 2023 (Figure 3.2.1).

The parental study included 407 patients of whom 317 were consented for enrolment into the FU study. As mentioned above, patients were selected for FU if they had a positive KSHV serology, independently of their SARS-CoV-2 serology status or whether they had received the COVID-19 vaccine. A total of 173 patients were eligible for FU based on their positive KSHV serology; however, only 88 of these individuals arrived for the 6-month FU visit at the clinic. The remaining 84 patients were therefore categorised as lost to follow-up (LTFU). At the 12-month FU timepoint, a further 42 individuals were LTFU and only 46 of the 88 individuals that presented at the 6-month FU also arrived for the 12-month FU visit. In addition, 13 individuals presented at the 12-month FU without prior 6-month FU visit (Figure 3.2.2). The demographics of the LTFU patients were similar to that seen for the patients that presented at their FU time points, thereby providing no obvious explanation for the high LTFU rate (Supplementary Table 2). For the purpose of consistency, the 46 individuals that presented at the initial enrolment, at the 6-month and at the 12-month FU were analysed and will be presented here forth.

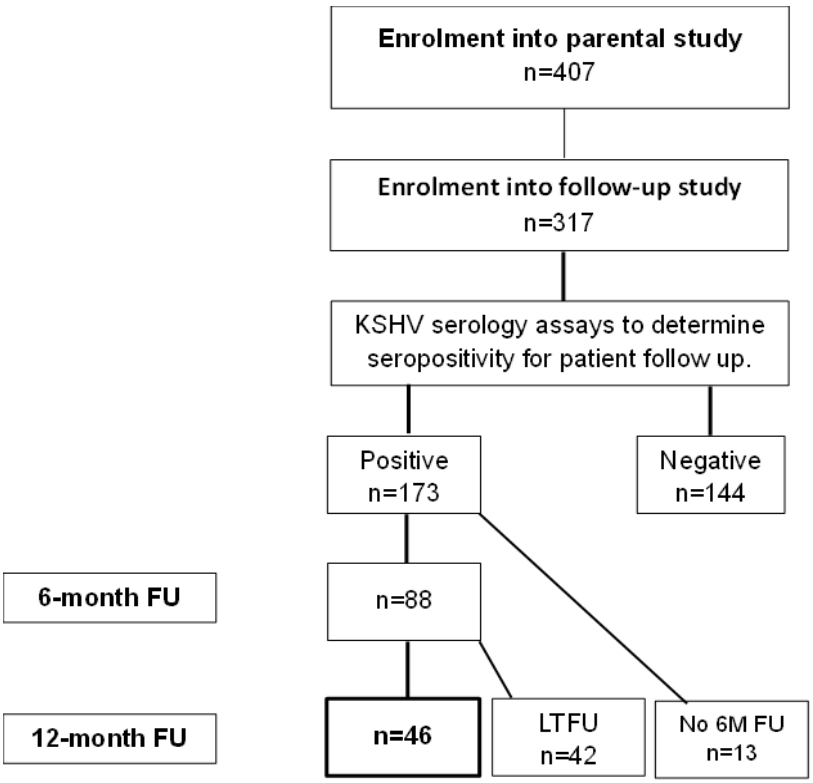

Figure 3.2.2: Schematic flow chart of the patients selected for FU analysis from initial recruitment.

3.2.1 Clinical characteristics and univariate analysis of FU cohort

To assess any differences in the clinical and study-related parameters of the FU patients at the three study visits (initial recruitment, 6- and 12-month FU), we analysed this cohort on a univariate level as presented in Table 3.2.1 and Supplementary Table 3. As already seen in the entire cohort (section 3.1), the majority of patients was female (78.3 %), with a median age of 41.5 years at enrolment and a similar median weight at each study visit. Overall, the COVID-19 vaccination status of the cohort followed an increasing and statistically significant trend throughout the FU period with a *p*-value of 0.004 (see also Figure 3.2.8 below).

At presentation, patients reported very few or no symptoms which did not change over the course of the FU period. As shown for the parent cohort, the majority of the FU individuals were on ART, with over 50 % of the group receiving Tenofovir/ Lamivudine/Dolutegravir (TDF/3TC/ DTG) and the remainder of the group receiving other regimens. The majority of the patients were on WHO HIV clinical stage 1 (>50 %) and 3 (>25 %) throughout the FU period. In terms of HIV VL, patients displayed a decreasing trend throughout the FU period with the majority having a HIV VL that was either suppressed or low-level viremic (<1000 copies/ml), while absolute CD4 median levels increased (Table 3.2.1 and section 3.2.2.1 below).

SARS-CoV-2 serology was determined by ELISA, revealing almost all patients (93.5 %) with a positive SARS-CoV-2 serology (i.e. high OD units) against both RBD and S1 already at initial recruitment, thus indicating high exposure to SARS-CoV-2 (see also section 3.1, Table 3.2.1 and section 3.2.2.2 below). KSHV serology was performed at the initial recruitment to determine a patient's eligibility for enrolment into the FU study. The median K8.1 OD for all enrolled FU patients was 1.47, while the median LANA OD was 3.06. Further assessment of KSHV serology at the FU visits revealed a decrease in K8.1 and LANA levels at the 6-month FU, with K8.1 antibodies increasing again at the 12-month FU time point and LANA levels further decreasing (Table 3.2.1 and section 3.2.2.3 below).

The median KSHV VL remained at 1 copy/10^6 cells at initial recruitment and at the 6-month FU but increased to 11172.45 copies/10^6 cells at the 12-month FU period. Interestingly, the percentage of individuals with detectable KSHV VL decreased over the FU period (Table 3.2.1 and section 3.2.2.3 below).

Absolute IL-6 and CRP concentrations did not change over the FU period, but some trends in laboratory blood parameters were found, such as sodium concentration (increased percentage of individuals within the normal range), creatinine concentration (decreased percentage of individuals within the normal range) and white cell count (increased percentage of individuals within the normal range, Supplementary Table 3 and section 3.2.2.4 below).

Table 3.2.1: Univariate analysis of demographic and virological variables of FU patients (n = 46). Data are presented as number and percentage of total or median and IQR where appropriate. *p*-values are by Kruskal-Wallis test for continuous variables and Chi-square test for categorical variables, as appropriate.

Parameter		**Initial Recruitment N (%) or Median (IQR)**	**FU 6-months N (%) or Median (IQR)**	**FU 12-months N (%) or Median (IQR)**	***p*-value**
Demographic information					
Age, years		41.50 (33.0 – 47.0)			–
Sex	Female	36 (78.3 %)	36 (78.3 %)	36 (78.3 %)	–
	Male	10 (21.7 %)	10 (21.7 %)	10 (21.7 %)	
Weight, kgs		69.0 (55.0 – 87.0)	70 (56.0 – 87.0)	70.2 (58.0 – 88.4)	0.864
COVID-19 vaccination	Yes	20 (43.5 %)	30 (66.7 %)	35 (76.1 %)	0.004
	No	26 (56.5 %)	15 (33.3 %)	11 (23.9 %)	
HIV-related parameters					
Time since HIV diagnosis, days		2731.0 (636.0 – 4623.0)			0.082
HIV VL, copies/ml		53.0 (10.0 – 803.5)	49.0 (1.0 – 430.0)	34.5 (1.00 – 150.5)	0.259
HIV VL	Suppressed/low level viremia <999 copies/ml	33 (75.0 %)	39 (84.8 %)	38 (86.4 %)	0.320
	Viremia ≥1000 copies/ml	11 (25.0 %)	7 (15.2 %)	6 (13.6 %)	
Absolute CD4, cells/µl		216.00 (126.00 – 303.00)	244.50 (180.00 – 364.00)	284.50 (200.00 – 375.00)	0.110
Absolute CD4	Within normal range (332 – 1642 cells/µl)	9 (19.6 %)	13 (28.9 %)	16 (34.8 %)	0.259
	Below normal range (<332 cells/µl)	37 (80.4 %)	32 (71.1 %)	30 (65.2 %)	
WHO clinical stage of HIV disease	1.00	24 (54.5 %)	24 (52.2 %)	22 (52.4 %)	1.000
	2.00	7 (15.9 %)	7 (15.2 %)	6 (14.3 %)	
	3.00	12 (27.3 %)	14(30.4 %)	13 (31.0 %)	
	4.00	1 (2.3 %)	1 (2.2 %)	1 (2.2 %)	

Parameter		Initial Recruitment N (%) or Median (IQR)	FU 6-months N (%) or Median (IQR)	FU 12-months N (%) or Median (IQR)	*p*-value
ART regimen[1]	TDF, FTC, EFV	9 (21.4 %)	3 (7.7 %)	0 (0.0 %)	0.503
	TDF, 3TC, DTG	24 (57.1 %)	27 (69.2 %)	27 (69.2 %)	
	AZT, 3TC, LPV/r	2 (4.8 %)	2 (5.1 %)	2 (5.1 %)	
	TDF, FTC, LPV/r	3 (7.1 %)	3 (7.7 %)	3 (7.7 %)	
	TDF, FTC, ATV/r	1 (2.4 %)	1 (2.6 %)	2 (5.1 %)	
	3TC, DTG, ABC	0 (0.0 %)	0 (0.0 %)	1 (2.6 %)	
	AZT, 3TC, DTG	0 (0.0 %)	0 (0.0 %)	1 (2.6 %)	
	3TC, ATV/r, ABC	1 (2.4 %)	2 (5.1 %)	2 (5.1 %)	
	AZT, 3TC, ATVr	1 (2.4 %)	1 (2.6 %)	1 (2.6 %)	
	AZT,3TC, NVP	1 (2.4 %)	0 (0.0 %)	0 (0.0 %)	
SARS-CoV-2 ELISA-related parameters					
RBD IgG, OD		4.42 (1.60 – 5.62)	3.47 (1.80 – 4.63)	3.48 (2.53 – 4.59)	0.274
S1 IgG, OD		4.70 (2.33 – 6.40)	3.22 (2.22 – 4.22)	4.38 (3.39 – 5.41)	0.026
KSHV-related parameters					
KSHV VL (copies/10^6 cells)		1.00 (1.00 – 1.00)	1.00 (1.00 – 1.00)	11172.45 (1.00 – 22343.91)	0.634
KSHV VL detectable[2]	Not detectable	34 (79.1 %)	41 (89.1 %)	41 (95.3 %)	0.065
	Detectable	9 (20.9 %)	5 (10.9 %)	2 (4.7 %)	
K8.1		1.47 (0.47 – 2.22)	0.79 (0.21 – 2.59)	1.77 (0.36 – 3.21)	0.266
LANA		3.06 (1.37 – 5.56)	1.02 (0.28 – 2.09)	0.84 (0.36 – 1.78)	<0.001

[1]Data collated from pharmacy records. [2] KSHV VL includes all detectable (and not quantifiable) samples which were assigned a value of 1.
Abbreviations: FTC, Emtricitabine; EFV, Efavirenz; TDF, Tenofovir; 3TC, Lamivudine; DTG, Dolutegravir, AZT, Zidovudine; LPV/r, Lopinavir/Ritonavir; ABC, Abacavir; ATV/r, Atazanavir/Ritonavir; NVP, Nevirapine; OD, optical density.

3.2.2 Trends in clinical and virological parameters during the FU study period

Following the univariate analysis presented in Table 3.2.1 and Supplementary Table 3, selected results were plotted graphically to observe trends visually and analysed using Dunn's test adjusted with Bonferroni correction for statistical analysis. These post-hoc statistical tests were conducted after univariate analysis to determine differences between groups at the different FU time points. Importantly, compared to the univariate analysis where we assessed the overall mean between groups, here we look at trends in the parameters in individual patients within the groups over the FU period.

3.2.2.1 HIV-related-parameters

As mentioned above, the median HIV VL decreased throughout the FU period while median CD4 counts increased (Table 3.2.1). At initial recruitment, median HIV VL was 53.0 copies/ml, but decreased at the 6-month FU to 49.0 copies/ml and further decreased at the 12-month FU to 34.5 copies/ml (Figure 3.2.3A). Median CD4 counts increased from initial recruitment (216.0 cells/µl) to 244.5 cells/µl at the 6-month FU, and further increased to 284.5 cells/µl at the 12-month FU (Figure 3.2.3B). While we observed no statistical significance between overall patient groups at the FU time points at a univariate level (Table 3.2.1), an observation of each individual's pattern of HIV VL and CD4 count showed different trends during FU (Figure 3.2.3); however, no statistical significances were detected either.

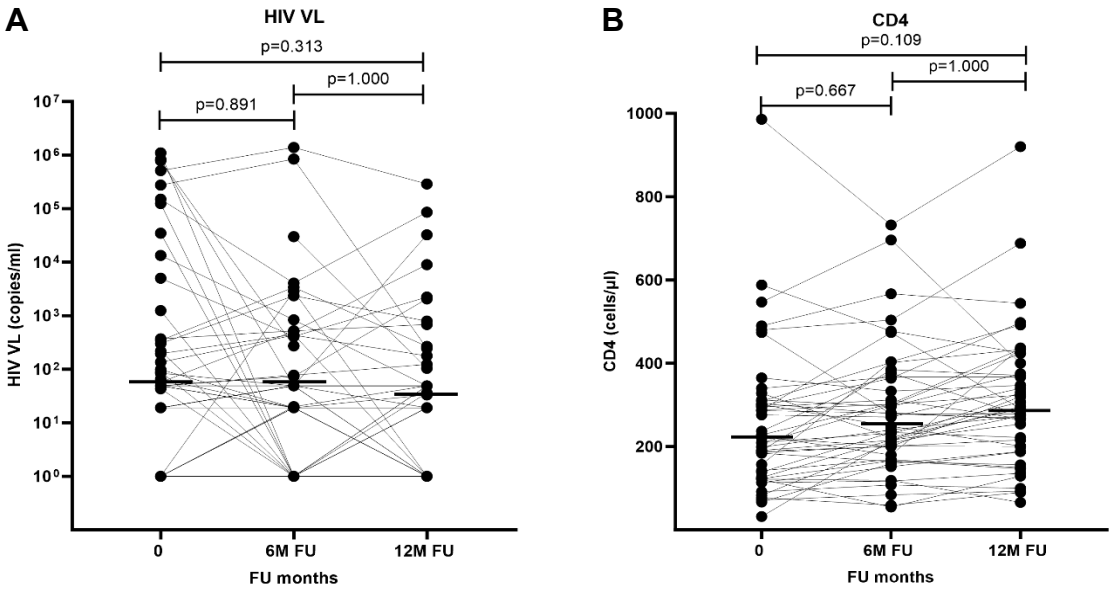

Figure 3.2.3: Change in patients' HIV-related parameters over the course of the FU period. A) HIV VL and **B)** CD4 count of each individual over the FU period with median indicated by the black solid lines, n = 46 per group. *p*-values are by Dunn's test adjusted with Bonferroni correction and each black circle represents an individual patient.

3.2.2.2 SARS-CoV-2-related parameters

Plotting individual patients' results for SARS-CoV-2-related parameters showed that most of the individuals presented with a decrease in RBD OD units from initial recruitment (median RBD OD = 4.42) to 6-month FU (median RBD OD = 3.47) and remained at a similar median OD at the 12-month FU (median RBD OD = 3.48), Figure 3.2.4A. At a univariate level, these changes in RBD levels over time were not significant and also revealed no significances upon post-hoc analyses. However, the fluctuation in S1 were found to be significantly different over time at univariate level with $p = 0.026$ (Table 3.2.1). Most individuals showed a decrease in S1 OD units from initial recruitment (median S1 OD = 4.70) to 6-month FU (median S1 OD = 3.22) with post-hoc statistical analysis revealing a significant difference between the S1 OD units at initial recruitment and the 6-months FU visit ($p = 0.034$). At the 12-month FU, most patients had an increase in S1 OD (median S1 OD = 4.38) which was not statistically significant (Figure 3.2.4B).

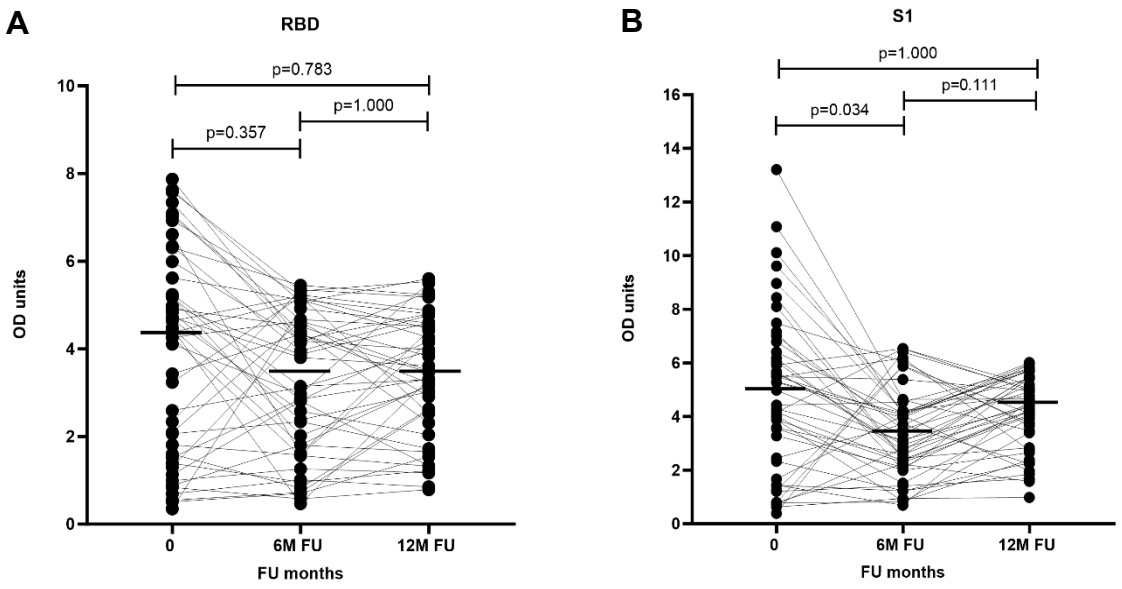

Figure 3.2.4: Change in patients' SARS-CoV-2 serology over the course of the FU period. A) RBD and **B)** S1 of each individual over the FU period with median indicated by the black solid lines, n = 46 per group. *p*-values are by Dunn's test adjusted with Bonferroni correction and each black circle represents an individual patient.

3.2.2.3 KSHV-related parameters

KSHV serology showed that K8.1 antibody levels decreased in most individuals from initial recruitment (median OD = 1.47) to 6-month FU (median OD = 0.69), followed by an increase at 12-month FU (median OD = 1.77). On a univariate level, the changes in K8.1 over the FU period (fluctuations in absorbance) were not significantly different over time with $p = 0.266$ (Table 3.2.1), neither was the post-hoc analysis (Figure 3.2.5A). Individual LANA results

showed most patients with a decrease in OD units between the initial recruitment (median OD = 3.06) and 6-month FU (median OD = 1.02), and a further decrease in OD units for most individuals between the 6- and 12-month FU (median OD = 0.84). Changes in LANA antibodies over time (decrease in OD) were significantly different at a univariate level with p <0.001 (Table 3.2.1). Additionally, after post-hoc analysis, there was a significant difference seen between the initial recruitment and 6-month FU group with a p <0.001 as well as between the initial recruitment and 12-month FU group with p <0.001 (Figure 3.2.5B).

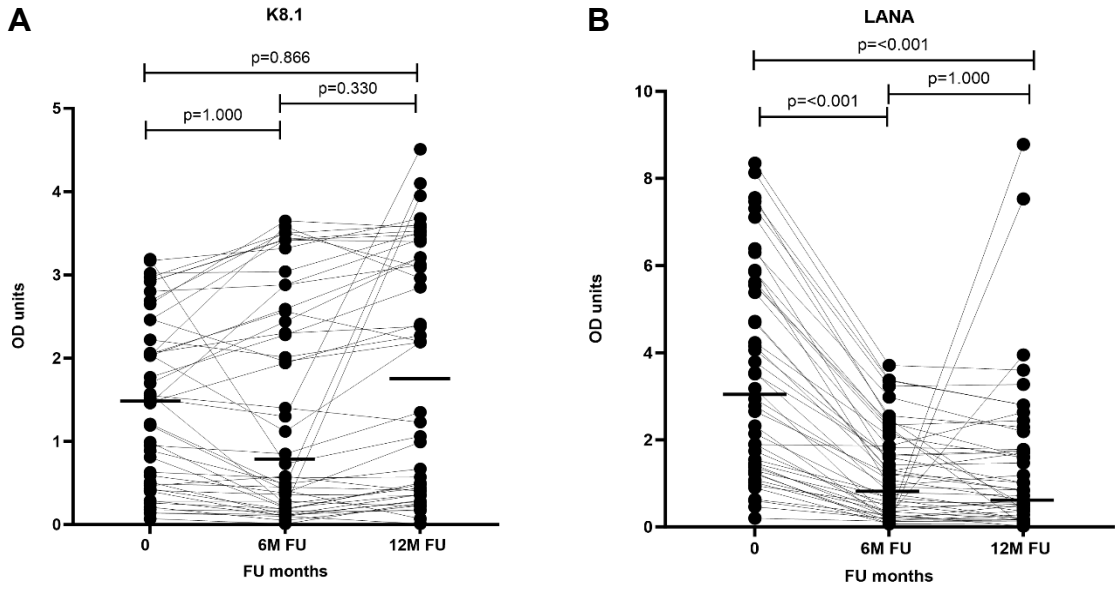

Figure 3.2.5: Change in patients' KSHV serology over the course of the FU period. A) K8.1 and **B)** LANA of each individual over the FU period with median indicated by the black solid lines, n = 46 per group. *p*-values are by Dunn's test adjusted with Bonferroni correction and each black circle represents an individual patient.

In addition to KSHV serology, KSHV VL was also determined in the patients over the 12-month FU period. As shown in Figure 3.2.6, the percentage of detectable KSHV VL decreased over the FU period, from an initial 20.9 % to 10.9 % at the 6-month FU and 4.7 % at the 12-month FU. However, the actual quantifiable VL went up at the 12-month FU time point (Table 3.2.1). At a univariate level, this relationship was not significantly different (p = 0.065); however, as mentioned above, a downward trend of individuals with detectable KSHV VL can be seen at the three time points.

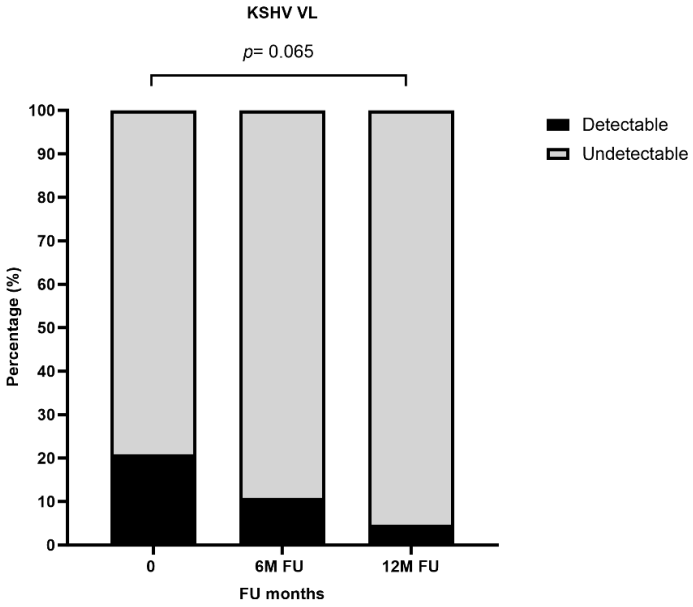

Figure 3.2.6: Percentage of patients with detectable and undetectable KSHV VL over the FU period (n = 46). *p*-values are by Chi-squared test.

3.2.2.4 Inflammatory markers

As mentioned above, absolute IL-6 concentrations did not change over the FU period, with the median level being 0 pg/ml for all three study visits, while individual patients displayed elevated levels following individual trends (Figure 3.2.7A). Similar results were also seen for CRP levels with the median CRP concentration staying at 4 mg/ml (Figure 3.2.7B).

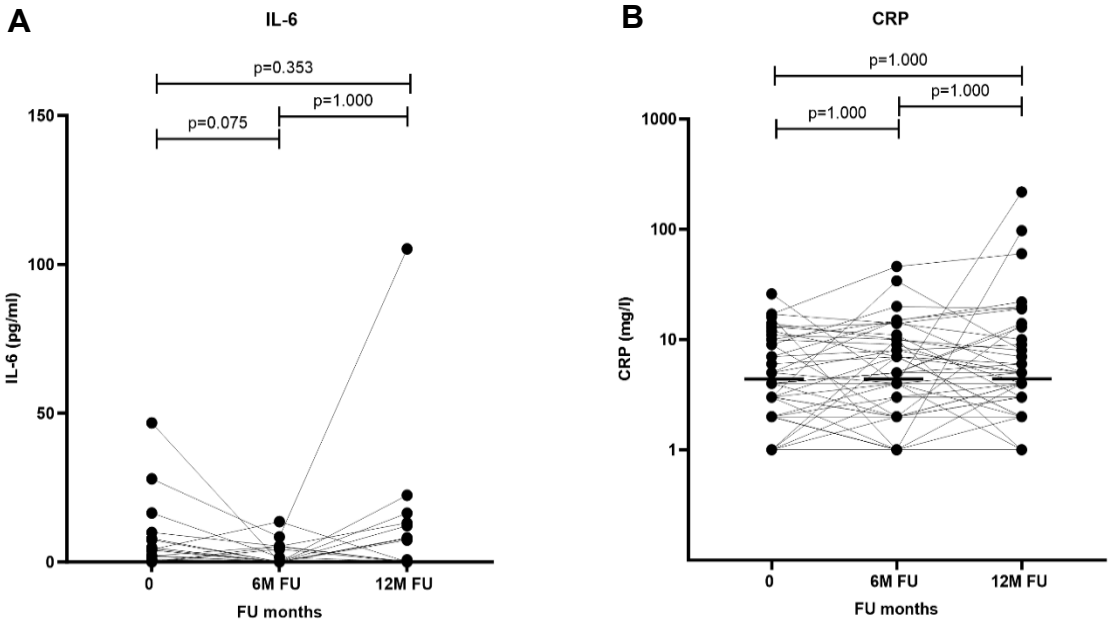

Figure 3.2.7: Change in patients' inflammatory parameters over the course of the FU period. A) CRP and **B)** IL-6 of each individual over the FU period with median indicated by the black solid lines, n = 46 per group. *p* values are by Dunn's test adjusted with Bonferroni correction and each black circle represents an individual patient.

Overall, and similar to the cross-sectional study presented in section 3.1, inflammation- associated markers did not change in the patient cohort over the FU period.

3.2.3 The impact of COVID-19 vaccination on clinical and virological parameters during the FU study period

As we observed a steady decrease in the number of patients with detectable KSHV VL over the FU period, which was different to the observed increase in the number of patients with detectable KSHV VL over the recruitment period in the cross-sectional study design described in section 3.1, we aimed to determine the impact of SARS-CoV-2 infection and/or COVID-19 vaccination on KSHV reactivation. We thus, as in section 3.1, divided the FU cohort (n = 46) into vaccinated and unvaccinated patients and analysed selected clinical and virological variables. The number of vaccinated patients was 20 (or 43.5 %), 31 (or 66.7 %) and 35 (or 76.1 %) at the initial recruitment, 6-month and 12-month FU time points, respectively. This steady increase in the number of vaccinated patients is depicted in Figure 3.2.8 and was found to be statistically significant (p = 0.004) (Table 3.2.1).

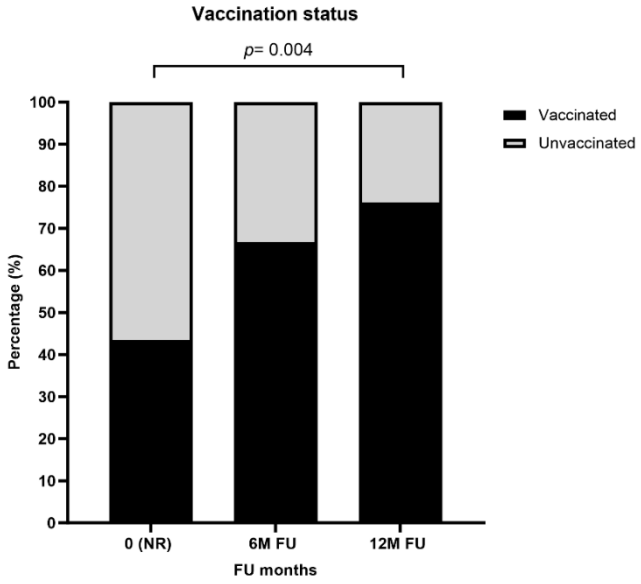

Figure 3.2.8: Percentage of patients that self-reportedly received the COVID-19 vaccine during the FU period (n = 46). p-values are by Chi-squared test.

After dividing the cohort into vaccinated and unvaccinated patients, we analysed the different trends seen between the FU groups within their vaccination status. Although no statistically significant differences in HIV-related parameters (HIV VL and CD4 count) over the FU study period in neither the vaccinated nor the unvaccinated groups were observed, there was an

overall decrease in HIV VL and increase in CD4 count, corroborating the trends seen for the entire FU cohort (Figure 3.2.3). In the vaccinated group, HIV VL median values for the patients at initial recruitment was 70.5 copies/ml which decreased to 49 copies/ml at both the 6- and 12-month FUs. This was different to the unvaccinated group, where HIV VL median values decreased consistently from initial recruitment which was 49 copies/ml to 19 copies/ml and then to 1 copy/ml at the 6- and 12-month FU, respectively (Figure 3.2.9A).

The median CD4 count in both groups (vaccinated and unvaccinated), were within a similar range at the different FU times with no significant associations between and within the groups (Figure 3.2.9B).

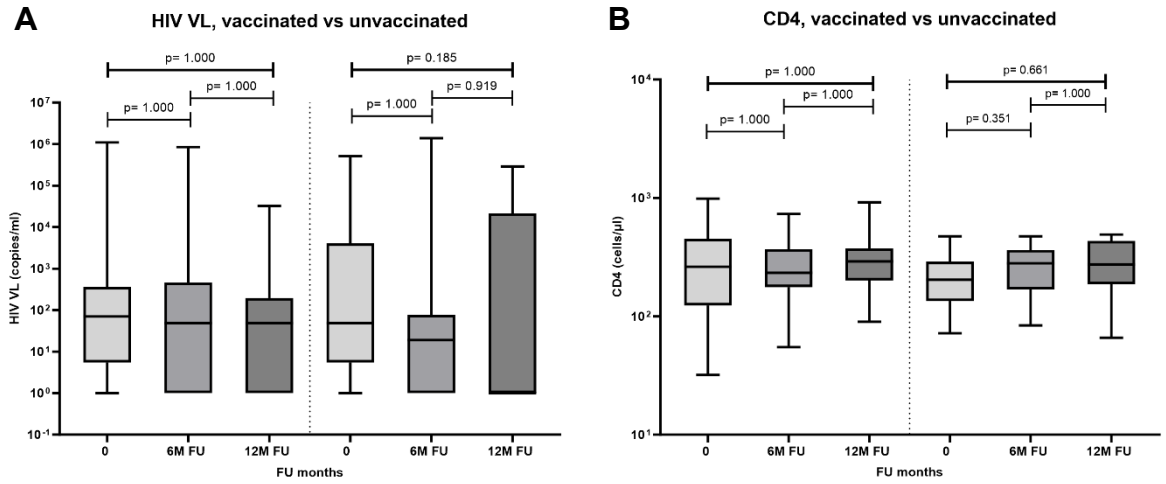

Figure 3.2.9: HIV-related parameters over the course of the FU period in vaccinated and unvaccinated patients. A) HIV VL and **B)** CD4 count of vaccinated (left) and unvaccinated (right) groups over the FU period with median, minimum and maximum values indicated within a box and whisker plot. Values are based on the number of individuals presenting at that specific time point. The percentage of individuals for these subgroups (0, 6-month and 12-month FU) can be seen in Figure 3.2.8, *p*-values are by Dunn's test adjusted with Bonferroni correction.

We then analysed SARS-CoV-2 serology and expectedly observed higher RBD and S1 OD values in the vaccinated groups compared to the unvaccinated groups. Interestingly, RBD levels decreased in the vaccinated patients from initial recruitment to the 6-month FU and then stayed at a similar level at the 12-month FU. The decrease in median RBD from initial recruitment to 6-month FU was significantly different ($p = 0.023$) and so was the median RBD from initial recruitment to the 12-month FU ($p = 0.020$). In the unvaccinated group, RBD levels showed a decrease from initial recruitment to 6-month FU and then increased from the 6- to the 12-month FU, thereby displaying a different trend to the vaccinated group. However, no significant differences were identified (Figure 3.2.10A).

With the OD units for S1 being generally higher than that of RBD, similar trends were seen in the vaccinated and unvaccinated groups compared to RBD. Again, a decrease from initial recruitment to 6-month FU in both the vaccinated and unvaccinated groups was observed, followed by an increase from 6- to 12-month FU in both groups. The difference between the median RBD values at initial recruitment and 6-month FU was shown to be statistically significant with a $p = 0.001$ for the vaccinated patients (Figure 3.2.10B).

Overall, the decreasing trend in SARS-CoV-2 antibodies seen in the vaccinated and unvaccinated groups from initial recruitment to the 6-month FU was likely due to antibodies starting to wane off after initial vaccination and/or natural infection, respectively. The increase in SARS-CoV-2 antibody levels seen from 6- to 12-month FU could be due to increased uptake of vaccinations, boosters, and/or acquisition of new infections as the period for this FU time point coincided with the 4th COVID-19 wave which was dominated by the omicron variant [177], one of the more virulent and transmissible SARS-CoV-2 variants (Figure 3.2.10).

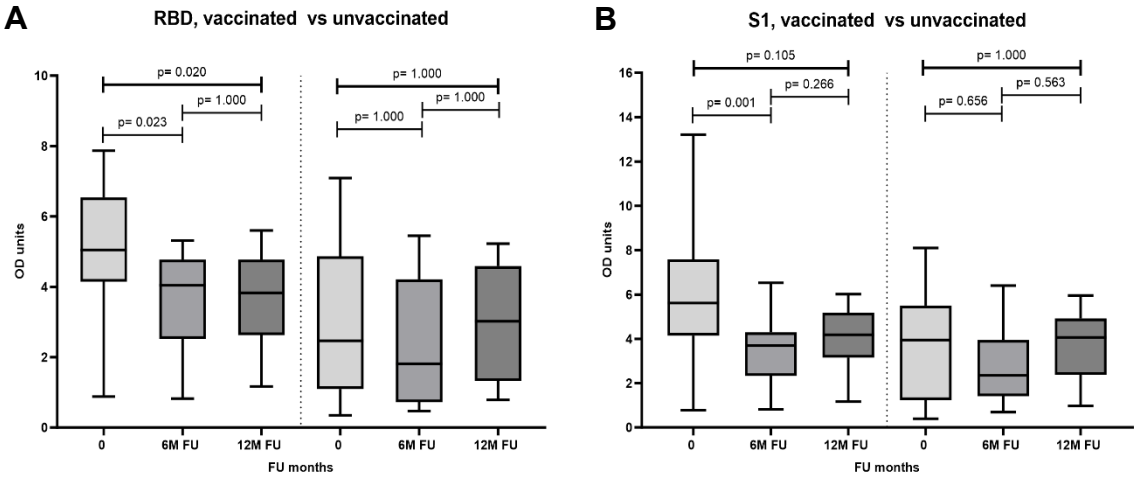

Figure 3.2.10: SARS-CoV-2 serology over the course of the FU period in vaccinated and unvaccinated patients. A) RBD and **B)** S1 of vaccinated (left) and unvaccinated (right) groups over the FU period with median, minimum and maximum values indicated within a box and whisker plot. Values are based on the number of individuals presenting at that specific time point. The percentage of individuals for these subgroups (0, 6-month and 12-month FU) can be seen in Figure 3.2.8, *p*-values are by Dunn's test adjusted with Bonferroni correction.

The KSHV antibody response revealed varying results for the lytic and latent proteins in both the vaccinated and unvaccinated groups. While both K8.1 and LANA antibodies decreased from initial recruitment to the 6-month FU visit, the antibody response to the lytic antigen K8.1 increased at the 12-months FU visit, while LANA serology remained low, independent of the vaccination status. Interestingly, absorbance values for K8.1 were generally lower in the unvaccinated compared to the vaccinated patients. However, these differences were not found to be significant. The decrease in LANA antibodies from initial recruitment to the 6- and

12-month FU visits, respectively, was found to be statistically significant with p <0.001. A similar trend was seen in the unvaccinated group; however, only the differences between the initial recruitment and 6-month FU was significant with a p = 0.014 (Figure 3.2.11).

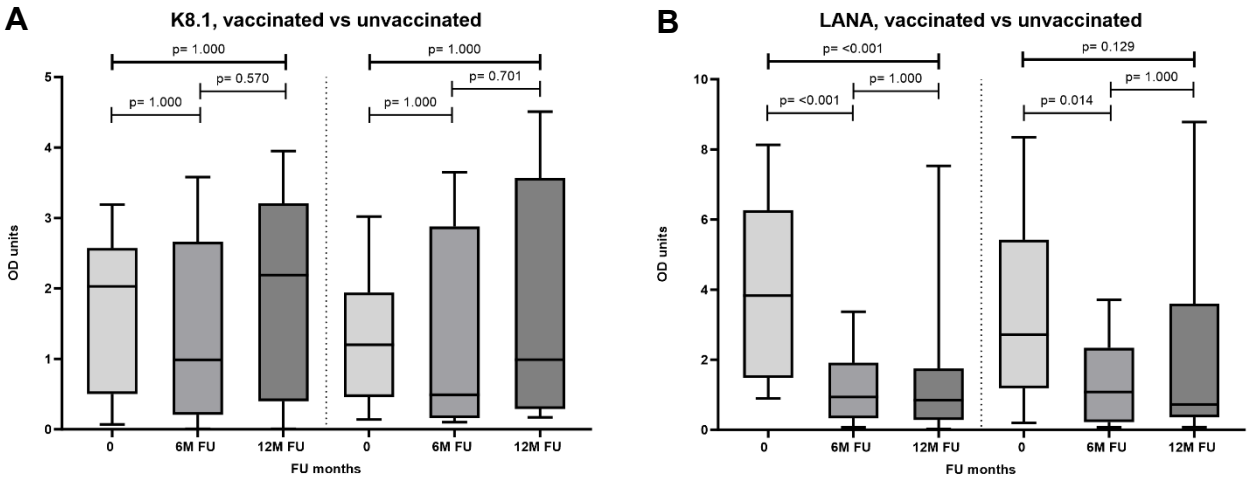

Figure 3.2.11: KSHV serology over the course of the FU period in vaccinated and unvaccinated patients. A) K8.1 and **B)** LANA of vaccinated (left) and unvaccinated (right) groups over the FU period with median, minimum and maximum values indicated within a box and whisker plot. Values are based on the number of individuals presenting at that specific time point. The percentage of individuals for these subgroups (0, 6-month and 12-month FU) can be seen in Figure 3.2.8, p-values are by Dunn's test adjusted with Bonferroni correction.

In addition to KSHV serology, the percentage of patients with detectable KSHV VL at the three study visits were also analysed, dependent on their COVID-19 vaccination status. In the vaccinated group, an overall decrease in the percentage of individuals with detectable KSHV VL from initial recruitment (15.8 %) to the 12-month FU (0 %) was observed. It should be noted that these percentages were calculated based on the number of individuals presenting at that specific time point. The percentage of individuals for these subgroups (0, 6-month and 12-month FU) can be seen in Figure 3.2.8. In the unvaccinated group, we see a different result: here, the percentage of individuals with detectable KSHV VL per specific time point was highest at initial recruitment (25.0 %) decreased at the 6-month study visit (13.3 %) and then increased again at the 12-month FU visit (22.2 %) (Figure 3.2.12). These differences were not significant but support the findings seen in section 3.1: vaccination seems to protect against KSHV reactivation while in the unvaccinated patients repeated SARS-CoV-2 exposure may cause reactivation of KSHV.

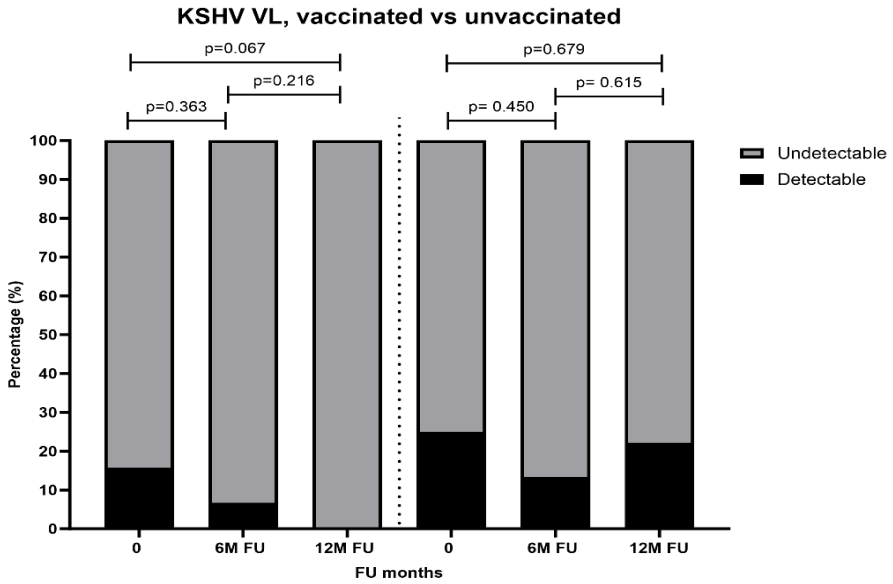

Figure 3.2.12: Percentage of patients with detectable and undetectable KSHV VL over the FU period in the vaccinated (left) versus unvaccinated (right) groups. These percentages were calculated based on the number of individuals presenting at that specific time point. The percentage of individuals for these subgroups (0, 6-month and 12-month FU) can be seen in Figure 3.2.8. *p*-values are by Dunn's test adjusted with Bonferroni correction.

When assessing the inflammatory parameters IL-6 and CRP between vaccinated and unvaccinated patients, no significant changes between the groups or between the study visit time points were observed. The median IL-6 levels for all groups (vaccinated and unvaccinated) at all FU categories was 0 pg/ml. Interestingly, more unvaccinated individuals presented with detectable IL-6 compared to the vaccinated group (Figure 3.2.13).

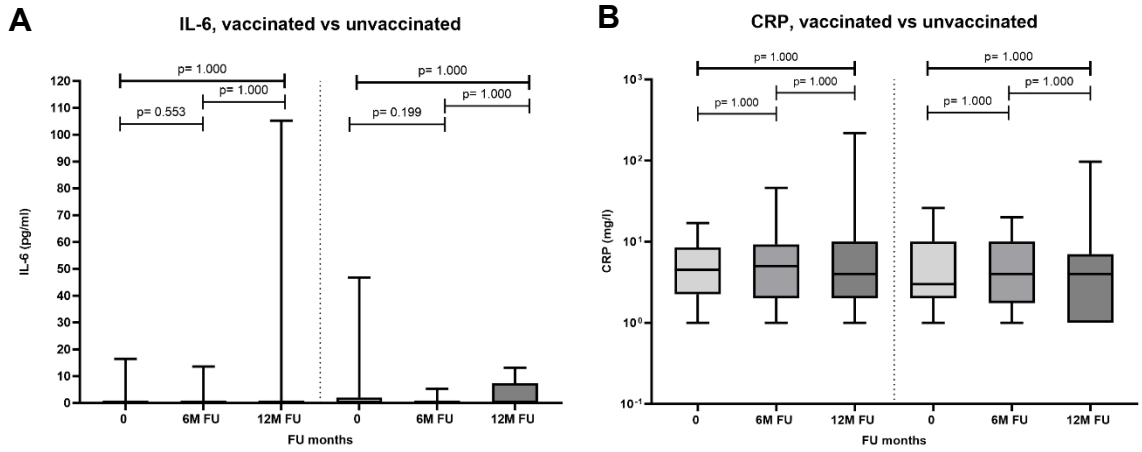

Figure 3.2.13: Inflammatory parameters over the course of the FU period in vaccinated and unvaccinated patients. A) IL-6 and **B)** CRP of vaccinated (left) and unvaccinated (right) groups over the FU period with median, minimum and maximum values indicated within a box and whisker plot. Values are based on the number of individuals presenting at that specific time point. The percentage of individuals for these subgroups (0, 6-month and 12-month FU) can be seen in Figure 3.2.8, *p*-values are by Dunn's test adjusted with Bonferroni correction.

Lastly, a cox regression model was used to assess the probability of having detectable KSHV over the FU period stratified by vaccination status. The cox regression model as shown in Figure 3.2.14 suggests that unvaccinated patients have a higher probability of KSHV detection over time compared to vaccinated patients. In other words, the chances of KSHV reactivation (detectable KSHV VL) are higher in the unvaccinated group than the vaccinated group (Figure 3.2.14).

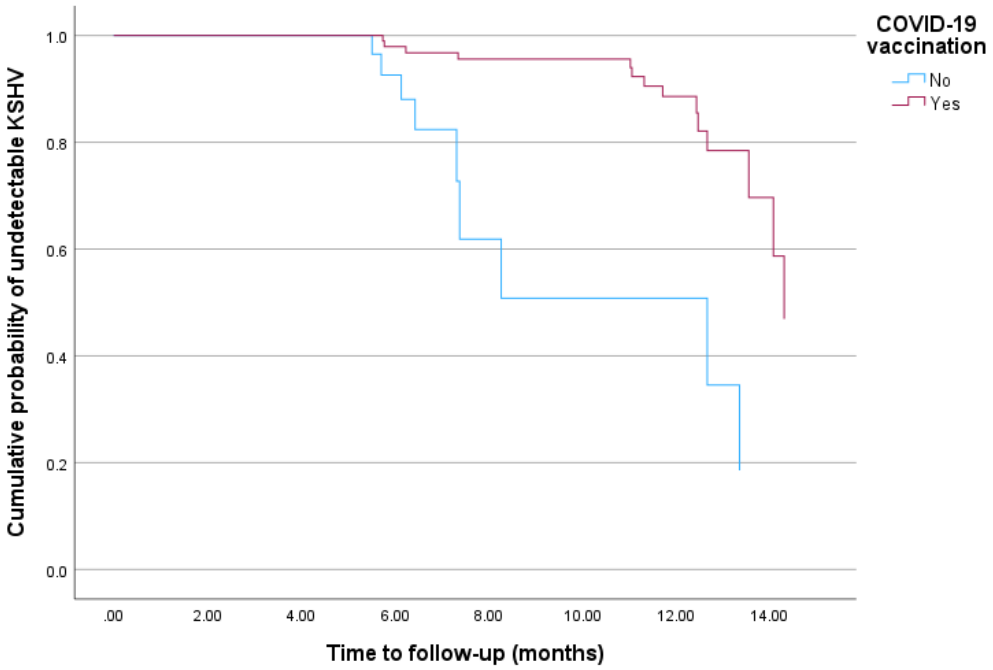

Figure 3.2.14: Cox regression curves of KSHV VL detection of the FU dataset stratified by COVID-19 vaccination status of the FU period.

In addition, we calculated the measure for risk as expressed by the hazard ratio (HR), with an HR of 1 indicating that the risk is the same for each patient. Our analysis to determine the HR between selected variables and the survival function (i.e. KSHV VL undetectability) included RBD IgG OD, age, absolute CD4 count, lymphocyte count and LANA serology (OD). As shown in Table 3.2.2, the HR for RBD was >1, which indicates a positive association with the event probability. In other words, this indicates that with a higher RBD OD there is an increase in the risk of detectable KSHV VL (p = 0.006). The HR for age and absolute CD4 was found to be around 1, indicating no effect on the event probability. Conversely, for lymphocyte count and LANA serology the HRs were <1, indicating a reduction in the hazard. However, this association was not significant.

Table 3.2.2: Cox regression analysis in the FU data set.

Characteristics	HR	95% CI	*p*
RBD IgG OD	1.884	1.200 – 2.97	0.006*
Age	1.017	0.974–1,061	0.448
Absolute CD4 count	1.002	0.999 – 1.004	0.238
Lymphocyte count	0.391	0.104 – 1.472	0.165
LANA OD	0.967	0.606 – 1.543	0.888

In summary, this FU study revealed a decrease in the number of individuals with detectable KSHV VL in the vaccinated group throughout the FU period while in the unvaccinated group there was a decrease initially in KSHV detectability, but this increased between the 6- and 12-month FU period. In addition, SARS-CoV-2 serology increased between the 6- and 12-month FU visits in both the vaccinated and unvaccinated patients. The relationship between KSHV detectability and SARS-CoV-2 responses in the unvaccinated group was confirmed in our cox regression model where a higher risk of KSHV detectability (reactivation of KSHV) in response to SARS-CoV-2 infection (increase in RBD titres) was identified. This relationship was not seen for the vaccinated patients.

3.3 Persistent viremia in a 34-year old HIV-infected male over a 2-year period during the COVID-19 pandemic: A case report

In sections 3.1 and 3.2, we reported on the impact of SARS-CoV-2 infection on the reactivation of KSHV in a cohort of 407 patients, using a cross-sectional and a FU study design, respectively. In the context of the results obtained for the entire cohort, we identified one case with an unusually high KSHV VL early in the recruitment phase, before national roll-out of COVID-19 vaccinations. This particular patient, who was designated as GUG116 in the patient cohort, was invited for FU visits every 6 months for a total of 2 years and will be presented in more detail in this section.

3.3.1 Patient demographics and medical history in the context of HIV treatment

At the time of enrolment, patient GUG116 was a 34-year-old black male, living in an urban area in the Western Cape, SA, with a confirmed HIV diagnosis since May 2020. He had been a regular patient at the Gugulethu Community Health Centre Antiretroviral clinic (Desmond Tutu HIV Centre, UCT) before his enrolment into this study on the 26 May 2021. His past medical history revealed that he was diagnosed with TB in 2015, which was unlikely to be HIV-related. The patient did not start ART upon HIV diagnosis in May 2020 as he was jailed for a few months. Instead, he started his ART regime Tenofovir/Emtricitabine/Efavirenz (TDF/FTC/EFV) in November 2020. At the time of diagnosis, the patient was categorised under WHO stage 2 and was clinically well. In March 2021, the patient had an HIV VL <20 copies/ml.

In May 2021, when the patient was enrolled into the study, he was still classified under HIV WHO stage 2 and had a HIV VL of <20 copies/ml and a CD4 count of 221 cells/µl which fitted the inclusion criteria for the study (<350 cells/µl). In November 2021, when the patient presented for his 6-month FU, the patient's HIV VL was <50 copies/ml, and he was doing clinically well. His ART regimen, however, changed to Tenofovir/Lamivudine/Dolutegravir (TDF/3TC/DTG) in July 2021 due to a change in national ART guidelines. In February 2022, he missed his general hospital visit as he was noted to be jailed again. In May 2022, the patient was diagnosed with TB, and in June 2022, he presented for his 12-month FU to the clinic where his ART regimen changed to Abacavir/Lamivudine/Dolutegravir (ABC/3TC/DTG) due to abnormal renal function indicated by high creatinine levels (Table 3.3.1). Since then, the patient has remained on this regimen. He was noted to not be taking his TB medication and was sent again to start TB treatment. His HIV VL remained suppressed. In October 2022, he

was noted to have missed a few months of ART. By December 2022 he completed his TB treatment course. In March 2023, the patient presented for FU (22 months after initial recruitment); his HIV VL had increased to 61 copies/ml and at this time a lump was noted on his chest. In April 2023, the patient's HIV VL was <50 copies/ml. In addition, he was put on TB treatment for one year as nodes on an abdominal ultra-sound were identified. In August 2023, the patient presented for his final FU for this study at 27-months where his HIV VL was recorded as 347 copies/ml. As of February 2024, this patient is still in care and presenting at the clinic for regular check-ups (Figure 3.3.1).

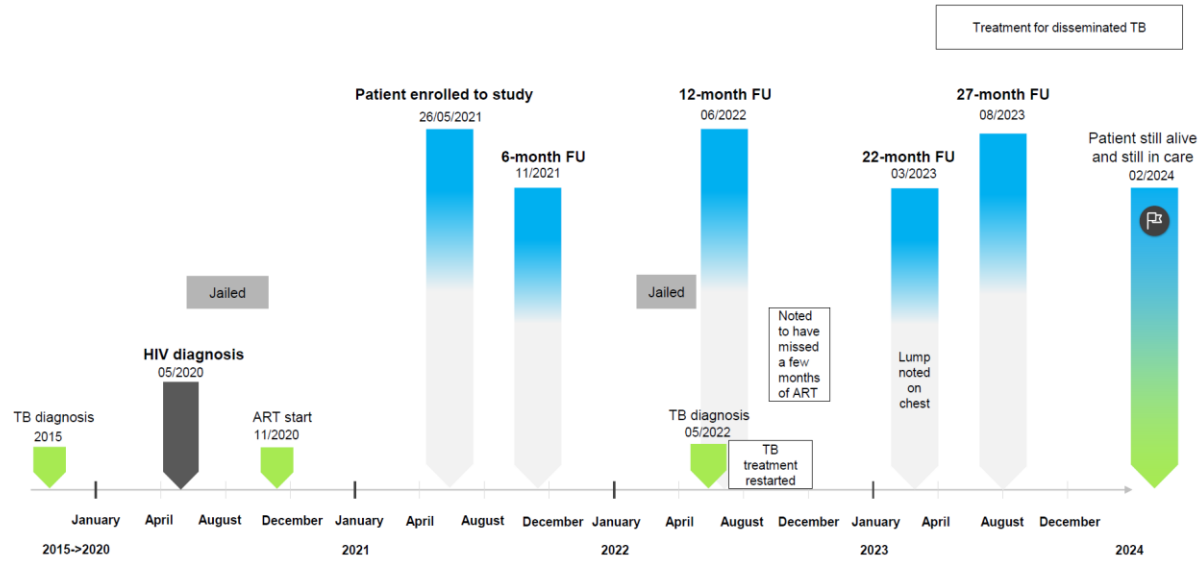

Figure 3.3.1: Medical history and course of events occurring in patient GUG116 from 2015 to 2024.

3.3.2 Clinical and virological parameters in the context of this study

3.3.2.1 Patient enrolment and laboratory results

At the time of enrolment in May 2021 and at subsequent FU visits of this patient, clinical examinations were conducted. Patient GUG116 was invited for FU visits at 6-, 12-, 18- and 24-months but had missed the correct later time points and presented at 22- and 27-months instead. Initially, the patient reported no symptoms; neither signs of an altered mental state nor radiographic abnormalities were recorded. At presentation, this individual had no symptoms associated with either KSHV or SARS-CoV-2 infection. Moreover, this patient reported no administration of the COVID-19 vaccination at presentation nor during the time of FU visits.

As described in section 2.2.3, peripheral blood was analysed by the NHLS on the day of presentation. This included absolute CD4 count, HIV VL, blood chemistry (sodium and creatinine concentrations), albumin, ALT and CRP concentrations, full blood count (haemoglobin, haematocrit, platelet, mean corpuscular haemoglobin, mean corpuscular volume, red and white cell count) and differential cell count (neutrophils, lymphocytes, monocytes, eosinophils, basophils). Table 3.3.1 presents all study-relevant variables throughout the FU period. Of note were the initial sodium blood chemistry of 135 mmol/l which was below the normal range, absolute CD4 count of 221 cells/µl (which was below the normal range but fell within the criteria of recruitment to the study), elevated CRP levels of 16 mg/l, and suppressed HIV VL of <20 copies/ml.

In November 2021, the patient presented for the 6-month FU and was doing clinically well; his weight was 61.6 kg. At this point, the patient presented with a few symptoms as indicated in Table 3.3.1, namely weight loss, night sweats, arthralgia, myalgia and respiratory symptoms/coughing. Blood analysis results showed a considerable decrease in platelet count to 184 $x10^9$/l and a decrease in the absolute CD4 count to 201 cells/µl, while HIV VL remained suppressed (Table 3.3.1). Thereafter, the patient was noted to be jailed in February 2022 during which time he missed his routine clinical visits. At the 12-month FU in June 2022, the patient weighed 43 kg, a substantial decrease in weight since his previous FU visit, and he presented with additional symptoms (Table 3.3.1). These additional symptoms, such as altered mental state, respiratory symptoms/coughing and cachexia could possibly be attributed to the patient's imprisonment during which he missed his clinical check-up. The patient had a negative TB diagnosis at the time of the visit but his symptoms at presentation led to the re-initiation of TB treatment. Blood analysis results showed that the patient's sodium concentration increased to 140 mmol/l and creatinine levels and platelet count increased notably to 115 µmol/l and 275 $x10^9$/l, respectively (Table 3.3.1). In March 2023, when the patient presented for FU at 22 months after initial recruitment, his HIV VL had increased to 61 copies/ml and he was now categorised under WHO stage 3, likely due to the patient missing a few months of ARTs and to the patient's clinical TB diagnosis. He presented with additional symptoms which included neuropathy (with pain) which was post TB treatment. At this stage, still with a negative TB diagnosis, the patient presented with a lump on the chest. Blood analysis results showed creatinine levels increased further to 175 µmol/l whereas his platelet count decreased to 245 $x10^9$/l. However, the patients' weight increased since the 12-month FU to 54 kg, and there was a considerable increase in absolute CD4 count to 359 cells/µl (Table 3.3.1).

At the 27-month FU in August 2023, the patient's weight had decreased again to 45 kg. At this time point, the patient has been positively diagnosed with TB where nodes were identified on

an abdominal ultra-sound. Blood analysis results showed his creatinine levels decreased slightly to 150 µmol/l and his platelet count decreased considerably to 162 $x10^9$/l. In addition, the patient's absolute CD4 count decreased notably to 143 cells/µl and his HIV VL increased substantially to 347 copies/ml (Table 3.3.1).

Taken together, these clinical and virological parameters indicate a continuous increase in the patient's symptoms, particularly respiratory-related symptoms as well as weight fluctuations. Sodium levels of the patient were within the same range throughout FU visits, but creatinine levels increased substantially. In addition, several missed clinical visits due to imprisonment resulted in the patient not receiving ARTs during that time which might have contributed to his weight fluctuations as well as his symptoms at presentation.

Table 3.3.1: Clinical and virological parameters of a 34-year-old male patient presenting with increasing KSHV VL. Abnormally low/high values are indicated in bold: sodium (<136 mmol/l); creatinine (>104 µmol/l); absolute CD4 count (<350 cells/µl); elevated KSHV VL (>100 copies/10^6 cells); elevated EBV VL (> median of entire cohort = 5580.4 copies/10^6 cells); elevated IL-6 (>1.8 pg/ml); elevated CRP (>10 mg/l). *Lower than detectable limit.

		Follow-up (months)				
		0	**6**	**12**	**22**	**27**
General:						
WHO stage		2	2	2	3	3
ART regimen		TDF/FTC/EFV	TDF/3TC/DTG	ABC/3TC/DTG		
Weight (kg)		No info	61.6	43	54	45
TB diagnosis		No	No	Yes	Yes	Yes
COVID-19 vaccination		No	No	No	No	No
Symptoms at presentation (self-reported)		None	Respiratory symptoms/coughing, weight loss, night sweats, arthralgia, myalgia	Fatigue, oedema, cachexia, respiratory symptoms/coughing, gastrointestinal disturbance, altered mental state, weight loss, night sweats, neuropathy, radiographic abnormalities, arthralgia, myalgia	Oedema, cachexia, gastrointestinal disturbance, weight loss, night sweats, arthralgia, myalgia, neuropathy with pain (post TB treatment)	Respiratory symptoms/coughing, night sweats, arthralgia, myalgia
Laboratory blood analysis (chemical pathology and haematology):						
Sodium (mmol/l)		**135**	136	140	138	**135**
Creatinine (µmol/l)		74	76	**115**	**175**	**150**
Platelet count (x10^9/l)		269	184	275	245	162
Absolute CD4 (cells/µl)		**221**	**201**	**200**	359	**143**
Virology (serology and VL):						
HIV VL (copies/ml)		<20	<50	1*	61	347
SARS-CoV-2 serology (OD units)	RBD	0.35	4.92	4.59	4.90	5.19
	S1	0.39	6.40	5.05	6.21	5.74

KSHV serology (OD units)	K8.1	2.65	4.74	4.56	4.69	4.66
	LANA	6.54	6.48	6.54	6.50	7.75
EBV serology (OD units)		2.38	1.73	1.46	1.37	1.49
KSHV VL (copies/10^6 cells)		**189946.3**	**325019.4**	**83107.2**	**20434.6**	**1278350.5**
EBV VL (copies/10^6 cells)		**6450.0**	**10800.0**	1120.0	1730.0	3000.0
Inflammatory markers:						
IL-6 (pg/ml)		**1.96**	**5.28**	**13.11**	**14.99**	**21.83**
CRP (mg/l)		**16**	1	**97**	**44**	**136**

3.3.2.2 HIV-related parameters

In terms of HIV variables, we observed some fluctuation throughout the FU period, with HIV VL staying relatively low (either just above or below suppressed VL levels around <50 copies/ml) for 22 months but increased to 347 copies/ml at 27 months which was still defined as low-level viremia (<1000 copies/ml) (Figure 3.3.2 A). CD4 count levels stayed relatively low (<221 cells/µl) up to the 12-month FU, increased to 359 cells/µl at the 22-month FU and then decreased to below 200 cells/µl at 27-month FU, indicating immunosuppression (Figure 3.3.2 B).

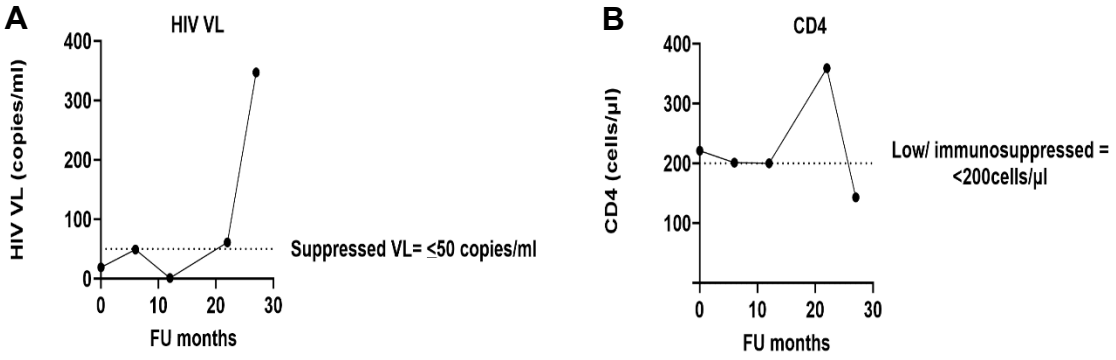

Figure 3.3.2: HIV-related variables of patient GUG116 throughout the 2-years FU period. **A)** HIV VL, dotted line indicates VL = 50 copies/ml. For graphical purposes, we recorded HIV VL lower than detectable limit as 1 copy/ml, <20 as 19 copies per ml and <50 as 49 copies/ml. **B)** Absolute CD4 count, dotted line indicates CD4 = 200 cells/µl.

3.3.2.3 Inflammatory markers

Although no striking observations were reported for the inflammatory markers IL-6 and CRP in the entire cohort (sections 3.1 and 3.2), patient GUG116 showed some interesting trends. At the initial recruitment, the patient's IL-6 concentration was 1.96 pg/ml whereas his CRP concentration was 16 mg/l, both being marginally elevated. At the 6-month FU, the IL-6 concentration had increased since initial enrolment and followed this upward trend at each subsequent time point, reaching 21.83 mg/l at the end of the study period (Figure 3.3.3A and Table 3.3.1). Overall, IL-6 levels showed a consistent increasing trajectory throughout the FU period where levels were clearly above the threshold for elevated IL-6 (1.8 pg/ml), with the exception of the 6-month FU time point. The trend for CRP concentrations throughout the study period was slightly different: while there was a decrease at the 6-month FU, the CRP levels at the 12-month FU had increased quite considerably. At the 22-month FU, CRP had decreased again, with the last FU at 27-month indicating a considerable increase in CRP levels to 136 mg/l (Figure 3.3.3B and Table 3.3.1).

Taken together, both inflammatory markers IL-6 and CRP displayed an increasing trend throughout the FU period (Figure 3.3.3).

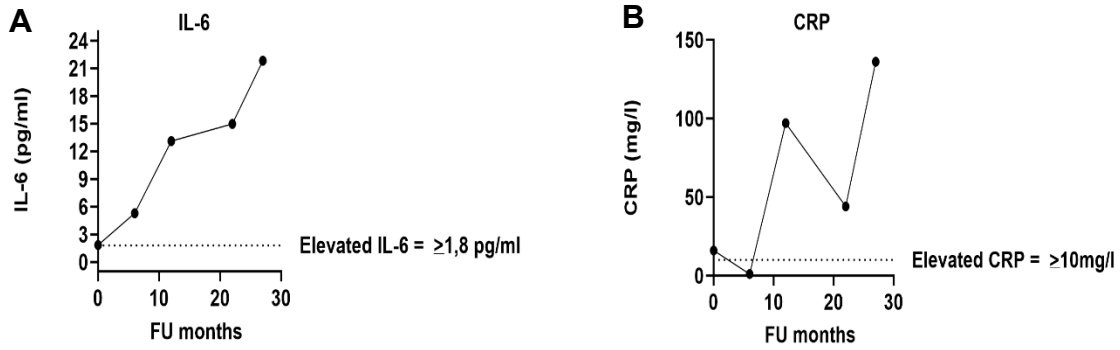

Figure 3.3.3: Inflammatory markers of patient GUG116 throughout the 2-years FU period. A) IL-6 plasma levels, dotted line indicates IL-6 = 1.8 pg/l; **B)** CRP levels, dotted line indicates CRP = 10 mg/l.

3.3.2.4 Co-infections with SARS-CoV-2, KSHV and EBV

SARS-CoV-2

This patient was enrolled into this study before the national roll-out of COVID-19 vaccinations in SA and self-reportedly never received a vaccine throughout the study period. He reported some unspecific symptoms at presentation (Table 3.3.1) which may have been COVID-19-related but could have overlapped with the symptoms of other chronic diseases such as HIV/AIDS and/or TB.

The patient's SARS-CoV-2 serology was negative at the time of enrolment (RBD = 0.35 and S1 = 0.39 OD units). These values were below the cut-off value for the entire cohort which was set at 1 (section 2.7). However, at the 6-month FU we saw a considerable increase in SARS-CoV-2 antibodies (RBD = 4.92 and S1 = 6.40 OD units). Thereafter, antibody levels stayed consistently high throughout the FU period (at 12 months, RBD = 4.59 and S1 = 5.05 OD units; at 22 months, RBD = 4.90 and S1 = 6.21 OD units; and at 27 months, RBD = 5.19 and S1 = 5.74 OD units) (Table 3.3.1). Interestingly, RBD and S1 OD values were found to be lower than the median value for the whole cohort (n = 407) at the time of enrolment; thereafter, antibody levels increased and stayed well above the median value for the whole cohort throughout the study period (Figure 3.3.4). These high levels of antibodies can be attributed

to natural SARS-CoV-2 infection(s), with the first infection occurring between recruitment and 6-month FU, as this individual had self-reportedly not received the COVID-19 vaccine.

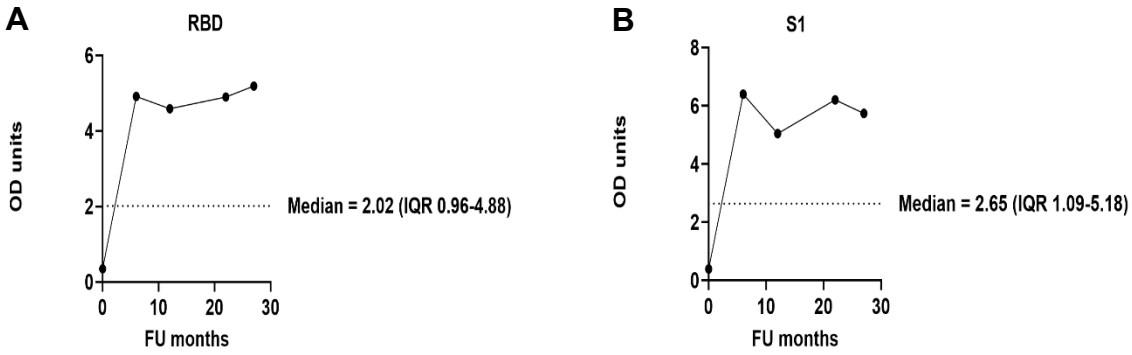

Figure 3.3.4: SARS-CoV-2 serology of patient GUG116 throughout the 2-year FU period. A) RBD and **B)** S1 IgG antibody levels. The dotted line indicates median with IQR for the whole cohort (n = 407).

KSHV

KSHV serology and KSHV VL in the patient's peripheral blood was determined by ELISA and qPCR, respectively. Compared to the median KSHV antibody levels in the entire cohort, patient GUG116 displayed considerably higher K8.1 and LANA levels at enrolment of 2.65 and 6.54 OD units, respectively. Thereafter K8.1 increased to OD units = 4.74 at the 6-month FU whereas LANA stayed at a similar level, OD units = 6.48. Both K8.1 and LANA then stayed relatively constant throughout the entire study period (Table 3.3.1 and Figure 3.3.5A and B). Overall, both K8.1 and LANA serology of patient GUG116 remained highly increased above the assay cut-off and considerably above the median value of the entire cohort.

These observations were further supported by the patient's KSHV virology. Compared to the entire cohort where blood KSHV VL was mostly either undetectable (79.0 %) or detectable but not quantifiable (21.0 %, see also section 3.1, Table 3.1.1), patient GUG116 stood out by displaying a very high KSHV VL of 189946.3 copies/10^6 cells at recruitment. At the 6-month FU, his KSHV VL almost doubled to 325019.4 copies/10^6 cells. Although he displayed some fluctuations in his KSHV VL throughout the study period, it remained substantially higher than the remainder of the cohort and was clearly above the threshold for elevated KSHV VL of 100 copies/10^6 cells as defined previously [8, 98]. The patient's KSHV VL were 83107.2, 20434.6 and 1278350.5 copies/10^6 cells at the 12-, 22- and 27-month FU, respectively (Table 3.3.1 and Figure 3.3.5C).

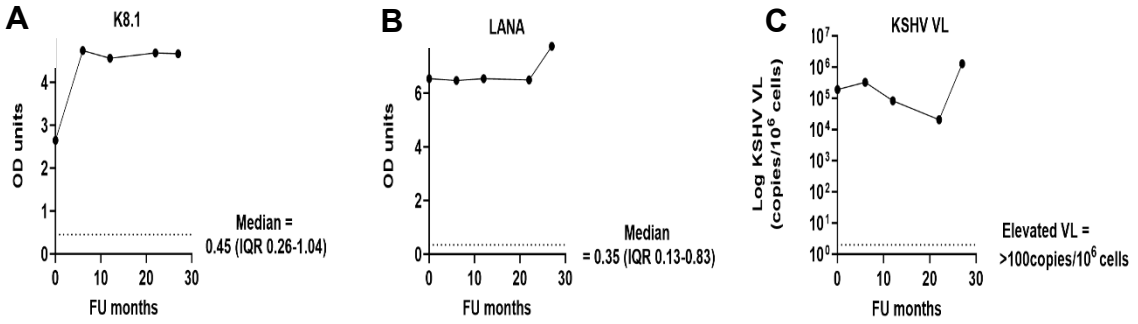

Figure 3.3.5: KSHV-associated variables of patient GUG116 throughout the 2-year FU period. A) and **B)** KSHV serology of K8.1 and LANA, respectively, dotted lines indicate median with IQR of the entire cohort. **C)** KSHV VL, dotted line indicates the threshold for elevated KSHV VL = 100 copies/10^6 cells.

EBV

In addition to KSHV-related parameters, we also determined the related oncogenic gammaherpesvirus EBV serology and VL. When assessing the serology of EBV nuclear antigen (EBNA) through ELISA, we found that patient GUG116 EBNA levels were slightly below the median of the entire cohort (median = 2.56) (unpublished data, kindly provided by Prishanta Chinna) with 2.38 OD units at initial recruitment. EBNA levels subsequently decreased further at the 6-, 12-, 22- and 27-month FU time points with OD units = 1.73, 1.46, 1.37 and 1.49, respectively, thereby remaining below the median value of the entire cohort throughout the study period (Figure 3.3.6A).

In addition, the patient's EBV VL were analysed. Compared to the median of the entire cohort (5580.4 copies/10^6 cells) (unpublished data, kindly provided by Prishanta Chinna), patient GUG116 had an EBV VL of 6450.0 copies/10^6 cells at inital recruitment. At the 6-month FU, his EBV VL almost doubled to 10800.0 copies/10^6 cells but decreased at 12-month FU to 1120.0 copies/10^6 cells and was 1730.0 copies/10^6 at 22-month (below the median of the entire cohort). At the 27-month FU, the EBV VL was found to have increased to 3000.0 copies/10^6 cells (Table 3.3.1 and Figure 3.3.6B). Despite these fluctuations throughout the recruitment period, it is important to note that the patient's EBV VL showed no striking differences compared to the remainder of the cohort, these fluctuations also occurred around the median EBV VL of the entire cohort (5580.4 copies/10^6 cells).

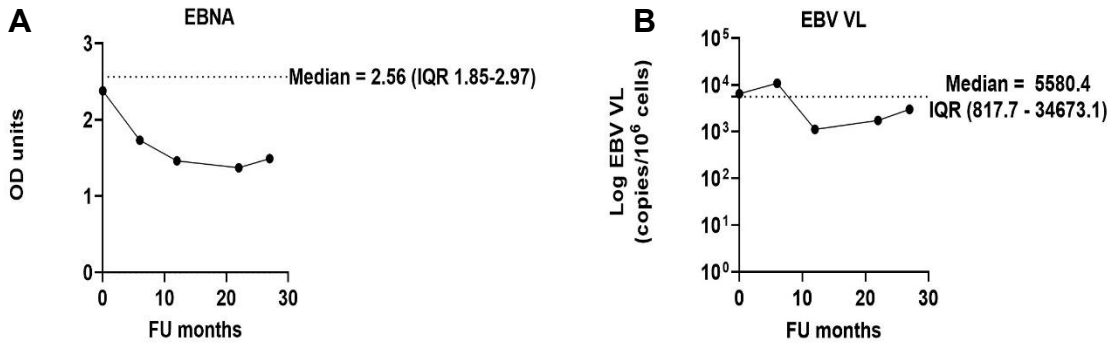

Figure 3.3.6: EBV-associated variables of patient GUG116 throughout the 2-year FU period. A) EBNA serology, dotted line indicates median with IQR of the entire cohort. **B)** EBV VL, dotted line indicates median with IQR of the entire cohort.

Taken together, SARS-CoV-2 serology of patient GUG116 was initially lower than the median value for the whole cohort at the time of enrolment but increased by the 6-month FU time point (indicating SARS-CoV-2 infection in the absence of COVID-19 vaccination) and stayed well above the median value of the whole cohort throughout the study period. KSHV showed elevated VL levels which remained well above the median while EBV VL fluctuated around the median of the entire cohort throughout the study period. In addition, while KSHV serology (both K8.1 and LANA) was found elevated, EBV serology (EBNA) remained below the median value of the remainder of the cohort.

3.3.3 Concluding remarks

Taken together, patient GUG116 was a unique case in the cohort as he presented with an unusually high KSHV VL early in the recruitment phase. He exhibited persistent KSHV viremia over the 27-month FU period and displayed an increase in severity of other clinical and virological parameters as well as inflammatory markers. The increased KSHV VL was also accompanied by elevated KSHV serology and increased SARS-CoV-2 antibodies indicating infection(s) (in the absence of COVID-19 vaccination), while EBV VL and EBV serology remained at or below median cohort levels, respectively. While the patient's living circumstances, his frequent non-adherence to his HIV/TB treatment and malnutrition may be the reason for his uncontrolled viremia (see section 3.1 and [92]), other underlying infections and specifically (repeated) SARS-CoV-2 infection may have played contributing roles. Cumulatively, this data emphasises the importance of further understanding the role of co-infections especially in the context of KSHV reactivation and ultimately KSHV-related malignancies in individuals presenting with unspecific symptoms and high KSHV VL.

4. Discussion

Since the emergence of SARS-CoV-2, the virus that caused the COVID-19 pandemic globally in 2019, the question of whether exposure to the virus had a long-term impact on pre-existing pathologies has remained. In the global South, the COVID-19 pandemic occurred within the context of a longstanding HIV epidemic, adding an additional burden to already vulnerable populations. In SA specifically, HIV-infected people account for approximately one fifth of PLWH globally [178, 179].

SARS-CoV-2 infection has been suggested to contribute to the reactivation of latent herpesviruses, such as KSHV. Although KSHV seroprevalence varies greatly based on geography, the overall seroprevalence in SSA is between 40 - 50 % [15, 36] and is particularly high in the context of HIV co-infection [2, 17], highlighting KSHV's relevance in the infectious disease landscape in this region.

Research in our laboratory has primarily focused on SARS-CoV-2 infection in critically ill hospitalised patients, showing an interesting association between the presence of KSHV VL in patients' blood and mortality from COVID-19 [28]. Some case studies have similarly suggested an interplay between KSHV reactivation and COVID-19 in HIV patients with low CD4 counts [157, 158]. Lytic reactivation of herpesviruses like EBV, HCMV and HHV-6 in hospitalised patients in response to SARS-CoV-2 infection have also been reported [25, 27, 28, 152, 154]. However, these studies showed correlations but could not prove causation. The question whether SARS-CoV-2 triggers KSHV reactivation or if KSHV directly exacerbates SARS-CoV-2-associated pathogenesis by means of the" cytokine storm" requires further investigation.

As it is currently unknown what the impact of SARS-CoV-2 exposure on HIV/KSHV co-infected non-hospitalised individuals is, we performed both a longitudinal cross-sectional observational study and a FU study on a cohort of non-hospitalised HIV-infected patients. Limited implementation of social distancing measures and non-pharmaceutical interventions resulted in high exposure to SARS-CoV-2 infection from the very beginning and throughout the COVID-19 pandemic. Importantly, this unique cohort comprised COVID-19 unvaccinated and vaccinated patients as the roll-out and steady uptake of vaccinations in SA overlapped with the study period.

4.1 The contribution of SARS-CoV-2 to the reactivation of KSHV in non-hospitalised HIV-infected patients – a cross-sectional study

From the beginning of patient recruitment and enrolment into the study in October 2020, we observed a high seroprevalence of SARS-CoV-2 infection of >75 %, which remained high and continued to further increase due to subsequent waves as well as vaccination roll-out, (Figure 3.1.2).

The overall KSHV seroprevalence of this study cohort was >50 % supporting seroprevalences of 30 – 50 % previously reported in other studies in SA [19, 28, 180, 181] and SSA [182].

To determine whether high SARS-CoV-2 exposure had an impact on reactivation of KSHV in this cohort, we measured KSHV VL, which served as a proxy for KSHV lytic reactivation. Initially, results revealed very few patients with detectable KSHV VL; however, from mid-2022, gradual increases in individuals with detectable KSHV VL was observed (Figure 3.1.3). Interestingly, this occurred two years after the COVID-19 pandemic started and one year after the roll-out of vaccinations in SA. These results led to the hypothesis that the increase in the number of patients with KSHV VL could be due to repeated SARS-CoV-2 infections throughout the pandemic and/or due to increased uptake of vaccinations. Interestingly, our results revealed that only unvaccinated patients with detectable KSHV VL showed significantly increased SARS-CoV-2 RBD antibody levels compared to patients without detectable KSHV VL. This phenomenon was not seen in SARS-CoV-2 vaccinated patients. In vaccinated patients, SARS-CoV-2 RBD antibody levels were high in individuals with both detectable and undetectable KSHV VL, supporting a previous study whereby increased SARS-CoV-2 antibodies were reported in response to vaccine-induced immunity [183].

Further logistic regression revealed a statistically significant association between SARS-CoV-2 RBD levels and the presence of KSHV VL in the unvaccinated but not the vaccinated group (Table 3.1.3). This suggests that unvaccinated patients with high SARS-CoV-2 antibody titres (as a proxy for previous SARS-CoV-2 infection(s)) have a higher risk of KSHV reactivation (as measured by detectable KSHV VL in the blood) compared to vaccinated patients who are protected from downstream SARS-CoV-2 effects. The observed increased KSHV lytic reactivation due to (repeated) SARS-CoV-2 infections was further supported by the significantly higher lytic protein K8.1 antibody titres in unvaccinated patients with detectable KSHV VL compared to the vaccinated group.

Observation of HIV variables showed that in the unvaccinated group with detectable KSHV VL (i.e. reactivated KSHV) patients were on ART for a shorter period compared to individuals with undetectable KSHV VL, suggesting some protective effect of ART on controlling KSHV

infection. HIV VL, absolute CD4 count, WHO clinical stage of HIV disease or ART regimen did not differ between the groups. There was, however, a significantly higher proportion of individuals with a low lymphocyte count (lymphopenia) in the unvaccinated group with detectable KSHV VL which was not seen in the vaccinated group. This suggests that lymphopenia may contribute to KSHV reactivation as lymphocytes are key players in immune homeostasis as well as the inflammatory response [184]. It additionally suggests that COVID-19 vaccination protects against the impact of (repeated) SARS-CoV-2 exposure on lymphopenia. Repeated SARS-CoV-2 infection may lead to T-cell dysregulation (T-cell exhaustion and interference with T-cell expansion), particularly in immunocompromised patients, potentially leading to KSHV replication due to the absence of KSHV-specific immune control [24, 92, 185].

Studies on KSHV reactivation in response to SARS-CoV-2 infection and/or COVID-19 disease are rare: to our knowledge, KSHV reactivation was either only studied in a small number (n=104) of critically ill hospitalised COVID-19 patients (with or without HIV infection), showing an association between detectable KSHV VL and COVID-19 outcome [28], or reported as case studies in the context of advanced HIV disease [157, 158]. Studies on other herpesviruses indicate that critically ill COVID-19 patients are prone to herpesvirus reactivations as 85 % patients developed EBV, and/or HCMV or HHV-6 viremia while in the intensive care unit [27, 152].

Our study was different to those mentioned above as we looked at KSHV reactivation in response to SARS-CoV-2 infection in a cohort of non-hospitalised, generally healthy HIV-positive individuals (mostly on and adhering to ARTs). While the results of our cross-sectional study showed an increase in KSHV lytic reactivation during the later stages of the COVID-19 pandemic, possibly due to multiple SARS-CoV-2 infections, in unvaccinated but not vaccinated patients, the detected KSHV VL was generally not elevated, suggesting no immediate consequences for KSHV-associated disease onset. Moreover, the increase in the number of patients with detectable KSHV VL after (most likely) multiple SARS-CoV-2 exposures indicate some accumulative long-term effect of the infection(s) and not a consequence of SARS-CoV-2-induced inflammation. Indeed, all inflammatory parameters studied in our cohort, indicating acute disease, did not significantly differ depending on vaccination status and/or detectability of KSHV VL.

Vaccination induces a protective immunity mediated by antibodies and memory B-cells, as well as helper and cytotoxic T cells as part of the adaptive immune response [186]. Even when antibody levels decline, memory T and B cells remain stable for months providing enduring immunity to infection, such as with SARS-CoV-2 [187, 188]. This may in turn protect against

other downstream effects of SARS-CoV-2 preventing COVID-19 disease severity and possibly (among others) lytic reactivation of latent herpesviruses. The mechanisms of these are unknown and need to be elucidated. Although COVID-19 vaccination had a protective effect against KSHV reactivation as seen here, it is also possible that the majority of the patients of this cohort were further protected by their adherence to ART which might explain the generally low KSHV VL even in unvaccinated individuals.

The observed KSHV lytic reactivation seen here in our specific patient cohort may represent a potential long-term impact of SARS-CoV-2 infection on KSHV-related disease development, particularly in HIV-infected, unvaccinated individuals, and warrants further investigation. Recent literature suggests that SARS-CoV-2 is able to modulate oncogenic pathways and promote inflammation potentially contributing to the development of cancer [92, 189], similar to known oncoviruses [189]. This may be of particular significance in conjunction with KSHV infection which, via lytic reactivation, has the potential to promote tumorigenesis [71, 189]. The high prevalence of KSHV infection in SA, particularly in the context of HIV co-infection emphasises the importance of following-up these individuals in the post-pandemic era to put necessary preventative measures in place and to avoid adverse outcomes [24, 92, 189].

4.2 The long-term impact of the COVID-19 pandemic on KSHV-seropositive HIV-infected patients – a follow-up study

The results derived from our cross-sectional study indicated the necessity of further elucidating the potential long-term impact of SARS-CoV-2 infection on KSHV lytic reactivation in patients with impaired immune functions. We therefore designed a FU study inviting KSHV-seropositive patients (with or without initial SARS-CoV-2 serology, and irrespective of their vaccination status) to assess the long-term impact of SARS-CoV-2 exposure on KSHV reactivation over time. We were particularly interested in determining whether KSHV VL increased over time or whether there was an increase in the number of patients with detectable KSHV VL.

The COVID-19 pandemic was characterised by waves driven by emerging variants and infection dynamics. These COVID-19 waves overlapped with the recruitment and FU periods of this study. The waves in SA were characterised by the wildtype (or ancestral) strain, dominating the first wave, beta (dominating the second wave), delta (dominating the third wave) and omicron (dominating the fourth wave). The omicron variant resulted in the steepest surge in the number of COVID-19 cases in SA [190]. Exposure to these waves has altered susceptibility to subsequent infection resulting in different disease profiles. Zar *et al.* [175] found that natural infection of SARS-CoV-2 was not enough to prevent infection (and/or

re-infection) by the omicron variant in most individuals but this was boosted by vaccination which led to significant protection [175]. Although our study did not collect information regarding when an individual was exposed to SARS-CoV-2 (and which variant), it is still important that the different variants at the different recruitment stages are taken into account when interpreting the observed results.

Enrolment for the FU study commenced in March 2021; with the 6-month and 12-month FU periods starting in October 2021 and April 2022, respectively, and ran until September 2023 (Figure 3.2.1). This coincided with the decline of the second wave (which was dominated by the beta variant) for enrolment into the FU study, the decline of the third wave for the 6-month FU (which was dominated by the delta variant) and the decline of the fourth wave for the 12-month FU (which was dominated by the omicron variant), respectively. A total of 173 eligible patients were identified for FU based on KSHV serostatus at initial recruitment, however, only 46 individuals presented for FU at all FU time points. High LTFU in clinical trials is a common phenomenon [191]. LTFU among ART-eligible PLWH in SA pre-COVID-19 was estimated to be 20 – 30 % [192-194]. A study on the impact of the COVID-19 lockdown on ART adherence in PLWH in three sub-districts in Cape Town, SA, reported 45 % of patients LTFU [195]. In the context of our study, we see a substantially higher percentage of individuals LTFU; potentially due to socioeconomic factors, the stigma behind HIV, COVID-19-related travel restrictions and/or difficulty in adhering to treatment. Nevertheless, our analyses revealed some interesting trends.

Corroborating the SARS-CoV-2 antibody titres seen in the cross-sectional study, antibodies against both S1 and RBD remained high throughout the FU period, with a slight decrease compared to the initial recruitment time point, indicating high exposure to SARS-CoV-2 throughout (Figure 3.2.4 and Table 3.2.1). This may also reflect the increasing trend in COVID-19 vaccination observed throughout the FU period (with an overall 32.6 % increase in vaccination from initial recruitment to the 12-month FU time point).

The overall median HIV VL in the patients showed a decreasing trend throughout the FU period, while CD4 count increased, compared to initial recruitment (Figure 3.2.3 and Table 3.2.1). This may be linked to an increasing time on ART as well as adherence to treatment as reported before [196, 197]. In addition, it may also indicate a recovery of health care systems after initial COVID-19 disruptions during the start of the pandemic where many services (including the provisions of ART) were interrupted due to pandemic restrictions.

To assess the impact of SARS-CoV-2 exposure on KSHV reactivation over time in the FU patient cohort, blood KSHV VL was determined. The median KSHV VL was 1 copy/10^6 cells at initial recruitment and 6-month FU which then increased to 11172.45 copies/10^6 cells at the

12-month FU. This increase in median KSHV VL may signify changes in disease severity or progression of disease; however, the percentage of individuals with a detectable KSHV VL decreased over the FU period. This is an interesting result as a decrease in the percentage of individuals with detectable KSHV VL indicates a reduction in active replication on the cohort level which could be possible due to effective antiviral treatment. Individual patients who have shown an increase in KSHV VL, however, warrant further investigation.

Seemingly different to the results observed in the cross-sectional study, the number of patients with detectable KSHV VL decreased over time during the FU period. As this may be attributed to the increased number of patients that received the COVID-19 vaccine, we stratified our analysis by vaccination status. Similar to the whole FU cohort, we saw a slight decreasing trend in SARS-CoV-2 serology both in the vaccinated and unvaccinated groups between initial recruitment and 6-month FU which could be attributed to the waning of antibodies after initial vaccination in the vaccinated group and/or natural infection in the unvaccinated group. There was then an increase in antibody levels between the 6- and 12-month FU which could be due to the increase in vaccination uptake, the addition of booster vaccinations as well as new infections (Figure 3.2.10), particularly with the omicron variant [177], which was the most transmissible variant.

Most interestingly, when stratified according to COVID-19 vaccination status, the percentage of individuals with detectable KSHV VL steadily decreased over time in the vaccinated group, whereas there was an overall increase in the number of patients with detectable KSHV VL in the unvaccinated patients, particularly from the 6- to the 12-month FU time points. These results support the findings of the cross-sectional study design wherein vaccination seems to have a protective effect against KSHV lytic reactivation. Unvaccinated individuals, however, may be more vulnerable to KSHV reactivation through repeated exposure to SARS-CoV-2 infection (particularly with the more transmissible variants) as seen by an increase in the number of individuals with detectable KSHV VL. This was further confirmed by cox regression analysis that determined the probability of KSHV reactivation depending on vaccination status (Figure 3.2.14). The analysis revealed that over time, unvaccinated patients had a higher probability of presenting with detectable KSHV VL (and therefore KSHV lytic reactivation) compared to patients that were vaccinated.

These results allow us to speculate that adhering to ART regimens and vaccination against COVID-19 may protect an individual from the risk of KSHV reactivation. This in turn emphasises the importance of educating individuals on adhering to ART regimens and scheduled clinical visit as well as monitoring patients at risk for KSHV reactivation (including those with immunodeficiencies such as unvaccinated HIV-positive patients) in the

post-pandemic era. In addition, these results support the introduction of KSHV VL testing as a diagnostic tool in routine clinical practice in HIV care clinics.

4.3 A 34-year-old HIV-infected male with sustained elevated KSHV VL over a 2-year period during the COVID-19 pandemic: A case report

During the course of recruitment, we identified one patient, a 34-year-old male, who had an exceptionally high KSHV VL and selected him for additional FU to assess his KSHV VL dynamics and/or associated disease progression over time. The patient exhibited persistent, highly elevated KSHV viremia throughout the 2-year FU period with a simultaneous increase in severity of clinical and virological parameters and, of note, an increase in KSHV serology and SARS-CoV-2 antibodies. In contrast to the increase in KSHV related parameters, serology and VL for the related oncogenic virus EBV remained below or at the level seen in the entire cohort, respectively. Additionally, the patient displayed an increase in inflammatory markers (IL-6 and CRP) throughout the FU period compared to the entire cohort where most patients had inflammatory markers that were within normal range. The patient was not vaccinated against COVID-19 and therefore his increasing (and persistently elevated) SARS-CoV-2 antibody levels were likely indicative of repeated natural infections. In the context of the remainder of the cohort, this individual was unique as he displayed elevated KSHV VL from initial recruitment over the 2-year FU period which was therefore not triggered (but most likely sustained), for example by concurrent infections (such as with SARS-CoV-2).

However, there were some challenges that might have impacted on the herein reported observations. This includes the patient being incarcerated at more than one time during the study, with the FU visits being scheduled around his jail time, resulting in missed check-ups at the clinic which in turn resulted in the patient not receiving or adhering to ARTs and/or TB treatment, leading to delayed HIV viral suppression [198, 199], progression of TB disease, and/or continuous lytic reactivation of KSHV, respectively [198]. While (repeated) SARS-CoV-2 infection(s) could be causative for the observed KSHV viremia and/or increased inflammatory markers, the patient's living circumstances, his frequent non-adherence to his HIV/TB treatment, malnutrition and potentially other underlying infections are very likely to have played a role. The individual also being clinically diagnosed with TB might have added an increased challenge to this case study as it involved other medications and symptoms of infection that are similar to lytic KSHV and/or SARS-CoV-2 infection [17, 19, 200, 201]. This is well representative of the situation of many HIV-infected patients in SA and is an example

of the challenge facing clinicians treating patients in the context of multiple overlapping and simultaneous infectious diseases.

Indeed, over the course of the FU period, the patient reported several unspecific symptoms which included weight loss, fatigue, oedema, cachexia, respiratory symptoms/coughing, gastrointestinal disturbance, altered mental state, weight loss, night sweats, neuropathy, radiographic abnormalities, arthralgia and myalgia (see Table 3.3.1). Despite the patient's sustained reactivation of KSHV, no diagnosis of any KSHV-related pathology was made during the FU period (which might have been too short for a conclusive diagnosis); however, this patient is still at an increased risk of developing a KSHV-related pathology. Interestingly, none of the above-mentioned challenges seem to have affected reactivation of the related oncovirus EBV, indicating a specific effect on lytic KSHV infection. This is supported by our previous study whereby KSHV, but not EBV, co-infection was associated with COVID-19 severity and outcome [28]. Moreover, EBV and KSHV have been described in AIDS-associated PEL to suppress each other's lytic replicative cycles during co-infection by physical interaction on a molecular level [202]. However, our study did not elucidate whether this is the case in the herein described patient.

Going forward, alternative and more effective modes of treatment delivery should be considered. This includes providing larger ART quantities to those individuals that are not able to always travel, or that individuals be allowed to refill their ARTs at any clinic nearby [199]. In general, the consistent education on the importance of ART adherence, as mentioned above is required.

Taken together and as discussed in the context of the entire cohort, the results for this case study emphasise the importance of monitoring individuals presenting with unspecific symptoms for increased KSHV VL and serology as it will assist in early diagnosis and in turn timely treatment. It emphasises the importance of further understanding the role of co-infections especially in the context of KSHV reactivation and highlights the need of implementing KSHV VL testing in a clinical setting, especially for patients at risk of developing KSHV-associated pathologies. Tailor-based treatment options based on early diagnosis would contribute to better and more effective treatment leading to improved outcomes.

4.4 Future directions, limitations and concluding remarks

In general, highly active ART is used in the treatment of KSHV-related pathologies like PEL and KS [59], with anti-herpes virus drugs and systemic treatment through chemotherapy

(bleomycin, vinistrine and doxorubicine) in limited-resource settings additionally being used for KS [59, 203]. However, the best strategy against KSHV-associated diseases would be prevention of infection as cancers with an infectious causative agent provide a unique opportunity for prevention. The introduction of a sterilising preventative vaccine could be introduced to interrupt transmission of KSHV [204], and RNA vaccines have already been designed against certain herpesviruses like EBV and HCMV [204, 205]. This may provide a platform for the development of a prophylactic KSHV vaccine.

In addition, reducing virus spread through adequate public health measures as well as educational dialogue about how to prevent transmission of the virus are conceivable. Prevention of infection from mother to child or between siblings would be the ultimate goal to manage KSHV; however, there are currently no known mechanisms to prevent this.

Considering the reactivation of KSHV through co-infection with SARS-CoV-2 and other pathogens such as Plasmodium *sp.*, HIV-1 and Mtb [2, 17, 60] will help clinicians to implement alternative monitoring and management strategies for patients at risk, such as those presenting with positive KSHV serology and unspecific symptoms at HIV care clinics (Figure 4.1).

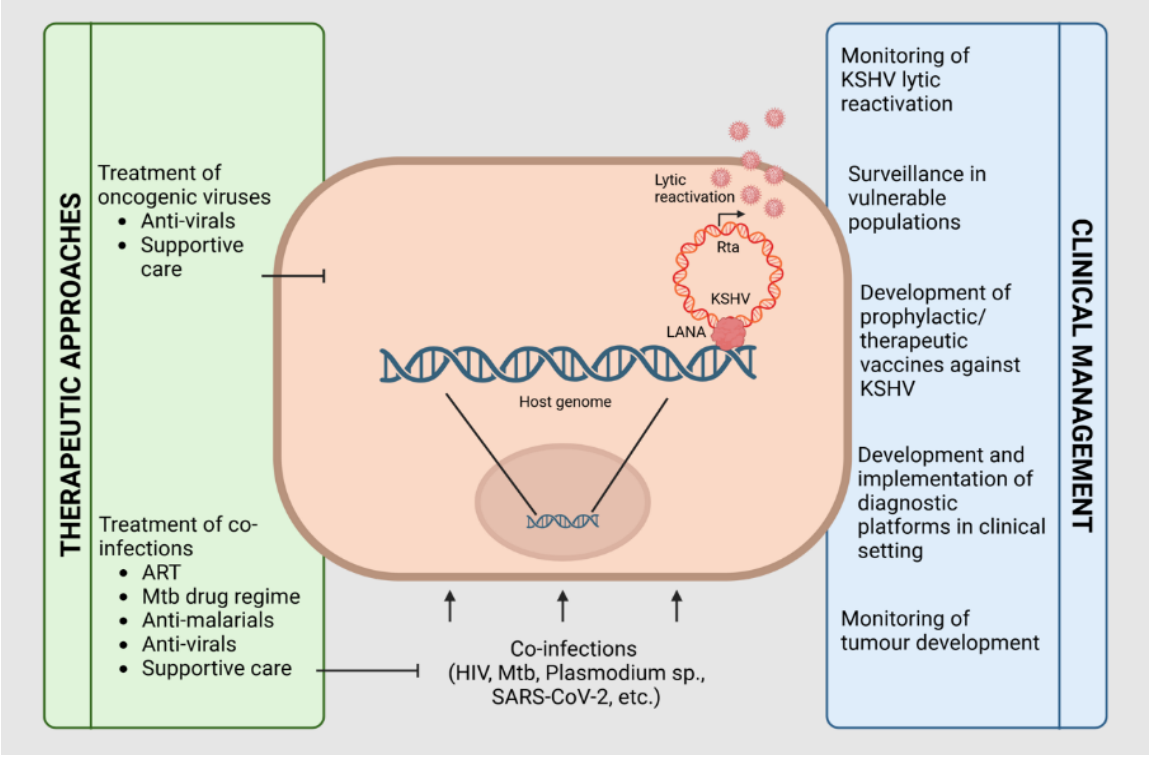

Figure 4.1: Co-infections impact KSHV lytic reactivation. Certain clinical and therapeutic approaches may improve KSHV-associated disease outcomes. Figure adapted and modified from Chinna *et al*. [17], figure modified with Biorender.com.

It should be emphasised that the herein presented cohort consisted of non-hospitalised patients who were generally healthy at presentation. Accordingly, we did not see increased inflammatory markers (IL-6 and CRP) and reactivation of KSHV was generally rather low (i.e. no or low KSHV VL in the cohort, with a few exceptions). The observed KSHV lytic replication in this cohort therefore might not have an immediate impact within the context of this study but we may observe a long-term impact on tumorigenesis/cancer development in the post- pandemic era. This creates an emphasis on establishing appropriate surveillance and FU systems in the clinical setting. KSHV infection is currently not monitored clinically at all nor considered as part of the diagnostic work up of HIV-positive patients. The work presented in this thesis thus adds to a growing body of evidence supporting the inclusion of KSHV VL testing and monitoring in routine HIV care in a clinical setting, with focus on patients at risk (such as immunosuppressed individuals) or in patients presenting with unspecific symptoms with negative TB or malaria diagnoses. This would inform more efficient monitoring and treatment strategies as KSHV VL in the peripheral blood could serve as a useful diagnostic and monitoring tool for KSHV lytic reactivation or KSHV-associated pathologies.

In general, our study had a few limitations which are important for the interpretation of our results. For both the initially recruited and FU patient samples with positive SARS-CoV-2 serology the exact time point of SARS-CoV-2 infection (or infections) was unknown, as was COVID-19 disease severity if symptomatic infection had occurred. We were thus only able to report on SARS-CoV-2 serology and self-reported vaccination status. We collected extensive data particularly on HIV; however, we did not have data on patient comorbidities and other clinical variables which may have posed confounding effects in the observed results. We have only looked at one gene as a proxy for lytic reactivation of KSHV after SARS-CoV-2 infection. In future, it would be beneficial to include gene expression analysis studies on a few KSHV lytic genes in order to have a more robust overview on the mechanisms involved in the potential lytic reactivation of KSHV in response to co-infections, including SARS-CoV-2. Additionally, there was a higher than expected number of individuals who were LTFU, and this decreased the sample size, which may have impacted the results and statistical power of the study. Addressing the issue of LTFU should be an area of focus to avoid the loss of patients and subsequently valuable information.

Going forward, and based on the clinical observations seen here, the “cause-effect” needs to be further studied. This should be done in appropriate cell culture models that are able to mimic the life cycle of KSHV which will allow further confirmation of the observed results and allow a better understanding of the effect of SARS-CoV-2 infection on KSHV lytic reactivation.

In conclusion, we leveraged a unique cohort of patients in a low-income area in the Western Cape, SA, to investigate the effect of SARS-CoV-2 infection on KSHV lytic reactivation. The data presented in this thesis supports the monitoring of KSHV VL of patients at risk in a clinical setting, particularly in the context of co-infections, as well as in the management of patients with KSHV infection in the future. In the long term, the insights gained in this study may be applied to diagnostic and prognostic guidelines in HIV centres with the aim to prevent the lytic reactivation of KSHV and associated pathologies.

5. References

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6. Appendix

6.1 Solution recipes

KSHV serology ELISA

Assay buffer:

2.5 % Bovine Serum Albumin (BSA)

2.5 % Normal Donor Goat serum (Equitech-Bio)

0.005 % Tween 20

0.005 % Triton X-100 (3 %)

In 1X PBS

Wash buffer:

0.05 % Tween 20 in 1X PBS

Stop buffer:

3M NaOH in dH_2O

IL-6 ELISA

Reconstitution buffer:

0.09 % Azide in 1x PBS

Coating buffer:

1X PBS, pH 7.2-7.4

Wash buffer:

0.05 % Tween20 in 1x PBS

Blocking buffer:

5 % BSA in 1x PBS

Standard and Secondary antibody dilution buffer:

1 % BSA in 1x PBS

HRP diluent buffer:

1 % BSA

0.1 % Tween20

in 1x PBS

6.2 Supplementary material

Supplementary Table 1: Univariate analysis of clinical variables in COVID-19 unvaccinated (n = 276) and vaccinated (n = 131) patients with undetectable compared to detectable KSHV VL. Data are presented as number and percentage of total or median and IQR where appropriate. For continuous variables, *p*-values are by Mann-Whitney U test or T-test (for normally distributed variables as indicated by #). For categorical variables, *p*-values are by Chi-square test, Fisher's Exact test or Fisher-Freeman-Halton test, as appropriate. All ranges are as per NHLS definition.

		COVID-19 unvaccinated (n = 276)			***COVID-19 vaccinated (n = 131)***		
Variable		**KSHV VL undetectable (n = 194) N (%) or Median (IQR)/Mean (SD)**	**KSHV VL detectable (n = 36) N (%) or Median (IQR)/Mean (SD)**	***p*-value**	**KSHV VL undetectable (n = 85) N (%) or Median (IQR)/Mean (SD)**	**KSHV VL detectable (n = 38) N (%) or Median (IQR)/Mean (SD)**	***p*-value**
Laboratory blood analysis							
Sodium, mmol/l		136 (135 – 138)	136.5 (135 – 138)	0.66	136 (135 – 138)	136 (135 – 139)	0.70
Sodium	Within normal range (136 – 145mmol/l)	121 (63.0 %)	24 (66.7 %)	0.68	55 (64.7 %)	23 (60.5 %)	0.66
	Below normal range (<136mmol/l)	71 (37.0 %)	12 (33.3 %)		30 (35.3 %)	15 (39.5 %)	
Creatinine, µmol/l		68.0 (57.5 – 80.0)	77.0 (60.5 – 93.0)	0.040	68.0 (59.0 – 78.0)	67.5 (59.0 – 87.0)	0.46
Creatinine	Within normal range (F:49 – 90µmol/l, M: 64 – 104µmol/l)	166 (86.5 %)	27 (75.0 %)	0.047	71 (83.5 %)	27 (71.1 %)	0.21
	Below normal range (F: <49µmol/l, M: <64µmol/l)	19 (9.9 %)	4 (11.1 %)		9 (10.6 %)	6 (15.8 %)	
	Above normal range (F: >90µmol/l, M: >104µmol/l)	7 (3.6 %)	5 (13.9 %)		5 (5.9 %)	5 (13.2 %)	
Albumin, g/l		42.0 (39.0 – 44.0)	42.0 (38.5 – 44.5)	0.76	42.0 (39.0 – 45.0)	43.0 (40.0 – 44.0)	0.36

		COVID-19 unvaccinated (n = 276)			*COVID-19 vaccinated (n = 131)*		
Variable		**KSHV VL undetectable (n = 194) N (%) or Median (IQR)/Mean (SD)**	**KSHV VL detectable (n = 36) N (%) or Median (IQR)/Mean (SD)**	***p*-value**	**KSHV VL undetectable (n = 85) N (%) or Median (IQR)/Mean (SD)**	**KSHV VL detectable (n = 38) N (%) or Median (IQR)/Mean (SD)**	***p*-value**
Albumin	Within normal range (35 – 52g/l)	174 (91.1 %)	33 (91.7 %)	0.85	79 (92.9 %)	37 (97.4 %)	0.44
	Below normal range (<35g/l)	14 (7.3 %)	3 (8.3 %)		6 (7.1 %)	1 (2.6 %)	
	Above normal range (>52g/l)	3 (1.6 %)	0 (0.0 %)		0 (0.0 %)	0 (0.0 %)	
ALT, IU/l		22.0 (17.0 – 33.0)	24.5 (18.5 – 33.0)	0.37	20.0 (13.0 – 28.0)	18.0 (15.0 – 25.0)	0.72
ALT	Within normal range (F: 7 – 35IU/l, M: 10 – 40IU/l)	153 (81.4 %)	29 (80.6 %)	0.85	76 (91.6 %)	33 (86.8 %)	0.54
	Below normal range (F: <7IU/l, M: <10IU/l)	1 (0.5 %)	0 (0.0 %)		1 (1.2 %)	0 (0.0 %)	
	Above normal range (F: >35IU/l, M: >40IU/l)	34 (18.1 %)	7 (19.4 %)		6 (7.2 %)	5 (13.2 %)	
IL-6, pg/ml		2.32 (0.81 – 6.70)	0.75 (0.30 – 6.85)	0.18	2.68 (1.18 – 6.93)	2.43 (0.75 – 4.84)	0.52
IL-6	Undetectable or less than reference value (<1.8pg/ml)	138 (71.1 %)	31 (86.1 %)	0.062	64 (75.3 %)	35 (92.1 %)	0.030
	Above reference value (>1.8pg/ml)	56 (28.9 %)	5 (13.9 %)		21 (24.7 %)	3 (7.9 %)	
CRP, mg/l		4.0 (2.0 – 10.0)	4.0 (2.0 – 10.0)	0.88	4.0 (1.0 – 8.0)	4.5 (2.0 – 7.0)	0.99

		COVID-19 unvaccinated (n = 276)			*COVID-19 vaccinated (n = 131)*		
Variable		**KSHV VL undetectable (n = 194) N (%) or Median (IQR)/Mean (SD)**	**KSHV VL detectable (n = 36) N (%) or Median (IQR)/Mean (SD)**	***p*-value**	**KSHV VL undetectable (n = 85) N (%) or Median (IQR)/Mean (SD)**	**KSHV VL detectable (n = 38) N (%) or Median (IQR)/Mean (SD)**	***p*-value**
CRP	Within normal range (<10mg/l)	139 (72.8 %)	26 (72.2 %)	0.95	66 (77.6 %)	32 (84.2 %)	0.40
	Elevated (>10mg/l)	52 (27.2 %)	10 (27.8 %)		19 (22.4 %)	6 (15.8 %)	
White cell count, $x10^9$/l		4.97 (3.94 – 6.42)	4.54 (3.65 – 6.28)	0.37	5.18 (4.09 – 6.91)	4.49 (4.06 – 5.81)	0.22
White cell count	Within normal range (F: 3.9 – 12.6 $x10^9$/l, M: 3.92 – 10.4 $x10^9$/l)	146 (76.8 %)	23 (63.9 %)	0.10	64 (75.3 %)	29 (76.3 %)	0.90
	Below normal range (F: <3.9$x10^9$/l, M: <3.92$x10^9$/l)	44 (23.2 %)	13 (36.1 %)		21 (24.7 %)	9 (23.7 %)	
Red cell count[#], $x10^{12}$/l		4.11 (0.59)	4.35 (0.62)	0.019*	4.10 (0.45)	4.23 (0.59)	0.17
Red cell count	Below or within normal range (F: <4.8 $x10^{12}$/l, M: <5.5 $x10^{12}$/l)	185 (97.4 %)	33 (91.7 %)	0.12	84 (98.8 %)	34 (89.5 %)	0.031
	Above normal range (F: >4.8$x10^{12}$/l, M: >5.5$x10^{12}$/l)	5 (2.6 %)	3 (8.3 %)		1 (1.2 %)	4 (10.5 %)	
Haemoglobin, g/dl		12.50 (11.30 – 13.70)	13.00 (12.10 – 14.55)	0.057	12.40 (11.40 – 13.60)	12.70 (12.00 – 13.70)	0.38
Haemoglobin	Within normal range (F: 12 – 15 g/dl, M: 13 – 17 g/dl)	111 (58.4 %)	27 (75.0 %)	0.061	54 (63.5 %)	26 (68.4 %)	0.60
	Below normal range (F: <12g/dl, M: <13g/dl)	79 (41.6 %)	9 (25.0 %)		31 (36.5 %)	12 (31.6 %)	

		COVID-19 unvaccinated (n = 276)			*COVID-19 vaccinated (n = 131)*		
Variable		**KSHV VL undetectable (n = 194) N (%) or Median (IQR)/Mean (SD)**	**KSHV VL detectable (n = 36) N (%) or Median (IQR)/Mean (SD)**	***p*-value**	**KSHV VL undetectable (n = 85) N (%) or Median (IQR)/Mean (SD)**	**KSHV VL detectable (n = 38) N (%) or Median (IQR)/Mean (SD)**	***p*-value**
Haematocrit, l/l		0.38 (0.35 – 0.42)	0.40 (0.37 – 0.43)	0.10	0.38 (0.35 – 0.41)	0.39 (0.37 – 0.42)	0.078
Haematocrit	Within normal range (F: 0.36 – 0.46 l/l, M: 0.4 – 0.5 l/l)	189 (99.5 %)	35 (97.2 %)	0.29	85 (100.0 %)	37 (97.4 %)	0.31
	Above normal range (F: >0.46l/l, M: >0.5l/l)	1 (0.5 %)	1 (2.8 %)		0 (0.0 %)	1 (2.6 %)	
Mean corpuscular volume, fl		93.30 (88.60 – 97.20)	93.20 (88.95 – 95.25)	0.56	91.40 (88.10 – 96.50)	94.20 (87.80 – 98.10)	0.22
Mean corpuscular volume	Within normal range (F: 78.9 – 98.5 fl, M: 83.1 – 101.6 fl)	147 (77.4 %)	31 (86.1 %)	0.64	66 (77.6 %)	29 (76.3 %)	0.94
	Below normal range (F: <78.9fl, M: <83.1fl)	15 (7.9 %)	2 (5.6 %)		7 (8.2 %)	3 (7.9 %)	
	Above normal range (F: >98.5fl, M: >101.6fl)	28 (14.7 %)	3 (8.3 %)		12 (14.1 %)	6 (15.8 %)	
Mean corpuscular haemoglobin, pg		30.70 (28.50 – 32.10)	30.85 (28.85 – 31.90)	0.99	30.40 (28.60 – 32.70)	31.00 (28.30 – 32.60)	0.85

		COVID-19 unvaccinated (n = 276)			*COVID-19 vaccinated (n = 131)*		
Variable		**KSHV VL undetectable (n = 194) N (%) or Median (IQR)/Mean (SD)**	**KSHV VL detectable (n = 36) N (%) or Median (IQR)/Mean (SD)**	***p*-value**	**KSHV VL undetectable (n = 85) N (%) or Median (IQR)/Mean (SD)**	**KSHV VL detectable (n = 38) N (%) or Median (IQR)/Mean (SD)**	***p*-value**
Mean corpuscular haemoglobin	Within or above normal range (F: >26.1 pg, M: >27.8pg)	174 (91.6 %)	34 (94.4 %)	0.75	78 (91.8 %)	33 (86.8 %)	0.51
	Below normal range (F: <26.1pg, M: <27.8pg)	16 (8.4 %)	2 (5.6 %)		7 (8.2 %)	5 (13.2 %)	
Mean corpuscular haemoglobin concentration, g/dl		32.50 (31.90 – 33.50)	33.20 (32.00 – 33.65)	0.22	33.10 (32.20 – 34.10)	32.80 (31.60 – 33.60)	0.026
Mean corpuscular haemoglobin concentration	Within normal range (F: 32.7 – 34.9 g/dl, M: 33 – 35g/dl)	78 (41.1 %)	22 (61.1 %)	0.080	44 (51.8 %)	18 (47.4 %)	0.22
	Below normal range (F: <32.7g/dl, M: <33g/dl)	103 (54.2 %)	13 (36.1 %)		32 (37.6 %)	19 (50.0 %)	
	Above normal range (F: >34.9g/dl, M: >35g/dl)	9 (4.7 %)	1 (2.8 %)		9 (10.6 %)	1 (2.6 %)	
Red cell distribution width, %		13.8 (13.1 – 14.9)	13.9 (13.5 – 15.1)	0.45	13.8 (13.1 – 14.6)	13.6 (13.2 – 14.6)	0.55

		COVID-19 unvaccinated (n = 276)			*COVID-19 vaccinated (n = 131)*		
Variable		**KSHV VL undetectable (n = 194) N (%) or Median (IQR)/Mean (SD)**	**KSHV VL detectable (n = 36) N (%) or Median (IQR)/Mean (SD)**	***p*-value**	**KSHV VL undetectable (n = 85) N (%) or Median (IQR)/Mean (SD)**	**KSHV VL detectable (n = 38) N (%) or Median (IQR)/Mean (SD)**	***p*-value**
Red cell distribution width	Within normal range (F: 12.4 – 17.3 %, M: 12.1 – 16.3 %)	165 (86.8 %)	34 (94.4 %)	0.25	70 (82.4 %)	31 (81.5 %)	0.56
	Below normal range (F: <12.4 %, M: <12.1 %)	10 (5.3 %)	1 (2.8 %)		7 (8.2 %)	5 (13.2 %)	
	Above normal range (F: >17.3 %, M: >16.3 %)	15 (7.9 %)	1 (2.8 %)		8 (9.4 %)	2 (5.3 %)	
Platelet count, $x10^9$/l		292 (245 – 338)	297 (216 – 351)	0.91	283 (233 – 346)	288 (214 – 332)	0.72
Platelet count	Within normal range (F: 186 – 454 $x10^9$/l, M: 171 – 388 $x10^9$/l)	162 (85.7 %)	29 (80.6 %)	0.52	74 (87.1 %)	37 (97.4 %)	0.25
	Below normal range (F: <186$x10^9$/l, M: <171$x10^9$/l)	15 (7.9 %)	3 (8.3 %)		6 (7.1 %)	1 (2.6 %)	
	Above normal range (F: >454$x10^9$/l, M: >388$x10^9$/l)	12 (6.3 %)	4 (11.1 %)		5 (5.9 %)	0 (0.0 %)	
Neutrophils[#], %		54.26 (12.58)	52.79 (12.32)	0.52	52.42 (12.92)	49.02 (12.21)	0.17
Neutrophil count, $x10^9$/l		2.62 (1.94 – 3.83)	2.36 (1.66 – 3.23)	0.37	2.70 (1.95 – 3.81)	2.08 (1.73 – 3.20)	0.097

		COVID-19 unvaccinated (n = 276)			*COVID-19 vaccinated (n = 131)*		
Variable		**KSHV VL undetectable (n = 194) N (%) or Median (IQR)/Mean (SD)**	**KSHV VL detectable (n = 36) N (%) or Median (IQR)/Mean (SD)**	***p*-value**	**KSHV VL undetectable (n = 85) N (%) or Median (IQR)/Mean (SD)**	**KSHV VL detectable (n = 38) N (%) or Median (IQR)/Mean (SD)**	***p*-value**
Neutrophil count	Within normal range (F: 1.6 – 8.3 x10^9/l, M: 1.6 – 6.98 x10^9/l)	154 (81.5 %)	27 (75.0 %)	0.32	65 (76.5 %)	31 (81.6 %)	0.70
	Below normal range (<1.6x10^9/l)	33 (17.5 %)	8 (22.2 %)		17 (20.0 %)	7 (18.4 %)	
	Above normal range (F: >8.3x10^9/l, M: >6.98x10^9/l)	2 (1.1 %)	1 (2.8 %)		3 (3.5 %)	0 (0.0 %)	
Lymphocytes, %		32.20 (25.20 – 41.30)	28.95 (25.20 – 39.60)	0.28	34.30 (24.20 – 42.50)	34.70 (27.40 – 42.40)	0.32
Lymphocyte count, x10^9/l		1.62 (1.24 – 1.97)	1.35 (1.14 – 1.79)	0.054	1.69 (1.27 – 2.21)	1.72 (1.28 – 2.30)	0.79
Lymphocyte count	Within normal range (F: 1.4 – 4.5 x10^9/l, M: 1.4 – 4.2 x10^9/l)	125 (66.1 %)	14 (38.9 %)	0.0050	54 (63.5 %)	25 (65.8 %)	0.79
	Below normal range (<1.4x10^9/l)[2]	63 (33.3 %)	21 (58.3 %)		30 (35.3 %)	13 (34.2 %)	
	Above normal range (F: >4.5x10^9/l, M: >4.2x10^9/l)	1 (0.5 %)	1 (2.8 %)		1 (1.2 %)	0 (0.0 %)	
Monocytes, %		8.00 (6.40 – 9.90)	10.25 (8.10 – 14.40)	0.0010	9.60 (8.00 – 11.30)	10.05 (8.60 – 12.70)	0.12
Monocyte count, x10^9/l		0.41 (0.31 – 0.52)	0.50 (0.36 – 0.71)	0.0010	0.48 (0.38 – 0.60)	0.50 (0.40 – 0.67)	0.53

		COVID-19 unvaccinated (n = 276)			*COVID-19 vaccinated (n = 131)*		
Variable		**KSHV VL undetectable (n = 194) N (%) or Median (IQR)/Mean (SD)**	**KSHV VL detectable (n = 36) N (%) or Median (IQR)/Mean (SD)**	***p*-value**	**KSHV VL undetectable (n = 85) N (%) or Median (IQR)/Mean (SD)**	**KSHV VL detectable (n = 38) N (%) or Median (IQR)/Mean (SD)**	***p*-value**
Monocyte count	Within normal range (F: 0.2 – 0.8 x10^9/l, M: 0.3 – 0.8 x10^9/l)	175 (92.6 %)	30 (83.3 %)	0.013	74 (87.1 %)	36 (94.7 %)	0.54
	Below normal range (F: <0.2x10^9/l, M: <0.3x10^9/l)	10 (5.3 %)	1 (2.8 %)		1 (1.2 %)	0 (0.0 %)	
	Above normal range (>0.8 x10^9/l)	4 (2.1 %)	5 (13.9 %)		10 (11.8 %)	2 (5.3 %)	
Eosinophils, %		2.10 (1.00 – 4.30)	2.05 (1.20 – 5.05)	0.51	2.50 (1.30 – 4.50)	2.25 (1.30 – 3.70)	0.35
Eosinophil count, x10^9/l		0.11 (0.05 – 0.22)	0.12 (0.06 – 0.19)	0.72	0.13 (0.07 – 0.24)	0.12 (0.05 – 0.18)	0.29
Eosinophil count	Within normal range (F: 0 – 0.4 x10^9/l, M: 0 – 0.95 x10^9/l)	181 (95.8 %)	35 (97.2 %)	1.00	76 (89.4 %)	36 (94.7 %)	0.50
	Above normal range (F: >0.4x10^9/l, M: >0.95x10^9/l)	8 (4.2 %)	1 (2.8 %)		9 (10.6 %)	2 (5.3 %)	
Basophils, %		0.50 (0.30 – 0.70)	0.45 (0.30 – 0.70)	0.45	0.50 (0.40 – 0.70)	0.50 (0.40 – 0.70)	0.80
Basophil count, x10^9/l		0.03 (0.02 – 0.04)	0.02 (0.02 – 0.03)	0.27	0.03 (0.02 – 0.04)	0.03 (0.02 – 0.04)	0.38
Basophil count	Within normal range (0 – 0.1 x10^9/l)	191 (100.0 %)	36 (100.0 %)	–	85 (100.0 %)	38 (100.0 %)	–
	Above normal range (>0.1x10^9/l)	0 (0.0 %)	0 (0.0 %)		0 (0.0 %)	0 (0.0 %)	
Immature cells, %		0.3 (0.2 – 0.4)	0.3 (0.25 – 0.4)	0.36	0.3 (0.2 – 0.4)	0.2 (0.2 – 0.4)	0.021

		COVID-19 unvaccinated (n = 276)			*COVID-19 vaccinated (n = 131)*		
Variable		**KSHV VL undetectable (n = 194) N (%) or Median (IQR)/Mean (SD)**	**KSHV VL detectable (n = 36) N (%) or Median (IQR)/Mean (SD)**	***p*-value**	**KSHV VL undetectable (n = 85) N (%) or Median (IQR)/Mean (SD)**	**KSHV VL detectable (n = 38) N (%) or Median (IQR)/Mean (SD)**	***p*-value**
Immature cell count, $x10^9/l$		0.02 (0.01 – 0.02)	0.01 (0.01 – 0.03)	0.91	0.02 (0.01 – 0.02)	0.01 (0.01 – 0.02)	0.048
Immature cells count	Normal	191 (100.0 %)	36 (100.0 %)	–	85 (100.0 %)	38 (100.0	–
	Abnormal	0 (0.0 %)	0 (0.0 %)		0 (0.0 %)	0 (0.0 %)	
CD45+ white cell count, $x10^9/l$		4.81 (3.85 – 6.17)	4.26 (3.59 – 6.04)	0.32	5.07 (3.98 – 6.99)	4.38 (3.73 – 5.47)	0.095
CD45+ white cell count	Above or within normal range (>$4x10^9/l$)	135 (71.4 %)	21 (58.3 %)	0.12	62 (73.8 %)	25 (67.6 %)	0.48
	Below normal range (<$4x10^9/l$)	54 (28.6 %)	15 (41.7 %)		22 (26.2 %)	12 (32.4 %)	
CD4 percentage of lymphocytes, %		14.57 (9.98 – 20.76)	17.49 (11.20 – 21.80)	0.42	16.81 (10.44 – 23.6)	17.17 (12.56 – 21.97)	0.57
CD4 percentage of lymphocytes	Within normal range (28 – 51 %)	16 (8.5 %)	4 (11.1 %)	0.54	13 (15.5 %)	7 (18.9 %)	0.64
	Below normal range (<28 %)	173 (91.5 %)	32 (88.9 %)		71 (84.5 %)	30 (81.1 %)	
Symptoms at presentation							
Fever	No	189 (99.0 %)	35 (100.0 %)	1.0	84 (100.0 %)	38 (100.0 %)	–
	Yes	2 (1.0 %)	0 (0.0 %)		0 (0.0 %)	0 (0.0 %)	
Fatigue	No	186 (97.4 %)	35 (97.2 %)	1.0	82 (96.5 %)	37 (100.0 %)	0.55
	Yes	5 (2.6 %)	1 (2.8 %)		3 (3.5 %)	0 (0.0 %)	
Edema	No	183 (96.8 %)	36 (100.0 %)	0.59	85 (100.0 %)	38 (100.0 %)	–
	Yes	6 (3.2 %)	0 (0.0 %)		0 (0.0 %)	0 (0.0 %)	
Cachexia	No	181 (96.8 %)	33 (91.7 %)	0.16	84 (98.8 %)	38 (100.0 %)	1.0
	Yes	6 (3.2 %)	3 (8.3 %)		1 (1.2 %)	0 (0.0 %)	

		COVID-19 unvaccinated (n = 276)			*COVID-19 vaccinated (n = 131)*		
Variable		**KSHV VL undetectable (n = 194)** **N (%) or Median (IQR)/Mean (SD)**	**KSHV VL detectable (n = 36)** **N (%) or Median (IQR)/Mean (SD)**	***p*-value**	**KSHV VL undetectable (n = 85)** **N (%) or Median (IQR)/Mean (SD)**	**KSHV VL detectable (n = 38)** **N (%) or Median (IQR)/Mean (SD)**	***p*-value**
Respiratory symptoms	No	177 (94.1 %)	32 (88.9 %)	0.27	82 (96.5 %)	38 (100.0 %)	0.55
	Yes	11 (5.9 %)	4 (11.1 %)		3 (3.5 %)	0 (0.0 %)	
Gastrointestinal disturbance	No	182 (96.8 %)	35 (97.2 %)	1.0	85 (100.0 %)	38 (100.0 %)	–
	Yes	6 (3.2 %)	1 (2.8 %)		0 (0.0 %)	0 (0.0 %)	
Arthralgia	No	182 (96.3 %)	33 (97.1 %)	1.0	82 (97.6 %)	36 (94.7 %)	0.59
	Yes	7 (3.7 %)	1 (2.9 %)		2 (2.4 %)	2 (5.3 %)	
Altered mental state	No	186 (97.9 %)	35 (100.0 %)	1.0	85 (100.0 %)	38 (100.0 %)	–
	Yes	4 (2.1 %)	0 (0.0 %)		0 (0.0 %)	0 (0.0 %)	
Neuropathy	No	184 (97.4 %)	34 (97.1 %)	1.0	85 (100.0 %)	38 (100.0 %)	–
	Yes	5 (2.6 %)	1 (2.9 %)		0 (0.0 %)	0 (0.0 %)	
Radiographic abnormalities	No	189 (100.0 %)	35 (100.0 %)	–	84 (100.0 %)	38 (100.0 %)	–
	Yes	0 (0.0 %)	0 (0.0 %)		0 (0.0 %)	0 (0.0 %)	
Coughing	No	179 (93.7 %)	32 (91.4 %)	0.71	82 (97.6 %)	38 (100.0 %)	1.0
	Yes	12 (6.3 %)	3 (8.6 %)		2 (2.4 %)	0 (0.0 %)	
Loss of weight	No	175 (92.1 %)	31 (88.6 %)	0.51	83 (98.8 %)	37 (97.4 %)	0.53
	Yes	15 (7.9 %)	4 (11.4 %)		1 (1.2 %)	1 (2.6 %)	
Night sweats	No	181 (94.8 %)	32 (91.4 %)	0.43	81 (96.4 %)	38 (100/0 %)	0.55
	Yes	10 (5.2 %)	3 (8.6 %)		3 (3.6 %)	0 (0.0 %)	

Supplementary Table 2: Univariate analysis of demographic variables of the LTFU patients compared to the remainder of the cohort. Data are presented as number and percentage of total or median and IQR where appropriate. *p*-values are by Mann-Whitney U test for continuous variables and Chi-square test for categorical variables, as appropriate. LTFU is defined as patients who did not present for neither the 6- nor the 12- month FU. The remainder of the cohort presented at one or both of the FU time points.

Parameter		**LTFU (n= 72) N (%) or Median (IQR)**	**Remainder of cohort (n=101) N (%) or Median (IQR)**	***p*-value**
Age, years		38.0 (32.0 – 43. 5)	40.0 (32.0 – 46.0)	0.126
Sex	Female	52 (72.2%)	66 (65.3%)	0.338
	Male	20 (27.8 %)	35 (34.7 %)	
Weight, kg		65.0 (58.2 – 79.0)	68.0 (59.0 – 80.0)	0.406
COVID-19 vaccination	Yes	32 (44.4 %)	39 (38. 6 %)	0.442
	No	40 (55. 6%)	62 (61.4%)	

Supplementary Table 3: Univariate analysis of clinical variables of FU patients (n = 46). Data are presented as number and percentage of total or median and IQR where appropriate. *p*-values are by Kruskal-Wallis test for continuous variables and Chi-square test for categorical variables, as appropriate. All ranges are as per NHLS definition.

Parameter		**Initial Recruitment N (%) or Median (IQR)**	**FU 6-months N (%) or Median (IQR)**	**FU 12-months N (%) or Median (IQR)**	***p*-value**
Laboratory blood analysis					
Sodium, mmol/l		136.50 (135.00 – 138.00)	137.00 (136.00 – 139.00)	138.00 (136.00 – 139.00)	0.180
Sodium	Within normal range (136 – 145mmol/l)	30 (65.2 %)	34 (75.6 %)	36 (78.3 %)	0.332
	Below normal range (<136mmol/l)	16 (34.8 %)	11 (24.4 %)	10 (21.7 %)	
Creatinine, µmol/l		63.50 (55.00 – 74.00)	68.00 (59.00 – 81.00)	69.00 (57.00 – 82.00)	0.332
Creatinine	Within normal range (F:49 – 90µmol/l, M: 64 – 104 µmol/l)	41 (89.1 %)	40 (88.9 %)	39 (84.8 %)	0.695
	Below normal range (F: <49µmol/l, M: <64 µmol/l)	4 (8.7 %)	3 (6.7 %)	3 (6.5 %)	
	Above normal range (F: >90µmol/l, M: >104 µmol/l)	1 (2.2 %)	2 (4.4 %)	4 (8.7 %)	
Albumin, g/l		42.00 (39.00 – 44.00)	42.00 (39.00 – 43.00)	41.50 (39.00 – 44.00)	0.959
Albumin	Within and above normal range (>35 g/l)	44 (95.7 %)	43 (95.6 %)	42 (91.3 %)	0.598
	Below normal range (<35 g/l)	2 (4.3 %)	2 (4.4 %)	4 (8.7 %)	
ALT, IU/l		20.00 (16.00 – 26.00)	20.50 (15.00 – 25.00)	17.00 (14.00 – 21.00)	0.261
ALT	Within and below normal range (F: 7 – 35IU/l, M: 10 – 40IU/l)	41 (91.1 %)	40 (90.9 %)	43 (93.5 %)	0.883
	Above normal range (F: >35IU/l, M: >40IU/l)	4 (8.9 %)	4 (9.1 %)	3 (6.5 %)	
IL-6, pg/ml		0 (0.00 – 1.96)	0.00 (0 – 0.00)	0.00 (0 – 0.00)	0.071

Parameter		Initial Recruitment N (%) or Median (IQR)	FU 6-months N (%) or Median (IQR)	FU 12-months N (%) or Median (IQR)	*p*-value
IL-6	Undetectable or less than reference value (<1.8 pg/ml)	33 (71.7 %)	41 (89.1 %)	38 (82.6 %)	0.098
	Above reference value (>1.8 pg/ml)	13 (28.3 %)	5 (10.9 %)	8 (17.4 %)	
CRP, mg/l		4.00 (2.00 – 9.00)	4.00 (2.00 – 9.00)	4.00 (2.00 – 9.00)	0.894
CRP	Within normal range (<10 mg/l)	35 (76.1 %)	34 (75.6 %)	35 (76.1 %)	0.998
	Elevated (>10 mg/l)	11 (23.9 %)	11 (24.4 %)	11 (23.9 %)	
White cell count, $x10^9$/l		4.62 (3.78 – 5.96)	4.99 (3.91 – 5.70)	5.40 (3.67 – 6.44)	0.634
White cell count	Within normal range (F: 3.9 – 12.6 $x10^9$/l, M: 3.92 – 10.4 $x10^9$/l)	32 (69.6 %)	35 (76.1 %)	33 (71.7 %)	0.776
	Below normal range (F: <3.9$x10^9$/l, M: <3.92$x10^9$/l)	14 (30.4 %)	11 (23.9 %)	13 (28.3 %)	
Red cell count, $x10^{12}$/l		4.06 (3.78 – 4.50)	4.06 (3.67 – 4.50)	4.15 (3.69 – 4.52	0.987
Red cell count	Within normal range (F: 3.8 – 4.8 $x10^{12}$/l, M: 4.5 – 5.5 $x10^{12}$/l)	28 (60.9 %)	30 (65.2 %)	28 (60.9 %)	0.992
	Below normal range (F: <3.8$x10^{12}$/l, M: <4.5$x10^{12}$/l)	16 (34.8 %)	14 (30.4 %)	16 (34.8 %)	
	Above normal range (F: >4.8$x10^{12}$/l, M: >5.5$x10^{12}$/l)	2 (4.3 %)	2 (4.3 %)	2 (4.3 %)	
Haemoglobin, g/dl		12.50 (11.60 – 13.50)	12.45 (11.60 – 13.60)	12.55 (11.10 – 13.50)	0.867
Haemoglobin	Within normal range (F: 12 – 15 g/dl, M: 13 – 17 g/dl)	29 (63.0 %)	28 (60.9 %)	27 (58.7 %)	0.913
	Below normal range (F: <12g/dl, M: <13g/dl)	17 (37.0 %)	18 (39.1 %)	19 (41.3 %)	
Haematocrit, l/l		0.38 (0.36 – 0.41)	0.38 (0.35 – 0.41)	0.38 (0.34 – 0.41)	0.963

Parameter		Initial Recruitment N (%) or Median (IQR)	FU 6-months N (%) or Median (IQR)	FU 12-months N (%) or Median (IQR)	*p*-value
Haematocrit	Within normal range (F: 0.36 – 0.46 l/l, M: 0.4 – 0.5 l/l)	32 (69.6 %)	31 (67.4 %)	26 (56.5 %)	0.467
	Below normal range (F: <0.36l/l, M: <0.4l/l)	14 (30.4 %)	15 (32.6 %)	19 (41.3 %)	
	Above normal range (F: >0.46l/l, M: >0.5l/l)	0 (0.0 %)	0 (0.0 %)	1 (2.2 %)	
Mean corpuscular volume, fl		92.85 (88.10 – 98.40)	93.15 (89.70 – 97.00)	92.45 (88.20 – 97.90)	0.967
Mean corpuscular volume	Within normal range (F: 78.9 – 98.5 fl, M: 83.1 – 101.6 fl)	33 (71.7 %)	36 (78.3 %)	34 (73.9 %)	0.832
	Below normal range (F: <78.9fl, M: <83.1fl)	4 (8.7 %)	5 (10.9 %)	4 (8.7 %)	
	Above normal range (F: >98.5fl, M: >101.6fl)	9 (19.6 %)	5 (10.9 %)	8 (17.4 %)	
Mean corpuscular haemoglobin, pg		30.85 (28.60 – 32.10)	30.95 (29.50 – 32.10)	30.45 (28.500 – 32.00)	0.804
Mean corpuscular haemoglobin	Within normal range (F: 26.1 – 33.5 pg, M: 27.8 – 34.8 pg)	37 (80.4 %)	35 (76.1 %)	37 (80.4 %)	0.448
	Below normal range (F: <26.1 pg, M: <27.8 pg)	3 (6.5 %)	6 (13.0 %)	7 (15.2 %)	
	Above normal range (F: >33.5 pg, M: >34.8 pg)	6 (13.0 %)	5 (10.9 %)	2 (4.3 %)	
Mean corpuscular haemoglobin concentration, g/dl		32.70 (32.00 – 33.40)	33.05 (32.30 – 33.70)	32.85 (31.90 – 33.50)	0.504
Mean corpuscular haemoglobin concentration	Within normal range (F: 32.7 – 34.9 g/dl, M: 33 – 35 g/dl)	20 (43,5 %)	27 (58.7 %)	26 (56.5 %)	0.554
	Below normal range (F: <32.7g/dl, M: <33g/dl)	23 (50.0 %)	18 (39.1 %)	18 (39.1 %)	
	Above normal range (F: >34.9g/dl, M: >35g/dl)	3 (6.5 %)	1 (2.2 %)	2 (4.3 %)	
Red cell distribution width, %		13.75 (13.20 – 14.60)	13.80 (13.20 – 14.60)	13.80 (13.10 – 14.70)	0.900

Parameter		Initial Recruitment N (%) or Median (IQR)	FU 6-months N (%) or Median (IQR)	FU 12-months N (%) or Median (IQR)	*p*-value
Red cell distribution width	Within normal range (F: 12.4 – 17.3 %, M: 12.1 – 16.3 %)	42 (91.3 %)	43 (93.5 %)	40 (87.0 %)	0.625
	Below normal range (F: <12.4 %, M: <12.1 %)	2 (4.3 %)	1 (2.2 %)	1 (2.2 %)	
	Above normal range (F: >17.3 %, M: >16.3 %)	2 (4.3 %)	2 (4.3 %)	5 (10.9 %)	
Platelet count, x10^9/l		289.00 (236.00 – 324.00)	258.50 (231.00 – 326.00)	271.50 (231.00 – 362.00)	0.482
Platelet count	Within normal range (F: 186 – 454 x10^9/l, M: 171 – 388 x10^9/l)	42 (91.3 %)	40 (87.0 %)	41 (89.1 %)	0.623
	Below normal range (F: <186x10^9/l, M: <171x10^9/l)	4 (8.7 %)	6 (13.0 %)	4 (8.7 %)	
	Above normal range (F: >454x10^9/l, M: >388x10^9/l)	0 (0.0 %)	0 (0.0 %)	1 (2.2 %)	
Neutrophils, %		53.25 (43.90 – 58.70)	52.05 (44.70 – 56.40)	50.45 (45.60 – 63.50)	0.759
Neutrophil count, x10^9/l		2.47 (1.64 – 3.18)	2.36 (1.73 – 3.66)	2.53 (1.78 – 3.78)	0.729
Neutrophil count	Within normal range (F: 1.6 – 8.3 x10^9/l, M: 1.6 – 6.98 x10^9/l)	37 (80.4 %)	39 (84.8 %)	35 (76.1 %)	0.600
	Below normal range (<1.6x10^9/l)	9 (19.6 %)	7 (15.2 %)	10 (21.7 %)	
	Above normal range (F: >8.3x10^9/l, M: >6.98x10^9/l)	0 (0.0 %)	0 (0.0 %)	1 (2.2 %)	
Lymphocytes, %		31.75 (27.30 – 43.40)	33.00 (28.40 – 39.80)	33.30 (25.30 – 38.50)	0.897
Lymphocyte count, x10^9/l		1.62 (1.19 – 2.21)	1.58 (1.25 – 1.91)	1.57 (1.31 – 2.03)	0.991
Lymphocyte count	Within normal range (F: 1.4 – 4.5 x10^9/l, M: 1.4 – 4.2 x10^9/l)	29 (63.0 %)	32 (69.6 %)	31 (67.4 %)	0.683
	Below normal range (<1.4x10^9/l)	16 (34.8 %)	14 (30.4 %)	15 (32.6 %)	
	Above normal range (F: >4.5x10^9/l, M: >4.2x10^9/l)	1 (2.2 %)	0 (0.0 %)	0 (0.0 %)	
Monocytes, %		9.00 (7.00 – 11.00)	10.00 (8.00 – 12.00)	9.00 (7.00 – 12.00)	0.333
Monocyte count, x10^9/l		0.00 (0.00 – 1.00)	0.50 (0.00 – 1.00)	0.00 (0.00 – 1.00)	0.511

Parameter		Initial Recruitment N (%) or Median (IQR)	FU 6-months N (%) or Median (IQR)	FU 12-months N (%) or Median (IQR)	*p*-value
Monocyte count	Within normal range (F: 0.2 – 0.8 x10^9/l, M: 0.3 – 0.8 x10^9/l)	43 (93.5 %)	43 (93.5 %)	42 (91.3 %)	0.532
	Below normal range (F: <0.2x10^9/l, M: <0.3x10^9/l)	2 (4.3 %)	0 (0.0 %)	1 (2.2 %)	
	Above normal range (>0.8 x10^9/l)	1 (2.2 %)	3 (6.5 %)	3 (6.5 %)	
Eosinophils, %		3.00 (1.00 – 6.00)	3.00 (2.00 – 6.00)	3.00 (1.00 – 5.00)	0.895
Eosinophil count, x10^9/l		0.00 (0.00 – 0.00)	0.00 (0.00 – 0.00)	0.00 (0.00 – 0.00)	0.398
Eosinophil count	Within normal range (F: 0 – 0.4 x10^9/l, M: 0 – 0.95 x10^9/l)	40 (87.0 %)	44 (95.7 %)	42 (91.3 %)	0.334
	Above normal range (F: >0.4 x10^9/l, M: >0.95 x10^9/l	6 (13.0 %)	2(4.3 %)	4 (8.7 %)	
Basophils, %		1.00 (0.00 – 1.00)	1.00 (0.00 – 1.00)	1.00 (0.00 – 1.00)	0.608
Basophil count, x10^9/l		0.00 (0.00 – 0.00)	0.00 (0.00 – 0.00)	0.00 (0.00 – 0.00)	1.000
Basophil count	Within normal range (0 – 0.1 x10^9/l)	46 (100.0 %)	46 (100.0 %)	6 (100.0 %)	–
	Above normal range (>0.1x10^9/l)	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)	
Immature cells, %		0.00 (0.00 – 0.00)	0.00 (0.00 – 0.00)	0.00 (0.00 – 0.00)	0.941
Immature cell count, x10^9/l		0.00 (0.00 – 0.00)	0.00 (0.00 – 0.00)	0.00 (0.00 – 0.00)	1.000
CD45+ white cell count, x10^9/l		4.45 (3.70 – 5.96)	4.90 (3.47 – 5.84)	4.95 (3.39 – 6.05)	0.876
CD45+ white cell count	Above or within normal range (4 – >10x10^9/l)	28 (60.9 %)	31 (67.4 %)	29 (63.9 %)	0.803
	Below normal range (<4x10^9/l)	18 (39.1 %)	15 (32.6 %)	17 (37.0 %)	
CD4 percentage of lymphocytes, %		15.83 (11.83 – 21.20)	16.59 (13.61 – 23.87)	19.25 (15.92 – 23.26)	0.063
CD4 percentage of lymphocytes	Within normal range (28 – 51 %)	6 (13.0 %)	7 (15.2 %)	7 (15.2 %)	0.943
	Below normal range (<28 %)	40 (87.0 %)	39 (84.8 %)	39 (84.8 %)	
Symptoms at presentation					
Fever	No	46 (100.0 %)	43 (97.7 %)	46 (100.0 %)	0.349
	Yes	0 (0.0 %)	1 (2.3 %)	0 (0.0 %)	

Fatigue	No	45 (97.8 %)	45 (97.8 %)	44 (97.1 %)	0.773
	Yes	1 (2.2 %)	1 (2.2 %)	1 (2.9 %)	
Edema	No	45 (97.8 %)	46 (100 %)	45 (97.8 %)	0.602
	Yes	1 (2.2 %)	0 (0.0 %)	1 (2.2 %)	
Cachexia	No	45 (97.8 %)	46 (100 %)	44 (95.7 %)	0.360
	Yes	1 (2.2 %)	0 (0.0 %)	2 (4.3 %)	
Respiratory symptoms	No	43 (93.5 %)	45 (97.8 %)	44 (95.7 %)	0.593
	Yes	3 (6.5 %)	1 (2.2 %)	2 (4.3 %)	
Gastrointestinal disturbance	No	45 (97.8 %)	46 (100 %)	44 (95.7 %)	0.360
	Yes	1 (2.2 %)	0 (0.0 %)	2 (4.3 %)	
Arthralgia	No	44 (95.7 %)	45 (97.8 %)	44 (95.7 %)	0.813
	Yes	2 (4.3 %)	1 (2.2 %)	2 (4.3 %)	
Altered mental state	No	46 (100 %)	46 (100 %)	46 (100 %)	–
	Yes	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)	
Neuropathy	No	46 (100 %)	46 (100 %)	44 (95.7 %)	0.131
	Yes	0 (0.0 %)	0 (0.0 %)	2 (4.3 %)	
Radiographic abnormalities	No	46 (100 %)	46 (100 %)	44 (95.7 %)	0.131
	Yes	0 (0.0 %)	0 (0.0 %)	2 (4.3 %)	
Coughing	No	43 (93.5 %)	45 (87.8 %)	44 (95.7 %)	0.593
	Yes	3 (6.5 %)	1 (2.2 %)	2 (4.3 %)	
Loss of weight	No	44 (95.7 %)	44 (95.7 %)	44 (95.7 %)	1.00
	Yes	2 (4.3 %)	2 (4.3 %)	2 (4.3 %)	
Night sweats	No	42 (91.3 %)	45 (97.8 %)	45 (95.7 %)	0.349
	Yes	4 (8.7 %)	1 (2.2 %)	2 (4.3 %)	