THE ROLE OF HUMAN HERPESVIRUS (HHV) INFECTIONS AND PERSISTENT IMMUNE ACTIVATION IN ANTIRETROVIRAL THERAPY-TREATED HIV-INFECTED INDIVIDUALS

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THE ROLE OF HUMAN HERPESVIRUS (HHV) INFECTIONS AND PERSISTENT IMMUNE ACTIVATION IN ANTIRETROVIRAL THERAPY-TREATED HIV-INFECTED INDIVIDUALS

ABSTRACT

Co-infections with human herpesvirus (HHV) have been associated with residual chronic inflammation in antiretroviral therapy (ART)-treated human immunodeficiency virus (HIV)-infected individuals. However, the burden of HHV co-infection among HIV-infected individuals is unknown in the developing country and the role of HHV in modulating the kynurenine pathway and clinical outcomes in HIV-infected individuals is poorly understood. Thus, we investigated (1) the seroprevalence of and the risk factors associated with four common HHVs among treated HIV-infected individuals, (2) the association of HHV with kynurenine/tryptophan (K/T) ratio and (3) other chronic immune activation markers and the impact of HHV infection on long-term CD4 T-cell recovery in HIV/HHV co-infected individuals. In this cross-sectional study, HIV-infected patients receiving suppressive ART for a minimum of 12 months were recruited from the University Malaya Medical Centre (UMMC), Malaysia. Stored plasma was analyzed for CMV, VZV, HSV-1 and HSV-2 IgG antibody levels, immune activation markers (interleukin-6, interferon-γ, neopterin), kynurenine and tryptophan concentrations. The influence of the number of HHV co-infection and K/T ratio on CD4 T-cell recovery was assessed using multivariate Poisson regression. A total of 232 HIV-infected individuals was recruited and all participants were seropositive for at least one HHV; 96.1% with CMV, 86.6% with VZV, 70.7% with HSV-1 and 53.9% with HSV-2. Multivariate analysis revealed that a longer duration on ART was associated with an increased odd of both HSV-1 (aOR: 1.12; 95% CI: 1.02-1.22) and HSV-2 (aOR: 1.12; 95% CI: 1.04-1.21) infection. HSV-2 seropositivity was also associated with being female (aOR: 3.03; 95% CI: 1.31-7.0) and with a history of AIDS defining illness (aOR: 2.01; 95% CI: 1.12- 3.60). Indian ethnicity was associated with a lower odds of CMV (aOR: 0.17; 95% CI: 0.04-

0.80) and VZV (aOR: 0.26; 95% CI: 0.09-0.71) seropositivity compared to ethnic Chinese. In addition, higher current CD4:CD8 ratio was also significantly associated with lower odds of CMV seropositivity (aOR: 0.21; 95% CI: 0.07-0.68). K/T ratio was significantly positively correlated with antibodies for CMV (rho=0.205, *p*=0.002), VZV (rho=0.173, *p*=0.009) and a tendency with HSV-2 (rho=0.120, *p*=0.070); with CMV antibody titers demonstrating the strongest modulating effect on K/T ratio among the four HHVs. In multivariate analysis, higher K/T ratio (*p*=0.03) and increasing number of HHV co-infections (*p*<0.001) were independently associated with poorer CD4 T-cell recovery following 12 months of ART initiation. These data suggested that co-infection with multiple HHV are common among ART-treated HIV-infected participants in the developing country setting and are associated with persistent immune activation and poorer CD4 T-cell recovery.

Keywords: Immune activation, K/T ratio, cytomegalovirus, CD4 T-cell recovery, antiretroviral therapy

PERANAN JANGKITAN VIRUS HUMAN HERPES (HHV) DAN PENGAKTIFAN IMUN YANG BERTERUSAN DI KALANGAN INDIVIDU YANG DIJANGKITI HIV YANG DALAM RAWATAN ANTIRETROVIRAL

ABSTRAK

Jangkitan virus human herpes (HHV) termasuk virus cytomegalo (CMV), virus varicella zoster (VZV) dan virus human simplex (HSV) terhadap individu adalah berkaitan dengan lebihan keradangan yang kronik bagi individu yang dijangkiti virus human immunodeficiency (HIV) yang menerima rawatan antiretroviral (ART). Namun, beban terhadap jangkitan HHV di kalangan individu yang dijangkiti HIV tidak diketahui di negara yang membangun dan sebab itu peranan HHV dalam memodulasi jalur kynurenin dan hasil klinikal bagi individu yang dijangkiti HIV agak sukar difahami. Kajian dijalankan untuk mengkaji (1) kelaziman dan faktor risiko yang berkaitan dengan empat jenis HHV di kalangan individu yang dijangkiti HIV, (2) perkaitan HHV dengan nisbah kynurenine/tryptophan (K/T) dan (3) penanda pengaktifan imun kronik yang lain, dan kesan jangkitan HHV terhadap pemulihan sel T CD4 untuk jangka masa panjang bagi individu yang telah dijangkiti HIV/HHV. Dalam kajian rentas ini, pesakit yang dijangkiti HIV yang menerima rawatan ART sekurang-kurangnya 12 bulan telah dipilih dari Pusat Perubatan Universiti Malaya, Malaysia. Plasma yang disimpan telah digunakan untuk mengkaji tahap CMV, VZV, HSV-1 dan HSV-2 antibodi IgG, penanda pengaktifan imun (interleukin-6, interferon-γ, neopterin) dan kepekatan kynurenine dan tryptophan. Seramai 232 individu yang dijangkiti HIV direkrut dan semua peserta positif untuk sekurang-kurangnya satu HHV dengan 96.1% CMV, 86.6% VZV, 70.7% HSV-1 dan 53.9% HSV-2. Analisis pelbagai variasi menunjukkan peningkatan risiko menjangkiti HSV-1 (aOR: 1.12; 95% CI: 1.02-1.22) dan HSV-2 (aOR: 1.12; 95% CI: 1.04-1.21) adalah berkaitan dengan masa yang lama seseorang individu itu menerima rawatan ART. Positif HSV-2 juga dikaitkan dengan jantina wanita (aOR: 3.03; 95% CI: 1.31-7.0) dan individu dengan sejarah penyakit AIDS (aOR: 2.01; 95% CI: 1.12- 3.60). Berbanding

etnik Cina, etnik India mempunyai risiko yang rendah dijangkiti CMV (aOR: 0.17; 95% CI: 0.04-0.80) dan VZV (aOR: 0.26; 95% CI: 0.09-0.71). Di samping itu, nisbah CD4:CD8 yang lebih tinggi berkaitan dengan peningkatan risiko menjangkiti CMV (aOR: 0.21; 95% CI: 0.07-0.68). Nisbah K/T mempunyai korelasi positif bagi antibodi IgG CMV (p=0.002) dan VZV (p=0.009), dengan CMV menunjukkan kesan modulasi tertinggi pada nisbah K/T berbanding empat HHV. Dalam analisis pelbagai variasi, nisbah K/T (p = 0.03) dan penambahan nombor jangkitan HHV bagi individu (p <0.001) dikaitkan dengan kelemahan dari segi pemulihan sel T CD4 dengan 12 bulan rawatan permulaan ART. Data-data ini mencadangkan bahawa jangkitan bersama iaitu jangkitan pelbagai HHV mudah dijangkiti di kalangan pesakit HIV di bawah rawatan ART dan ini saling kaitan dengan pengaktifan imun yang berterusan dan kelemahan proses pemulihan sel T CD4.

Kata kunci: pengaktifan imun, nisbah K/T, virus cytomegalo, pemulihan sel T CD4, rawatan antiretroviral

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Yap Siew Hwei, 2018

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LIST OF SYMBOLS AND ABBREVIATIONS

AIDS	:	Acquired immune deficiency syndrome
aOR	:	Adjusted odds ratio
ART	:	Antiretroviral therapy
&	:	And
CDC	:	Centers for Disease Control and Prevention
CMV	:	Cytomegalovirus
CI	:	Confidence interval
CV	:	Coefficient of variation
mm^3	:	Cubic millimeter
DNA	:	Deoxyribonucleic acid
EBV	:	Epstein-Barr virus
ELISA	:	Enzyme-linked immunosorbent assay
ES-CBA	:	Enhanced sensitivity-cytometry bead array
ESI	:	Electrospray ionization
FDA	:	Food and Drug Administration
FRC	:	Fibroblastic reticular cell
GALT	:	Gut-associated lymphoid tissue
HCV	:	Hepatitis C virus
HHV	:	Human herpesvirus
HLA-DR	:	Human leukocyte antigen – antigen D related
HIV	:	Human immunodeficiency virus
HPLC	:	High performance liquid chromatography
hsCRP	:	High-sensitivity C-reactive protein
HSV	:	Human simplex virus

IDO	:	Indoleamine-2,3-deoxygenase
IDU	:	Intravenous drug user
ICH	:	International Conference on Harmonization
IFN-γ	:	Interferon-gamma
IgG	:	Immunoglobulin G
IL-6	:	Interleukin-6
IL-7Rα	:	Interleukin-7 receptor alpha
IS	:	Internal standard
IQR	:	Interquartile range
Kyn	:	Kynurenine
K/T ratio	:	Kynurenine/tryptophan ratio
>	:	Larger than
≥	:	Larger than or equal to
LC-MS/MS	:	Liquid chromatography-tandem mass spectrometry
L	:	Liter
LLOQ	:	Lower limit of quantification
p	:	Level of significance
m/z	:	Mass divider by charge number
µL	:	Microliter
mg	:	Milligram
mL	:	Milliliter
MRM	:	Multiple reaction monitoring
n	:	Sample size
ng	:	Nanogram
nmol	:	Nanomol
NNRTI	:	Non-nucleoside reverse-transcriptase inhibitors

OR	:	Odds ratio
PI	:	Protease inhibitors
PCR	:	Polymerase chain reaction
PLWH	:	People living with HIV
pg	:	Picogram
%	:	Percent
QC	:	Quality control
RNA	:	Ribonucleic acid
rpm	:	Revolutions per minute
<	:	Smaller than
≤	:	Smaller than or equal to
sCD14	:	Soluble CD14
SMART	:	Strategies for Management of Antiretroviral Therapy
SNAE	:	Serious non-AIDS event
SOM	:	Self-organising map
SPSS	:	Statistical Package for the Social Sciences
START	:	Strategic Timing of Antiretroviral Therapy
STD	:	Sexually transmitted disease
sTNFR	:	Soluble tumor necrosis factor receptor
TFA	:	Trifluoroacetic acid
T_H17 cells	:	T helper 17 cells
TNF-α	:	Tumor necrosis factor-alpha
T_{reg} cell	:	T regulatory cell
Trp	:	Tryptophan
UMMC	:	University of Malaya Medical Center
VZV	:	Varicella zoster virus

CHAPTER 1: INTRODUCTION

1.1 Introduction

Human immunodeficiency virus (HIV) infections are still a widespread epidemic in many countries (UNAIDS, 2013). In Malaysia, 3,330 new HIV infections were reported in 2015; contributing to a plateauting epidemic which peaked in 2002 (6,978 cases). By the end of 2015, Malaysia had reported a cumulative 108,519 HIV cases, 22,485 acquired immunodeficiency syndrome (AIDS) cases and 17,916 HIV/AIDS related deaths. The number of people reported to be living with HIV in 2015 was 92,895 with key affected populations including people who inject drugs, female sex workers, transgender people and men who have sex with men, reporting rates of infections exceeding 5% (Disease Control Division, Ministry of Health Malaysia, 2016).

The survival among HIV-infected individuals has significantly improved with the introduction of antiretroviral therapy (ART). Combination antiretroviral treatment has resulted in significant increases in peripheral CD4 T cells counts in patients with sustained undetectable HIV replication (Lima et al., 2007; Moore & Keruly, 2007). However, a subset of patients (from 15% to 40%) continue to experience sub-optimal CD4 T-cell recovery despite years of suppressive ART (Gazzola et al., 2009; Horta et al., 2013; Negredo et al., 2010; Onen et al., 2009). Sub-optimal immune reconstitution has been associated with an increased risk of morbidity and mortality related to serious non-AIDS events (SNAEs), including cardiovascular disease, non-AIDS malignancies, liver disease, bone-related abnormalities and cognitive impairment (Brouwer et al., 2014; Kelley et al., 2009; Kowalska et al., 2012; Lau et al., 2007). The development of SNAEs was initially attributed to ART use but the findings of Strategies for Management of Antiretroviral Therapy (SMART) trial have refuted this notion. The SMART trial compared episodic use of ART guided by achieving thresholds of CD4 T-cell counts with the current practice of continuous ART. The study aim was to assess if treatment-sparing strategies might

provide the benefits of minimizing the risk of adverse events and other risks associated with long-term ART use. Nevertheless, patients on episodic ART were found to have 1.8-fold increase in mortality and 1.7-fold increase in SNAEs when compared to those on continuous ART (El-Sadr et al., 2006). This indicates that the risk of SNAEs initially attributed to ART was not reduced with the episodic regime. Instead, it was found that episodic ART was associated with increased chronic immune activation and inflammation as measured by increased soluble CD14 (sCD14), interleukin-6 (IL-6) and D-dimer (Kuller et al., 2008; Tebas et al., 2008). Following the SMART trial, numerous other studies have confirmed that chronic immune activation and inflammation are associated with SNAEs and sub-optimal CD4 T-cell recovery (Kelley et al., 2009; Nakanjako et al., 2011; Teixeira et al., 2001). Thus, understanding the mechanisms that modulate immune recovery in individuals with sustained viral suppression remains a high priority in the management of HIV; especially in resource limited setting where morbidity related to HIV remains high.

Immune activation is a major contributor to the pathogenesis in HIV. Chronic immune activation has been associated with impaired CD4 T-cell recovery by increasing apoptosis and intrinsic T-cell death even in patients receiving suppressive ART (Nakanjako et al., 2011). Multiple factors have been shown to be associated with persistent immune activation including microbial translocation, residual viral replication, dysregulation of the immune system with shifts in the balance of T helper 17 (T_H17) to regulatory T (Treg) cells, and co-infections (Brenchley et al., 2006; Favre et al., 2010; Mavigner et al., 2009). Co-infections are potentially more important in driving chronic immune activation and its associated morbidity in developing countries given that endemic infections are generally more prevalent in this setting (Lichtner et al., 2014; Sheth et al., 2008).

Human herpesvirus (HHV), which constitutes eight types of viruses (described in the next section), is among the most prevalent infections particularly among

immunocompromised individuals in the developing world (Conde-Glez et al., 2013; Romanowski et al., 2009; Schaftenaar et al., 2014). Co-infections with HHVs including cytomegalovirus (CMV), herpes simplex virus (HSV), human herpesvirus 8 (HHV8), and varicella zoster virus (VZV), are associated with residual chronic inflammation in HIV infected individuals receiving suppressive ART (Masiá et al., 2014; Sheth et al., 2008; Smith, et at., 2013; Wittkop et al., 2013). In particular, CMV has been found to be directly associated with SNAEs, cardiovascular morbidity, stroke, poorer neurocognitive performance, frailty and disruption of epithelial junctions in the gut in HIV-infected individuals, while HSV and VZV infections have been associated with an increased risk of stroke in HIV patients and general population (Hechter et al., 2012; Leng & Margolick, 2015; Letendre et al., 2012; Lichtner et al., 2014; Maidji et al., 2017; Parrinello et al., 2012; Yang et al., 2016; Yen et al., 2016). An increasing burden of multiple HHV co-infections were also found to be associated with increased atherosclerosis in the Multicenter AIDS Cohort Study (MACS) (Hechter et al., 2012). Thus targeting the immune activation pathways associated with HHV infections in HIV may be a potential therapeutic option to augment CD4 T-cell recovery and reduce morbidity in HIV-infected individuals in resource limited settings. Few studies to date, however, have explored the role of HHV co-infections on immune reconstitution following ART. One such cross-sectional study conducted in Canada found that CMV seropositive group in ART treated HIV-infected individuals had significantly lower CD4/CD8 T-cell ratios and more phenotypic evidence of immune senescence, which potentially implies that CMV infection may be associated with poorer immune reconstitution in HIV infection (Barrett et al., 2014).

Innate and cell-mediated responses play a vital role in the control of HHV infections and interferon-γ (IFN-γ) is one of the main antiviral cytokines produced following these viral infections (Singh et al., 2003; Berg et al., 2010). Besides, IFN-γ is the main inducer

of the indoleamine-2,3-deoxygenase (IDO) enzyme, a rate limiting enzyme which catabolizes the essential amino acid tryptophan to kynurenine (Bipath et al., 2015). The IDO activity, which is measured by changes in the ratio of tryptophan and kynurenine (herein referred to as K/T ratio), remains elevated in HIV-infected patients despite ART (Chen et al., 2014) and is associated with increased mortality, reduced T-cell proliferation, poorer CD4 T-cell reconstitution of the gut-associated lymphoid tissue (GALT), and neurological dysfunction (Boasso et al., 2007; Baran et al., 2012; Byakwaga et al., 2014; Jenabian et al., 2015). While the role of HHV and particularly CMV in driving T-cell activation and senescence is well described in HIV (Naeger et al., 2010; Barrett et al., 2014; Redd et al., 2015), its exact role in modulating the IDO pathway in treated HIV-infected individuals especially in the developing country setting where seroprevalence is high, is less well studied.

1.2 Research hypothesis

Chronic latent infections with common HHVs (CMV, HSV, and VZV) are associated with derangement in tryptophan metabolism and chronic immune activation. This in turn may influence long-term immune recovery in HIV-infected individuals receiving suppressive ART.

1.3 Objectives of the study

Our specific objectives are:

i. To investigate the prevalence and risk factors associated with HHV infection in HIV-infected patients.
ii. To study the association between HHV infection with kynurenine/tryptophan ratio and other markers of chronic immune activation.

iii. To assess if HHV infection is associated with CD4 T-cell recovery following long-term suppressive ART.

1.4 Significance of the study

i. This study will provide useful insights into the mechanistic immune activation pathways associated with HHV co-infections in HIV-infected patients receiving long-term suppressive ART.

ii. The work will also contribute to a better understanding of the potential drivers modulating the kynurenine pathway and its associated effects on other immune activation markers and in the setting of HHV-HIV co-infections.

iii. The results of these investigations will provide evidence of the impact of HHV infections in modulating CD4 T-cell recovery in settings where HHV-HIV co-infections are high and the potential utility of interventions that target this pathway to augment sub-optimal CD4 T-cell recovery in individuals living in resource limited settings.

CHAPTER 2: LITERATURE REVIEW

2.1 Epidemiology of HIV

HIV infection has remained an epidemic in many countries and the most affected region is eastern and southern Africa with 19.0 million people living with HIV (PLWH) in 2015. In Asia and the Pacific region, total of 5.1 million people are living with HIV infection, with 300,000 of new cases reported in 2015. AIDS-related deaths across this region has declined 25% since 2010 to an estimated 180,000 in 2015 with more than 2 million people on ART (UNAIDS, 2016).

In Malaysia, there was an estimated 92,895 PLWH at the end of 2015 with 3,330 cases were reported new HIV infections. By the end of 2015, 25,600 PLWH were on life-saving ART, which translated to only 27.6% of treatment coverage. Thus, more strategic plans should be implemented to prevent and reduce the risk and spread of HIV infection and to improve the coverage of ART to PLWH (Disease Control Division, Ministry of Health Malaysia, 2016).

2.2 Natural history of HIV infection

2.2.1 Transmission risks

AIDS was a widespread disease in the 1980s and intense research led to the discovery of HIV as the cause of this transmissible disease. The disease is marked by a profound deficiency in cellular immune functions that most commonly manifest clinically as opportunistic infections. There are 3 major routes of HIV transmission: through semen and blood during intimate sexual contact, sharing needles or other drug equipment, and mother-to-child transmission (Livingston, 1992). Factors that increase the risk of HIV transmission are the number of HIV ribonucleic acid (RNA) copies per mL of plasma (viral load) (Quinn et al., 2000), co-infection with other sexually transmitted infections

(HSV-2 infection and bacterial vaginosis) (Røttingen, Cameron, & Garnett, 2001), pregnancy (Mugo et al., 2011), receptive anal intercourse (Baggaley, White, & Boily, 2010) and sexual behavior (many sexual partners and concurrent partnerships) (Epstein & Morris, 2011; Tanser et al., 2011). Populations which have higher risk of acquiring HIV include intravenous drug user (IDU), female sex workers, men who have sex with men and transgenders.

2.2.2 HIV disease pathogenesis

The main target of HIV is activated CD4 T lymphocytes, whereby HIV interacts with the CD4 receptor and the chemokine co-receptors, CCR5 or CXCR4. However, other cells expressing CD4 and chemokine receptors may also be infected, including resting CD4 T-cells, monocytes and macrophages, and dendritic cells, albeit a lesser extent. Once HIV binds to CD4 and co-receptor, HIV fuses into the T-cell, where reverse transcription of viral RNA genome takes place. The double-stranded deoxyribonucleic acid (DNA) of the virus integrates into host cell DNA. Transcription and translation of viral genome subsequently occur to produce more viruses, which eventually releases new HIV virions into the blood stream (Walker & Colledge, 2013). Thus, viral load is extremely high in the early phase of HIV infection.

CD4 T-cells are eliminated by direct infection, and bystander effects of syncytia formation, immune activation, proliferation and senescence. Besides, following acute viral infection, T-cell homoeostasis is affected not only in the periphery but also in the GALT which carries more than 80% of the bodies T lymphocytes (as shown in figure 2.1). In addition to loss of total CD4 T-cell, profound changes in T-cell subsets happen, including preferential loss of T-helper-17 cells, and mucosal-associated invariant T cells, which are crucial defence against bacteria in the GALT. The profound depletion of lymphoid cells in the GALT, together with enterocyte apoptosis, and enhanced

gastrointestinal tract permeability, leads to continuous local and systemic exposure of microbial products, such as lipopolysaccharides. Hence, both direct HIV-infection and indirect mechanisms result in CD4 T-cell loss and broad immunodeficiency in HIV-infected patients.

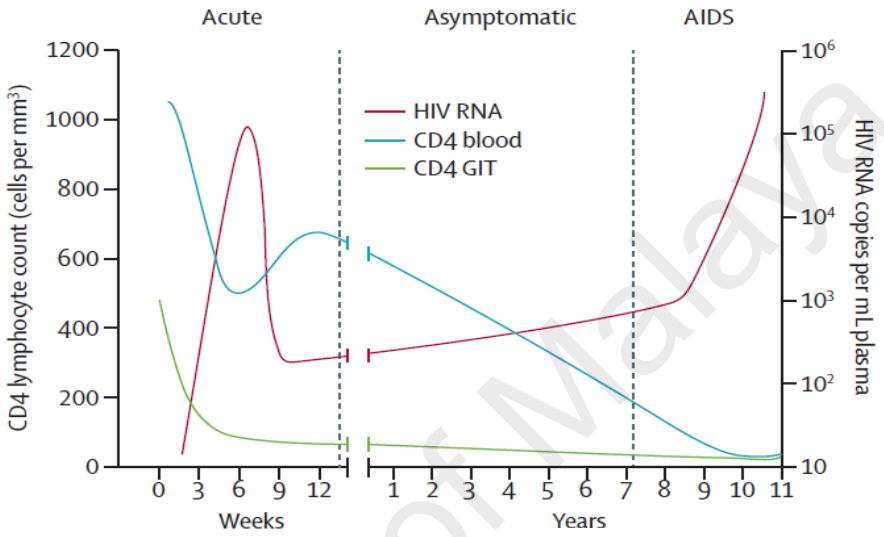

Figure 2.1: Natural history of HIV infection. As HIV enters and replicates within CD4 T cells in the immune system, the viral load increases sharply and there is a corresponding dip in the number of CD4 T-cell. The CD4 T cells in the gastrointestinal tract (GIT) are rapidly depleted early on (Adapted from Maartens, Celum, & Lewin, 2014).

AIDS is the advanced stage of untreated HIV infection. The Centers for Disease Control and Prevention (CDC) have identified illnesses that are considered AIDS-defining, including tuberculosis, CMV, candidiasis, Kaposi's sarcoma among others, and if left untreated will lead to death. Several factors acting concurrently appear to initiate this advanced stage. The gradual drop in CD4 T-cells eventually results in an immunodeficient state, where opportunistic infections are easily acquired by the patient. Activation of virally infected T-cells by antigen results in stimulation of viral transcription and progeny formation. This leads to accelerated T-cell death and exacerbating the immunodeficient state. Rapid viral replication also increases the viral

mutation rate, allowing escape from any HIV-specific immune controls that might remain (Coico & Sunshine, 2009).

2.3 ART and immune recovery following ART

ART regimens were developed in the late 1990s and have improved morbidity and mortality in HIV-infected individuals who achieve viral suppression while on ART (Palella et al., 1998). HIV has since become a chronic manageable disease instead of a progressive illness with a fatal outcome. Standard ART regimens include the combination of two nucleoside reverse transcriptase inhibitors with a non-nucleoside reverse transcriptase inhibitor (NNRTI), protease inhibitor (PI) or integrase inhibitor. After the initiation of ART, most HIV-infected individuals will achieve viral suppression to undetectable plasma levels of HIV RNA (Maartens et al., 2014) with a rapid increase in the peripheral CD4 T-cell counts. Besides, the amount of circulating inflammatory cytokines and chemokines such as tumor necrosis factor-α (TNF-α), IL-6 and monocyte chemoattractant protein-1 (MCP-1), which are elevated during infection, reduces. The number of CD8 T cells, natural killer cells and B cells are also increased in the peripheral blood after ART, which appears to reflect nonspecific mechanisms such as cell redistribution. This is because HIV replication in lymph nodes and GALT results in the sequestration of activated lymphocytes in these tissues. Arrested viral replication upon ART results in the nonspecific release of lymphocytes into peripheral blood. Because only a slight reduction in viral load is required to generate this T-cell redistribution, recovery of the CD4 T-cell compartment is sometimes observed despite only a modest reduction in viral load. Thus in the first 2-3 months after ART, the increased number of CD4 T-cells primarily reflect recovery from immunopathological alterations and inflammatory responses to the infection, but there is no or very little *de novo* production of immune cells (Guihot et al., 2011).

Over several years of ART, the extent of recovery depends mostly upon the magnitude and sustainability of viral control but not on the extent of CD4 T-cell depletion before treatment. Mechanistically, this results mostly from regeneration of the naïve CD4 T-cell population, which restores the diversity of the CD4 T-cell receptor repertoire. Moreover, before ART is initiated, the conversion of naïve CD4 T cells to memory cells is accelerated to partially compensate for the functional defects and loss of activated memory CD4 T cells that result from uncontrolled virus replication. ART stops this phenomenon, reducing differentiation of naïve CD4 T cells and allowing the naïve CD4 T-cell compartment to expand. Therefore, production of CD4 T cells in the thymus and passive accumulation of resting naïve CD4 T cells in the periphery contribute to immune reconstitution.

2.3.1 Sub-optimal immune recovery

Despite improved access to ART, a subset of patients continue to experience sub-optimal CD4 T-cell recovery (15-40%) despite suppression of HIV replication (Gazzola et al., 2009; Horta et al., 2013; Negredo et al., 2010; Onen et al., 2009). Studies have shown that patients who initiate treatment at high baseline CD4 T-cell counts are more likely to achieve normalized CD4 T-cell count thresholds (>500 cells/µl). In a study by Kelley et al. involving 366 ART-treated individuals, 95% of patients who started ART when their CD4 cell count was more than 300 cells/mm^3, were able to attain a CD4 T-cell count >500 cells/mm^3. However, in a subset of patients, especially those starting ART at CD4 T-cell count <200 cells/mm^3, CD4 T-cell count failed to reach >500 cells/mm^3 despite a mean duration of follow up of 7.5 years (Kelley et al., 2009). This group of individuals are often referred to as immunological non-responders (Horta et al., 2013) and they have higher risks of clinical progression to AIDS and SNAEs and death (Guihot

et al., 2011). Multiple factors have been associated with suboptimal CD4 T-cell recovery following ART, detailed in the following sections.

2.3.2 Factors associated with sub-optimal immune recovery

2.3.2.1 Chronic immune activation

Chronic immune activation is an established characteristic of chronic HIV disease. Persistent immune activation may be measured by the presence of activation markers, including CD38 and human leukocyte antigen (HLA)-DR, on the surface of T-cells and increased levels of circulating proteins, including neopterin, beta-microglobulin, soluble TNF receptor (sTNFR) II, immunoglobulin (Ig) A, D-dimer, IL-6 and C-reactive protein (CRP) (Fahey et al., 1990; Mildvan et al., 2005; Neuhaus, Jacobs, et al., 2010). The cause of chronic immune activation in HIV is multifactorial and includes:

(a) Microbial translocation

As mentioned in previous section, HIV destroys the CD4 T cells in GALT in the early phase of infection and these changes (both structural and immunological) do not normalize even after years of ART. This destruction results in leaky immune barrier, causing the translocation of microbial products from the gut lumen to the systemic circulation (Figure 2.2). Studies from many groups including ours have found that plasma lipopolysaccharide levels, an indicator of microbial translocation, were significantly increased in chronically HIV-infected individuals (Brenchley et al., 2006; Jiang et al., 2009; Rajasuriar et al., 2010). Microbial products have been linked to the increased cytokine production, direct activation of plasmacytoid dendritic cell, and direct activation of CD4 and CD8 cells (Brenchley et al., 2006). Systemic microbial products remain elevated even after effective ART in HIV-infected patients and plasma bacterial 16S rDNA levels is negatively correlated with the rate of increase in CD4 T-cell count, which

suggests that the association between microbial translocation and CD4 T-cell homeostasis in more closely related to the cellular turnover of chronic infection than to the rapid first-phase increase in circulating CD4 T cells after the application of ART (Jiang et al., 2009).

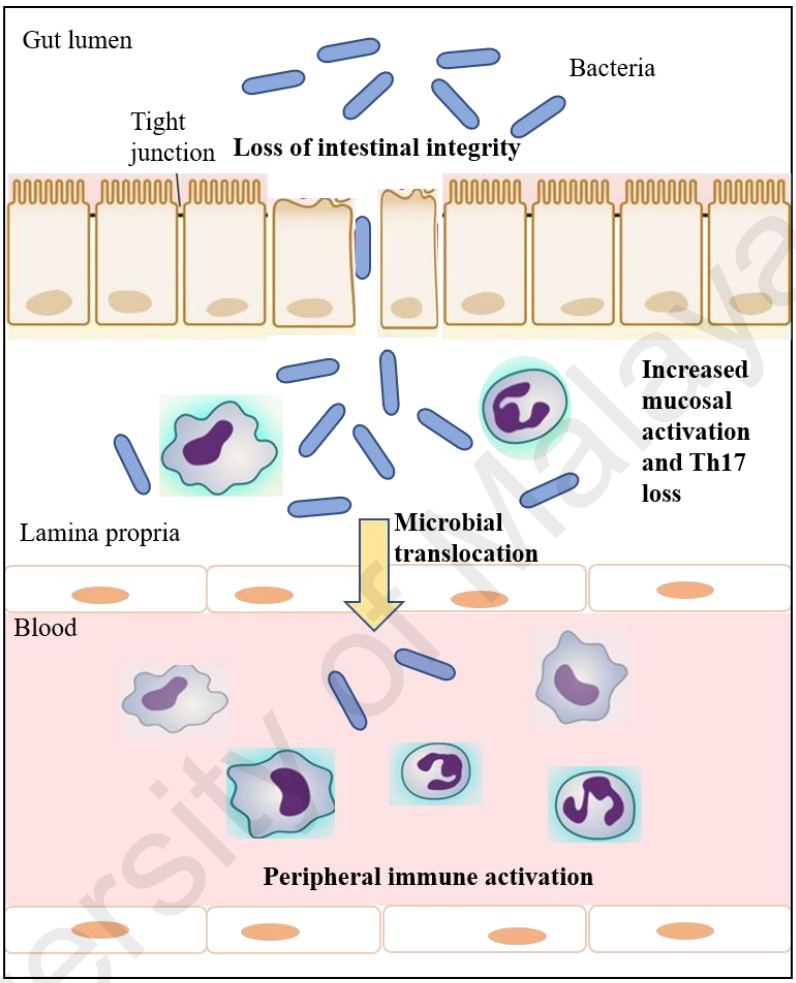

Figure 2.2: Illustration of microbial translocation from the gut to the blood circulation.

(b) Residual viral replication

Persistent production of viral particles by reservoir cells in aviremic treated patients even in the absence of ongoing replication may induce immune activation. This is because of the anti-HIV immune response and of the fact that various HIV components are able to stimulate the immune system. Furthermore, in most individuals with clinically undetectable HIV RNA on ART (<20 copies/mL in conventional polymerase chain reaction (PCR)-based assay), low level viremia (<1 copy/mL) can be detected in plasma

using more sensitive single-copy research based assays (Palmer et al., 2008). Residual plasma virus load was found to be higher in poor immunological responders than in the good immunological responders in HIV-infected patients who received sustained effective ART for a median duration of seven years, implying that low-level virus production may contribute to persistent T-cell activation and poor immune reconstitution despite ART (Mavigner et al., 2009).

(c) Altered T_H17/T_{reg} ratio

Studies have also shown that the degree of microbial translocation and T-cell activation in progressive HIV disease is tightly associated with the skewed maturation of the cells in the T_H17/T_{reg} axis (Favre et al., 2010). T_H17 cells are important in controlling bacterial growth in mucosal tissues. Favre et al. demonstrated that noncontrollers with more advanced disease (with CD4 T-cell count of <350 cells/μL and viral load of >10,000 copies/mL) has a reduced proportion of memory T_H17 cells than HIV-negative subjects and an increased proportion of memory T_{reg} cells compared to controllers (with CD4 T-cell count of >500 cells/μL and viral load of <2,000 copies/mL), HIV-infected subjects with suppressed viral loads (with undetectable viral load or ART), and HIV-negative (Favre et al., 2010). T_H17/T_{reg} ratio was about 5 and 10 times lower in noncontrollers with advanced CD4 depletion when compared with noncontrollers with preserved CD4 counts and HIV-negative individuals, respectively (Favre et al., 2010).

(d) Co-infections

The other possible sources of immune activation come from infectious agents other than HIV. For example, hepatitis C virus (HCV) co-infection in HIV-infected patients have high levels of CD8 and CD4 T-cell immune activation despite effective ART-mediated suppression of HIV (Gonzalez et al., 2009). Furthermore, HIV-infected patients

who experienced a Mycobacterium avium complex infection during their follow up and started an ART regimen, presented with a poorer increase in their total lymphocytes, CD4 and CD8 T-cell counts despite similar virological response (Lazaro et al., 2006). Thus, co-infection with other viruses may influence for immune reconstitution following ART.

(e) Derangement of K/T ratio

Numerous studies have been performed to investigate the involvement of tryptophan metabolism in driving immune activation and T-cell proliferation among HIV infected individuals (Appay & Sauce, 2008). Tryptophan is an essential amino acid for protein synthesis and formation of serotonin and melatonin. IDO is an intracellular, non-secreted enzyme, which catabolizes tryptophan to produce kynurenine (Figure 2.3). IDO is the rate limiting enzyme in tryptophan catabolism. After the conversion of tryptophan to kynurenine, kynurenine is metabolized further to kynurenine acid or 3-hydroxykynurenine, which is converted to either xanthurenic acid or 3-hydroxyanthranilic acid (Figure 2.3). Up-regulation of tryptophan-kynurenine pathway affects the formation of serotonin due to competition for the availability of tryptophan. It also leads to an increase production of kynurenine, which is an intermediate of the end product quinolinic acid (Guillemin et al., 2005). IFN-γ is the main cytokine that regulates the IDO enzyme in increased cellular immune activation (Frumento et al., 2002). IFN-γ also stimulates the production of neopterin by macrophages. Blood K/T ratio is a marker of IDO activity in human studies (Oxenkrug, 2011). It has been implicated in the overactivation of tryptophan catabolism in HIV-1 infection. Multiple factors have been shown to induce IDO activity in HIV-infected including the presence of AIDS-defining illness (Huengsberg et al., 1998), HIV-1 Tat proteins (Samikkannu et al., 2009) and increased levels of cytokines such as IFN-α and -β, TNF-α, and platelet activating factor, which are all elevated in HIV infection (Yasui et al., 1986). Terness et al. (2002)

demonstrated that L-kynurenine and other tryptophan metabolites such as 3-hydrokynurenine and 3-hydroxyanthranilic acid were able to completely inhibit the proliferation of T-cells stimulated by allogeneic dendritic cells and suppressed proliferation of CD3-stimulated T-cells. They found that all T-cell died at suppressive concentrations of kynurenine, 3-hydrokynurenine and 3-hydroxyanthranilic acid in *in vitro* experiments (Terness et al., 2002). Higher IDO activity was also observed in HIV-infected patients naïve to therapy compared to healthy controls, and ART could decrease IDO activity, but did not normalize the activity of IDO (Chen et al., 2014). Besides, study has also found that higher pre-ART K/T ratio in plasma was strongly associated with lower CD4 T-cell counts and a higher plasma K/T ratio, before ART and during ART, strongly predicted lower late CD4 T-cell recovery and mortality in HIV-infected individuals (Byakwaga et al., 2014).

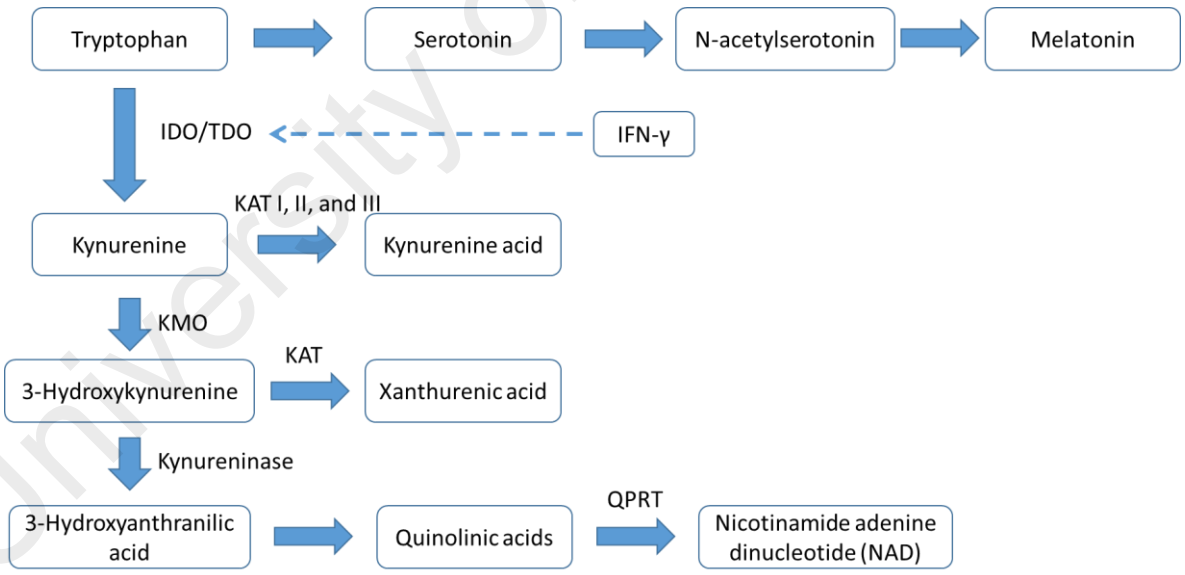

Figure 2.3: The Kynurenine pathway. Tryptophan is converted to kynurenine either by indoleamine 2,3-dioxygenase (IDO) or by tryptophan 2,3-dioxygenase (TDO). Kynurenine is metabolized further to kynurenine acid by kynurenine aminotransferase (KAT) or 3-hydroxtkynurenine by kynurenine mono-oxygenase (KMO). 3-hydroxykynurenine is converted to either 3-hydroxyanthranilic acid by kynurenase or xanthurenic acid by KAT. 3-hydroxyanthranilic acid is further metabolized to quinolinic acids and quinolinic acids is then converted to nicotinamide adenine dinucleotide (NAD) by quinolinate phosphoribosyl transferase (QPRT).

2.3.2.2 Lymph node fibrosis

HIV infection associated chronic immune activation and persistent inflammation has been found to induce profound damage to secondary lymphoid tissues that limit restoration of CD4 T-cell populations (Diaz et al., 2011; Estes, 2009; Estes et al., 2007; Nies-Kraske et al., 2009; Schacker et al., 2006; Zeng et al., 2011). Studies have shown that lymphoid depletion was seen in HIV-infected patients who had developed AIDS (Paiva et al., 1996). The link between CD4 T-cells and lymphoid tissue damage was further confirmed by the observation that the extent of lymphoid tissue fibrosis was inversely correlated with the size of the CD4 T-cell population within the T-cell zone compartment of lymph nodes in HIV-infected patients (Diaz et al., 2010, 2011; Schacker et al., 2002). Fibrosis is a condition marked by increased extracellular matrix constituents, primarily the fibrillary collagens, produced by resident tissue fibroblasts and is often results from a chronic inflammation insult (Wight & Potter-Perigo, 2011). The functional component of the tissue is gradually replaced by the accumulating extracellular matrix proteins, disrupting normal tissue structure and function (Wight & Potter-Perigo, 2011). Besides, HIV infection is associated with stage-specific progressive decreases in the fibroblastic reticular cell (FRC) network, the major source of the T-cell survival factor interleukin 7 (IL-7). The depletion of FRCs correlates with an increase in collagen deposited outside the FRC network. As infection progresses, fewer T cells have access to IL-7 on the FRC network compared with uninfected populations (Zeng et al., 2011, 2012). Furthermore, the loss of FRC network and collagen deposition significantly increased the apoptosis of naïve T-cell, due to the restriction of IL-7 access and depletion of the source of IL-7 (Zeng et al., 2011, 2012). Thus, progressive damage to these tissues could have deleterious effects on lymphocyte populations and function.

2.3.2.3 Other factors

(a) Low CD4 nadir

Low nadir CD4 T-cell count has been found to be associated with unsatisfactory immune recovery in discordant patients (Negredo et al., 2010). Moreover, patients with incomplete CD4 T-cell responses commenced ART at lower CD4 T-cell counts than patients with complete CD4 T-cell responses and the annual increases in CD4 T-cell count were significantly smaller for incomplete responders, compared with complete responders (Kaufmann et al., 2005).

(b) Old Age

A study conducted to determine whether age is related to immune recovery among 1956 HIV-infected patients with ART, found that patients who were older (>37.1 years) were significantly less likely to experience an increase of $\geq 200 \times 10^6$ CD4 cells/L than were the youngest age groups (Viard et al., 2001). Besides, patients who were older had a higher background of immune activation than younger ones (Desai, Grolleau-Julius, & Yung, 2010).

(c) IL-7 receptor alpha (IL-7Rα) polymorphisms

Genetic markers, specifically the IL-7Rα genotype, has been found to be associated with immune recovery following ART. For instance, IL-7Rα has 4 common haplotypes and IL-7Rα haplotype 2 (which contains thymine at exon 6) were found to independently predict faster CD4 T-cell recovery to counts of >500 cells/μL (Rajasuriar et al., 2010).

2.3.3 Clinical consequences of sub-optimal CD4 T-cell recovery

2.3.3.1 AIDS-related events

The introduction of ART has significantly improved morbidity and mortality in HIV-infected patients (Palella et al., 1998). However, despite treatment the risk of getting different AIDS-related events was not zero even with ART. The Strategic Timing of Antiretroviral Therapy (START) study was conducted by the International Network for Strategic Initiatives in Global HIV Trials (INSIGHT) to investigate the risks and benefits of immediate initiation of ART in HIV-positive patients who have a CD4+ count of more than 500 cells/mm^3, as compared with deferring initiation until the CD4+ count is 350 cells/mm^3. This study found that in deferring-initiation group, the hazard ratio was higher compared to immediate-initiation group for serious AIDS-related events, which included death from AIDS or any AIDS-defining event including tuberculosis, Kaposi's sarcoma and malignant lymphomas (INSIGHT START Study Group et al., 2015). This study showed that the initiation of treatment early at higher CD4 T-cell counts was essential in reducing the risks of an AIDS-related event.

2.3.3.2 SNAEs

The SMART trial has proven that SNAEs are associated with sub-optimal CD4 T-cell recovery (El-Sadr et al., 2006; Kelley et al., 2009; Nakanjako et al., 2011). Lau and colleagues (2007) demonstrated that there is a decline in AIDS-related mortality but the decline was offset by an increase in non-AIDS related mortality. Even with higher CD4 T-cell counts, SNAE occurs more frequently and are associated with a greater risk of death than AIDS events (Neuhaus et al., 2010). SNAEs include cardiovascular disease, renal disease, liver disease, non-AIDS malignancies, bone-related abnormalities and cognitive impartment (Belloso et al., 2010; Brouwer et al., 2014; Kowalska et al., 2012; Neuhaus, Angus, et al., 2010; Wester et al., 2011). Additionally, the recent landmark

START study, which evaluated the outcomes of the immediate initiation and deferred initiation of ART, showed that lower hazard ratio was observed in immediate-initiation group compared to deferred-initiation group for a serious non-AIDS-related event, which suggests that ART should be recommended for patients with HIV regardless of the CD4 T-cell count (INSIGHT START Study Group et al., 2015).

2.4 HHV co-infections in HIV-infected patients

2.4.1 Types of HHV and prevalence in HIV versus the general population

Members of *Herpesviridae* family have been identified in a variety of animals and they all share certain features, including an ability to establish latency following primary infection, as well as a potential to reactivate and cause further disease (Grinde, 2013). *Herpesviridae* are composed of a double-stranded DNA genome contained within a nucleocapsid surrounded by a lipid envelope (Dollard et al., 2010). There are eight known HHVs that infect and cause disease in humans, which includes HSV, VZV, CMV, Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated viruses, as shown in Table 2.1. Most HHVs are likely to be acquired during childhood or during sexual debut, and they infect most of the human population worldwide, with some regional differences (Dollard et al., 2010). As HIV-infected individuals are prone to opportunistic infections due to their progressive loss of immune function, almost all individuals infected with HIV are also co-infected with one or more HHV (Gianella et al., 2015). The estimated prevalence for HSV-1, HSV-2, CMV and VZV in United States are 50-70%, 20-50%, 40-70% and 85-95%, respectively. Comparatively, the estimated prevalence for HSV-1, HSV-2, CMV, and VZV in HIV-infected individuals are higher than healthy adults, which are 90-100%, 50-90%, 90-100% and 90-100%, respectively (Gianella et al., 2015).

Table 2.1 Characteristics of HHV infections.

Type of HHV	Subfamily	Target cells	Route of transmission
HSV-1	α	Mucoepithelial	Oral or STD
HSV-2	α	Mucoepithelial	STD
VZV	α	Mucoepithelial	STD and respiratory
EBV	γ	B cells and epithelial cells	Saliva, STD, transfusions/transplant, congenital
CMV	β	Monocytes, lymphocytes and epithelial cells	Saliva, STD, transfusions/transplant, congenital
HHV-6	β	T lymphocytes and others	Oral
HHV-7	β	T lymphocytes and others	Oral
HHV-8	γ	Endothelial cells	Oral or STD

Abbreviations: CMV, cytomegalovirus; EBV, Epstein-Barr virus; HHV, human herpesvirus; HSV, herpes simplex virus; STD, sexually transmitted disease; VZV, varicella zoster virus.

2.4.2 Difference in HHV prevalence and risk factors in developed and developing country settings

HHV seroprevalence among HIV-infected and ART-naïve adults in rural South Africa has been found to be high, as shown in Table 2.2 (Schaftenaar et al., 2014). In contrast to the developed country setting, in Canada, the seroprevalence for HSV-1 and HSV-2 in HIV-infected patients was lower compared to developing country (Table 2.2) (Romanowski et al., 2009). On the other hand, the seroprevalence for CMV in Iran is quite similar to South Africa, both being developing countries (Mehrkhani et al., 2011). Seropositive status of HHVs are associated with several risk factors, which are listed in Table 2.2. The risk factors are different in developing and developed countries probably due to the distinction in socioeconomic factors and geographical location.

Table 2.2 Seroprevalence of and risk factors for HHV, mainly HSV-1, HSV-2, CMV and VZV infections in developing and developed countries.

HHV type	Seroprevalence (%)			Risk factors
	Rural South Africa[a]	Iran[b]	Canada[c]	
HSV-1	98%	-	78.1%	Education[a], income[a], accessible to drinking water and latrine[a], country of birth[c], race[c] and risk behavior[c],
HSV-2	87%	-	54.6%	Age[a], ethnicity[a], accessible to drinking water and latrine[a], gender[c], country of birth[c], race[c] and risk behavior[c],
CMV	100%	94%	-	Did not analyze
VZV	87%	-	-	Age, education and marital status

Abbreviations: CMV, cytomegalovirus; HHV, human herpesvirus; HSV, herpes simplex virus; VZV, varicella zoster virus.
[a] From Schaftenaar et al. (2014) study.
[b] From Mehrkhai et al. (2010) study.
[c] From Romanowski et al. (2009) study.

2.4.3 Associations between HHV infection and persistent immune activation in HIV

HHV is much more frequent among immunocompromised individuals including those infected with HIV compared to healthy individuals and causes more severe HHV-related disease. For instance, HHV-8 infection is associated with increased inflammation and immune activation in virologically suppressed HIV-infected patients as HHV-8-infected patients had higher high sensitivity CRP (hsCRP) levels, higher CD4/CD38/HLA-DR, higher CD8/CD38/HLA-DR and lower plasminogen activator inhibitor (PAI-1) levels (Masiá et al., 2014). In addition, seropositive status of CMV was significantly associated with a higher level of immune activation, as measured by the median percentage of CD8 T-cells that expressed HLA-DR/CD38 (Wittkop et al., 2013). This indicates that CMV-specific immune response may play a role in chronic immune activation in HIV-infected

patients. Moreover, coinfection with HSV-2 in HIV-infected, therapy-naïve men was associated with reduced HIV-specific CD8 T-cell IFN-γ and proliferative responses (Sheth et al., 2008). Besides, a randomized placebo controlled study in Uganda found that HIV-1 and HSV-2 co-infected individuals treated with acyclovir twice daily decreased HIV-1 disease progression by 27%, which indicates that HSV-2 is associated with HIV-1 disease progression (Delany et al., 2009). Furthermore, a prospective study conducted in Spain by Masiá et al. has shown that VZV IgG antibodies level positively correlated with IL-6 and intercellular adhesion molecule 1 (ICAM-1), which are markers of inflammation, in HIV-infected patients with virological suppression (Masiá et al., 2013). In addition to that, a randomized placebo-controlled trial of valganciclovir among HIV-infected individuals with persistently low CD4 T-cell counts despite ART found that valganciclovir suppressed CMV DNA levels and significantly reduced the frequency of activated CD8 T cells and hsCRP levels than placebo (Hunt et al., 2011). These has suggested that CMV is a substantial cause of *in vivo* T-cell activation among treated HIV and CMV-coinfected individuals.

2.4.4 HHV infection and derangement of K/T ratio

HHV has also been shown to modulate the K/T pathway (Adams et al., 2004). IDO has been shown to be involved in the antiviral effect of IFN-γ against HSV-2 and that TNF-α enhances the IFN-γ-induced antiviral effect (Adams et al., 2004). A contribution of IDO in control of viral infections was suggested by *in vitro* experiments demonstrating that the inhibition of human CMV replication was induced by IFN-γ and IFN-β. This virostatic effect could be reverted by addition of exogenous tryptophan indicating an involvement of IDO (Bodaghi et al., 1999). However, how HHV may modulate IDO activity in the setting of HIV is currently unclear.

2.4.5 Clinical consequences of HHV co-infection in HIV

In addition to its role in driving chronic immune activation, HHV infection has also been associated with multiple clinical events in the setting of HIV-infection. In a study which recruited a total of 291 HIV-infected individuals with 109 subclinical coronary atherosclerosis cases and 182 controls, HSV-2 seropositivity was found to be significantly associated with subclinical coronary atherosclerosis in treated HIV-infected men (Hechter et al., 2012). Furthermore, patients with a history of herpes zoster infections have increased subsequent stroke risk (Yang et al., 2016). Similar associations have been found with CMV infections in HIV-infected individuals. A nationwide longitudinal study in Taiwan using insurance registry to identify the risk of stroke with prior opportunistic infections in 21,375 patients and 85,500 controls, found that the risk of incident stroke was significantly higher in HIV-infected patients reporting prior CMV disease among others (Yen et al., 2017). This is further supported by Masiá et al. (2013), where they found that there was a significant positive correlation between carotid intima-media thickness and CMV IgG antibodies in treated HIV-infected patients, linking CMV infection to the pathogenic process of atherosclerosis. The same study also showed that high serological response to VZV was associated with subclinical atherosclerosis, even after adjusting for serological response to CMV, indicating that it was independent from CMV immune effects (Masiá et al., 2013). Moreover, HHV-8 has been identified to be the underlying infectious cause of Kaposi's sarcoma, a form of cancer, whereby the relative hazard of developing Kaposi's sarcoma was 3.04 higher in HHV-8 seropositive subjects compared to HHV-8 seronegative subjects (O'Brien et al., 1999).

CHAPTER 3: METHODOLOGY

3.1 Patient population and samples

This study utilized stored plasma samples from an established cross-sectional cohort of HIV-infected patients receiving suppressive ART in University Malaya Medical Centre (UMMC) from the year of 2012 to 2014. The samples were from a prior study designed to assess the influence of host factors on immune recovery and collected from HIV-infected patients fulfilling the following criteria:

1. Men or women aged at least 18 years.
2. First antiretroviral regimen composed of ART defined by at least three antiretroviral drugs.
3. CD4 T-cell count <500/µL at commencement of ART.
4. Patients achieve controlled viremia within 12 months following commencement of ART. Controlled viremia was defined as HIV RNA of ≤50 copies/mL measured using a real time-PCR based assay.

All participants provided written informed consent. The study protocol was approved by the University Malaya Medical Centre Ethics Review Board (MEC Ref. No 896.32). Clinical data on HIV transmission-risk behavior, duration of ART, current ART prescribed and all CD4 count and HIV RNA measures from treatment initiation to the date of recruitment were abstracted from patient medical records.

3.2 HHV serology in subjects

Whole blood was collected and plasma separated as previously described (Rajasuriar et al., 2015). Plasma samples were stored at -80^{o}C until further analysis. HSV-1 and HSV-2 IgG levels were analyzed using HerpeSelect® enzyme-linked immunosorbent assay (ELISA) IgG kits (Focus Diagnostics, Cypress, CA, USA) as previously described

(Performed in the laboratory of Prof. Marie Larsson, Linkoping University as a collaboration) (Crisci et al., 2016). Briefly, 100µL of each diluted specimen (1 in 2 dilution), control or calibrator was dispensed into the appropriate 96-well plate. The plate was covered with sealing tape and incubated for 60 minutes at room temperature. The contents of the wells were emptied and filled with wash buffer solution for washing steps after the incubation. 100µL of IgG conjugate was dispensed to all wells and incubated with sealing tape for 30 minutes at room temperature. The washing steps were repeated. 100µL of Substrate Reagent was added to all wells and incubated for 10 minutes at room temperature. The reaction was stopped by adding 100µL of Stop Reagent to all wells. The absorbance was measured for each well at 450nm with microwell spectrophotometer.

The Elecsys® kit (Roche, Switzerland) was used to quantify CMV IgG class antibodies. Briefly, 20µL of the patient sample were incubated with a mix of biotinylated and ruthenylated monomeric CMV antigens. Streptavidin-coated paramagnetic microparticles were added and the reagent mixture was transferred to the measuring cell, which was then measured using a photomultiplier. VZV IgG class antibodies were quantified using the Siemens Enzygnost® (Siemens Healthcare GmbH, Germany) kit. Both quantitations of CMV and VZV IgG levels were done at the clinical diagnostic laboratory, UMMC.

Sample optical density readings were compared with reference cutoffs to determine positive, negative, or equivocal status. A cut-off of >1.1 was considered as HSV-1 and HSV-2 seropositive. A cut-off of >1.0 and a cut-off of >0.2 were used for CMV and VZV seropositivity, respectively, according to the manufacturer's instructions. Equivocal samples were considered as negative.

3.3 Measurement of tryptophan and kynurenine concentrations using liquid chromatography-tandem mass spectrometry (LC-MS/MS)

3.3.1 Materials

Tryptophan (Trp) and kynurenine (Kyn) reference compounds were purchased from Sigma-Aldrich (MO, USA). Trp-d5 was obtained from CDN Isotopes (Quebec, Canada) and Kyn-d4 was obtained from Buchem BV (Apeldoorn, The Netherlands). Acetonitrile, methanol, and trifluoroacetic acid (TFA, 99%) were high performance liquid chromatography (HPLC) grade and were purchased from Fisher Scientific, Inc.

3.3.2 Internal standard (IS) stock and working solutions preparation

Stock solutions of Trp and Kyn and IS stock solutions of Trp-d5 and Kyn-d4 were prepared at 1mg/mL in 50% acetonitrile in phosphate buffered saline with pH of 7.4. IS working solution containing 3.5µg/mL of Trp-d5 and 1.1µg/mL of Kyn-d4 was prepared by diluting the IS stock solution with ultrapure water and was stored at 4^{o}C.

3.3.3 Preparation of stripped plasma

Human plasma was purified using activated charcoal powder. 1.2g of charcoal activated powder was added in 20mL of plasma and shake for 2 hours on a shaker, then was centrifuged at 13300g for 10 minutes to obtain the supernatant.

3.3.4 Preparation of sample and standard solutions

Sample preparation was done as previously described (Huang et al., 2013). The plasma samples, calibrators and quality control (QC) samples were aliquoted into individualized test tube and IS working solution were added to each and mixed for 30s. Blood protein precipitation was done by adding TFA and vortexed for 1 min, followed by centrifugation

at 3000rpm for 10 min. The supernatants were transferred to autosampler vials and 5µL of the supernatants were injected into the LC-MS/MS system (Agilent, 6400 series).

3.3.5 LC-MS/MS

The HPLC conditions were as follows: the column was a Synergi Polar RP column (75 mm x 4.6 mm) from Phenomenex (CA, USA) and an isocratic mobile phase composed of 7% of a acetonitrile and methanol mixture. The flow rate was set at 1.0ml/min and the run time for each sample was 6 minutes. The autosampler was set at 4^{o}C. Data analysis was performed using Agilent MassHunter Workstation Software. The mass spectrometer was set to electrospray ionization (ESI) in positive multiple reaction monitoring (MRM) mode. The precursor/product transitions (*m/z*) were at *m/z* 204.9 -› 188.0 for Trp, *m/z* 209.0 -› 192.1 for Kyn, *m/z* 210.0 -› 150.1 for Trp-d5 and *m/z* 213.0 -› 196.0 for Kyn-d4.

3.3.6 HPLC method validation

HPLC method development and optimization were done as previously described (Huang et al., 2013) before analyzing the plasma samples. Method validation was performed according to the Food and Drug Administration (FDA) guidelines for biological samples method validation (US DHHS, FDA, & CDER, 2001), and the International Conference on Harmonization (ICH) guidelines (ICH Harmonised Tripartite Guideline, 2005).

3.3.6.1 Calibration curve

The calibrators were generated using surrogate matrix for Trp and Kyn from standard working solutions in stripped plasma. Each concentration of the calibrators was run in duplicate. The peak area ratios were calculated by dividing the peak area of the analyte

with the internal standard and plotted against the concentrations of the analyte to obtain the calibration curve. The calibration curve for Trp and Kyn is shown in Figure 3.1 and 3.2, respectively. Table 3.1 shows the linear regressions and regression coefficients of the calibration curves for Trp and Kyn.

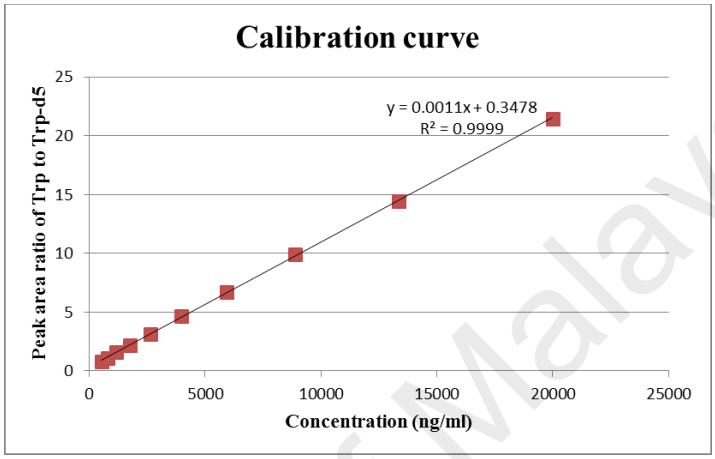

Figure 3.1: Calibration curve for tryptophan (Trp).

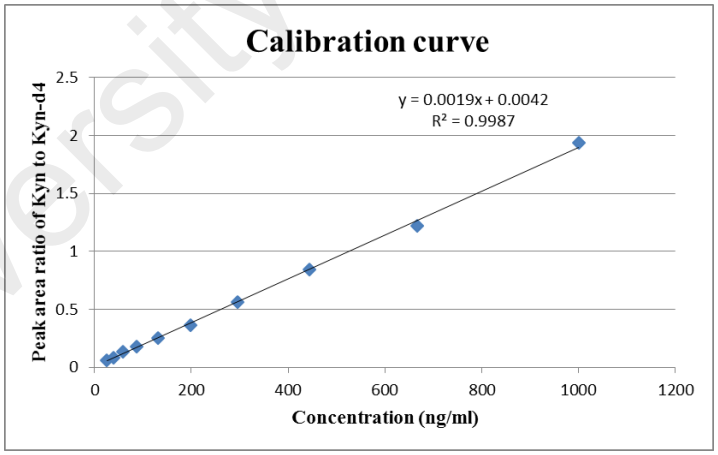

Figure 3.2: Calibration curve for kynurenine (Kyn).

Table 3.1 Linear regressions and regression coefficients of calibration curves for tryptophan and kynurenine analytes.

Analyte	Sensitivity (m)	Constant (c)	Regression coefficient (R^2)
Tryptophan	$0.0012 \pm 5.18 \times 10^{-5}$	0.3688 ± 0.1032	0.9987 ± 0.0010
Kynurenine	0.0017 ± 0.0002	0.0259 ± 0.0051	0.9991 ± 0.0007

3.3.6.2 Lower limit of quantification (LLOQ)

According to the FDA guidelines for biological method validation (US DHHS et al., 2001), the analyte response at the LLOQ should be at least 5 times the response compared to blank response. The LLOQ for Trp and Kyn were estimated at approximately 52.0246 and 26.0123 ng/ml, respectively and the coefficient of variation (CV) is less than 20%.

3.3.6.3 Assay precision and accuracy

Assay precision was evaluated using intra-day and interday analytical runs. The low, medium and high concentrations of stripped human plasma QC samples were generated at 5000, 10000 and 15000ng/mL for Trp and at 150, 300 and 600ng/mL for Kyn. According to the FDA guidelines for biological samples method validation (US DHHS et al., 2001), the precision determined at each concentration level should not exceed 15% of the CV. The concentrations and CVs for each analyte in intra-day and inter-day precision are shown in Table 3.2 and 3.3 respectively and the CVs are less than 15%. The inaccuracy for both Trp and Kyn analysis are less than 15% of the actual value and it is shown in Table 3.4. The extraction efficiencies (recovery) for Trp and Kyn spiked into stripped human plasma are above 87%, shown in Table 3.5.

Table 3.2 Intra-day precision (n=6) for analysis of tryptophan and kynurenine in human plasma.

Analyte	Low QC		Med QC		High QC	
	Concentration (ng/ml)	CV (%)	Concentration (ng/ml)	CV (%)	Concentration (ng/ml)	CV (%)
Tryptophan	5401.46 ± 141.03	2.61	10844.01 ± 222.71	2.05	15739.61 ± 312.13	1.98
Kynurenine	256.64 ± 7.20	2.80	519.71 ± 19.69	3.79	766.54 ± 11.80	1.54

Table 3.3 Inter-day precision (n=6) for analysis of tryptophan and kynurenine in human plasma.

Analyte	Low QC		Med QC		High QC	
	Concentration (ng/ml)	CV (%)	Concentration (ng/ml)	CV (%)	Concentration (ng/ml)	CV (%)
Tryptophan	5396.04 ± 410.13	7.60	11175.49 ± 607.03	5.43	16388.99 ± 701.77	4.28
Kynurenine	233.77 ± 8.62	3.69	474.60 ± 20.79	4.38	707.81 ± 30.74	4.34

Table 3.4 Percentage inaccuracy for analysis of tryptophan and kynurenine in human plasma.

Analyte	Percentage inaccuracy (%)		
	Low QC	Med QC	High QC
Tryptophan	3.93 ± 4.12	3.91 ± 4.60	2.22 ± 2.33
Kynurenine	-5.00 ± 2.87	-1.84 ± 5.22	-1.38 ± 1.62

Table 3.5 Recovery for analysis of tryptophan and kynurenine in human plasma.

Analyte	Low QC		Med QC		High QC	
	Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)
Tryptophan	98.38 ± 4.12	4.19	101.14 ± 4.60	4.55	100.37 ± 2.33	2.33
Kynurenine	87.07 ± 2.87	3.30	94.20 ± 5.22	5.54	95.97 ± 1.62	1.69

3.3.6.4 Freeze and thaw stability

The stock solutions of Trp and Kyn were frozen to -80^{o}C and thawed to room temperature for five cycles. The CVs for this experiment were less than 15% for both Trp and Kyn concentrations (Table 3.6).

Table 3.6 Freeze-thaw stability for analysis of tryptophan and kynurenine in human plasma.

Analyte	Low QC		Med QC		High QC	
	Concentration (ng/ml)	CV (%)	Concentration (ng/ml)	CV (%)	Concentration (ng/ml)	CV (%)
Tryptophan	4959.22 ± 539.52	10.88	10580.19 ± 1318.36	12.46	13892.11 ± 1764.81	12.70
Kynurenine	228.86 ± 22.34	9.76	440.31 ± 16.36	3.72	680.53 ± 2.78	2.78

3.4 Measurement of markers of chronic immune activation

Markers of chronic immune activation including sCD14 (R&D Systems, Minnesota) (Rajasuriar et al., 2015) and neopterin (Thermo Scientific, Hennigsdorf) were measured using ELISA kits following the manufacturer's instructions. Samples for both analyses were measured in duplicates. HsCRP was measured by immunochemiluminometric assay by the clinical diagnostic laboratory, UMMC. The markers IFN-γ and IL-6 were measured using the enhanced sensitivity cytometry bead array (ES-CBA) (Becton Dickinson, San Jose, CA) according to the manufacturer's instructions.

3.4.1 Measurement of neopterin concentration using ELISA

3.4.1.1 Procedure

Briefly, 50µL of the standards, controls and plasma samples were mixed with 150µL of the enzyme conjugate in an uncoated microtitre plate. An aliquot of 150µL of the mixture was transferred into a neopterin-antibody coated microtitre plate. The plate was incubated for 2 hours at room temperature in the dark. The incubation volume was decanted and washing steps were performed with washing solution. 100µL of the dissolved substrate was added into all wells including the blank. The plate was incubated for 30 minutes at room temperature. Then, 100µL of stop solution was added into all

wells to stop the enzyme reaction. The optical density was measured at a wavelength of 405nm in a photometer (BioTek EON, U.S.).

3.4.1.2 Method validation

A similar method validation was performed for all soluble analytes measured. Here, the validation steps for neopterin is shown as an example of the approach used.

(a) Standard curve

The standard curve was generated using the standards inside the kits. The standard curve for neopterin is shown in Figure 3.3, with a regression coefficient (R^2) of 0.99.

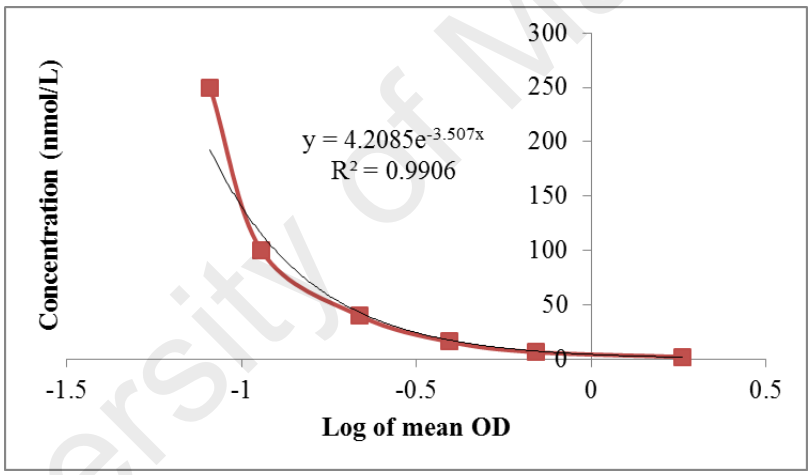

Figure 3.3: Standard curve for neopterin.

(b) Matrix dilution factor and linearity

To assess the matrix effect, human plasma samples were diluted two-, four- and eight-times with enzyme conjugate. As Figure 3.4 shows, the linear R^2 of neopterin indicate good dilution linearity, which supports the idea that there are no significant matrix effects for this assay.

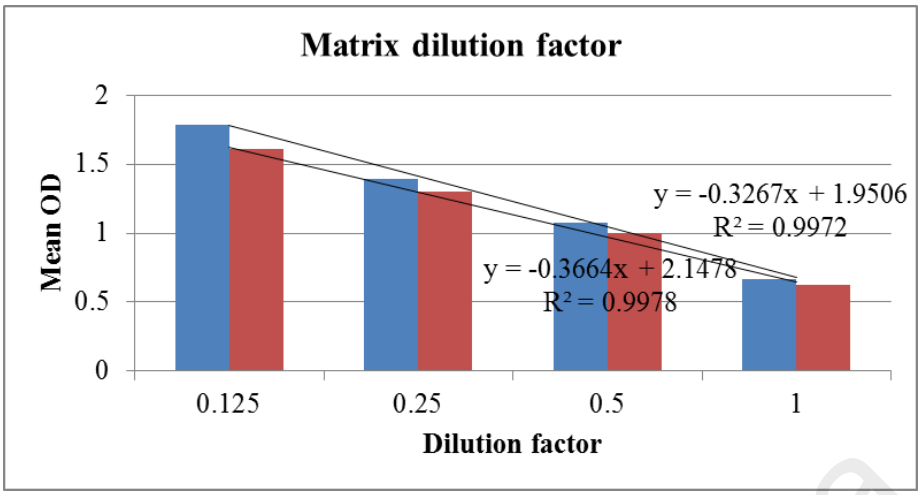

Figure 3.4: Matrix dilution factor and linearity for quality control (QC) 1 (blue bar) and 2 (red bar) for neopterin analysis.

(c) Intra-day variability and inter-day variability

The concentrations and CVs for each analyte in intra-day and inter-day precision are shown in Table 3.7 and 3.8, respectively. The CVs are less than 10% for intra-day precision and 20% for inter-day precision.

Table 3.7 Intra-day precision (n=7) for analysis of neopterin in human plasma.

Analyte	QC 1		QC 2	
	Concentration (nmol/L)	CV (%)	Concentration (nmol/L)	CV (%)
Neopterin	4.52 ± 0.26	5.68	5.11 ± 0.39	7.59

Table 3.8 Inter-day precision (n=7) for analysis of neopterin in human plasma.

Analyte	QC 3		QC 4	
	Concentration (nmol/L)	CV (%)	Concentration (nmol/L)	CV (%)
Neopterin	3.98 ± 0.39	9.93	3.85 ± 0.64	16.63

(d) Freeze-thaw stability

The human plasma samples were frozen to -80^{o}C and thawed to room temperature for five cycles and the CV for this experiment is less than 10% (Table 3.9).

Table 3.9 Freeze-thaw stability analysis for neopterin in human plasma samples.

Analyte	QC 1		QC 2	
	Concentration (nmol/L)	CV (%)	Concentration (nmol/L)	CV (%)
Neopterin	3.96 ± 0.37	9.44	4.64 ± 0.30	6.37

3.4.2 Measurement of IFN-γ and IL-6 concentration using ES-CBA

Briefly, 50µL of the standards, controls and plasma samples were mixed with 20µL of the mixed capture beads in the appropriate assay tubes. The tubes were incubated for 2 hours at room temperature. 20µL of the mixed Human Detection Reagent were added to each assay tube and the tubes were incubated for 2 hours at room temperature. 1mL of wash buffer were added to each tube and the tubes were centrifuged at 200g for 5 minutes. The supernatant were discarded and 100µL of Enhanced Sensitivity Detection Reagent were added to each tube. The tubes were incubated for 1 hour at room temperature. After that, 1mL of wash buffer were added to each tube and the tubes were centrifuged at 200g for 5 minutes. The supernatant were discarded and 300µL of wash buffer were added. The samples were acquired on a FACS Canto II (Becton Dickinson, San Jose, CA). Analysis was performed using the FCAP Array software (Becton Dickinson, San Jose, CA).

3.5 Data analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) 20.0 for Windows (IBM, United State) and Stata Statistical Software: Release 14 (StataCorp LP, College Station, TX). Mann-Whitney was used to identify the differences

between HHV-specific seropositive and seronegative groups. Risk factors for HHV infections were identified by performing univariate and multivariate analyses with a forward and backward stepwise logistic regression. Correlations between immune activation markers and K/T ratio were tested by non-parametric Spearman rank correlation coefficients. Comparison of immune activation markers in individuals with different numbers of HHV co-infection were assessed using Poisson Regression models adjusting for age, gender and ethnicity. Results were considered significant at *p*-value <0.05. Population averaged panel data models based on generalized linear regression were used on both univariate and multivariate basis to assess the risk factors associated with CD4 T-cell recovery between the 0 to 12 month period and >12 months following ART initiation. Log transformations were applied where appropriate. CD4 T-cell counts for the assessment of CD4 T-cell recovery only included periods of confirmed viral suppression and observations were censored when; (1) treatment interruption of > 2 weeks occurred; (2) frequency of plasma HIV RNA level determinations declined to <1/year; or (3) patients experience virological rebound defined as two consecutive plasma HIV RNA >1000 copies/ml. Variables with *p*-value of <0.250 or those covariates considered clinically important were included in the multivariate models. Interactions were tested and considered significant at the 0.05 level.

The clustering of four HHVs and associating K/T ratio was mapped out using a self-organizing map (SOM). Viscovery® SOMine version 5.0 (Eudaptics Software GmbH, Vienna, Austria) was used for the Kohonen's self-organizing map (SOM) analysis (Kohonen, 2001) clustering the positive or negative of four HHVs and featuring K/T ratio as an outcome. Briefly, SOM is a feedforward-type neural network learning model comprises of one input layer and one output layer, and an array of nodes located in the output layer. The SOM algorithm is based on unsupervised, competitive learning steps.

The network ultimately associates the output nodes with groups or patterns of input vectors by repeating the learning. For mapping, the weight (w) of four markers was assigned a value of 1 (w = 1), but no weight (w = 0) was adapted for K/T ratio. For graphical display, the four-dimensional characteristic spaces (four HHVs) and one outcome (K/T ratio) were projected onto an output layer comprising 500 nodes. The level of K/T ratio can be studied from the density of color shades in the nodes network.

CHAPTER 4: RESULTS

4.1 Characteristics of study participants and seroprevalence of HHV infections

A total of 232 treated HIV-infected individuals were included in the analyses. The majority were male (85.3%) and of Chinese ethnicity (75.9%). At the time of enrolment, the median age was 37 years (interquartile range [IQR], 32-43) and the median baseline CD4 T-cell count was 110 cells/μL (IQR, 26.5-232.0 cells/μL). The transmission risks was mainly through heterosexual contact (56.0%) with more than two-thirds (68.1%) of the cohort having history of AIDS-defining illness. The median current CD4 T-cell counts were 583 cells/uL (IQR, 428-763 cells/uL) whereas the median duration on ART was 7 years (IQR, 4.6-10.3 years). A high prevalence of seropositivity for HHV (CMV=96.1%, HSV-1=70.7%, HSV-2=53.9%, VZV= 86.6%) was observed in this cohort (Table 4.1). The number of HHV co-infections were quantified to reflect the cumulative number of HHV seropositivity in a subject with 3.0% having one HHV infection, 17.2% with 2, 49.1% with 3 pathogens and 30.6% seropositive for all 4 HHVs. All participants were seropositive for at least one HHV.

Table 4.1: Clinical and demographic characteristics of the cohort (n=232).

Characteristics	N (%) / Median (IQR)
Gender, n (%)	
Male	198 (85.3)
Female	34 (14.7)
Ethnicity, n (%)	
Chinese	176 (75.9)
Indian	21 (9.1)
Malay	35 (15.1)
HIV Transmission Risk, n (%)	
Homosexual contact	48(20.7)
Injection drug use	6 (2.6)
Heterosexual contact	130 (56.0)
Heterosexual contact & IDU	3 (1.3)
Receipt of blood/blood products	1 (0.4)
Other	44 (19.0)

Table 4.1 (continued)

Characteristics	N (%) / Median (IQR)
Smoking Status, n (%)	
No	86 (37.1)
Current smoker	98 (42.2)
Previous Smoker	1 (0.4)
Not recorded	47 (20.3)
History of Syphilis, n (%)	
Negative	148 (63.8)
Positive	58 (25.0)
Not recorded	26 (11.2)
ART Treatment Regimen, n (%)	
NNRTI-based	225 (97.0)
PI-based	7 (3.0)
History of AIDS-defining illness, n (%)	
No	74 (31.9)
Yes	158 (68.1)
Hepatitis B Antigen	
Negative	209 (90.1)
Positive	14 (6.0)
Hepatitis C Antibody	
Negative	210 (90.5)
Positive	18 (7.8)
Age (year)	37 (32-43)
hsCRP (mg/L)	0.26 (0.10-0.50)
Baseline CD4 T-cell count (cells/µL)	110 (26.5-323.0)
Baseline Viral Load (copies/mL)	102347 (63208-269175)
Current CD4 T-cell count (cells/µL)	583 (428-763)
Current CD4/CD8 ratio	0.64 (0.43-0.89)
Duration of ART (year)	7.0 (4.6-10.3)
Neopterin (nmol/mL)	13.18 (10.41-16.58)
sCD14 ($X10^6$ pg/mL)	1.98(1.63-2.30)
K/T ratio	0.0219 (0.0168-0.0260)
HSV-1 seropositive, n (%)	164 (70.7)
Median IgG levels (IQR), AU/mL	4.200 (0.335-4.894)
HSV-2 seropositive, n (%)	125 (53.9)
Median IgG levels (IQR), AU/mL	1.666 (0.340-6.097)
CMV seropositive, n (%)	223 (96.1)
Median IgG levels (IQR), AU/mL	500.000 (247.400-500.000)
VZV seropositive, n (%)	201 (86.6)
Median IgG levels (IQR), AU/mL	1.200 (0.720-1.590)
Number of HHV seropositivity, n (%)	
1	7 (3.0)
2	40 (17.2)
3	114 (49.1)
4	71 (30.6)

Abbreviations: AIDS, acquired immunodeficiency syndrome; ART, antiretroviral therapy; CMV, cytomegalovirus; HHV, human herpesvirus; HIV, human immunodeficiency virus; hsCRP, high sensitivity C-reactive protein; HSV, herpes

simplex virus; IDU, intravenous drug user; IgG, immunoglobulin G; K/T ratio, kynurenine/tryptophan ratio; NNRTI, non-nucleoside reverse-transcriptase inhibitors; PI, protease inhibitor; sCD14, soluble CD14; VZV, varicella zoster virus.

4.2 Risk factors associated with HSV-1, HSV-2, CMV and VZV seropositivity

Logistic regression analysis was performed to determine risk factors associated with individual HHV infections. In univariate logistic regression analysis for HSV-1 seropositivity, a longer duration on ART was associated with increased odds of HSV-1 seropositivity (OR: 1.117 95% CI: 1.023-1.219) and even after adjustments for clinical parameters in the multivariate model (aOR: 1.12 95% CI: 1.02-1.22) (Table 4.2). However, there were no significant association between HSV-1 seropositivity and gender.

Table 4.2: Risk factors associated with HSV-1 seropositivity in univariate and multivariate regression analysis.

Variable	Univariable model		Multivariable model[#]	
	OR (95% CI)	***p*-value**	**aOR* (95%CI)**	***p*-value**
Gender				
Male	1.000			
Female	1.717 (0.719-4.159)	0.231		
Ethnicity				
Chinese	1.000			
Indian	0.575 (0.224-1.476)	0.250		
Malay	0.531 (0.249-1.130)	0.100		
HIV Transmission Risk				
Homosexual contact	1.000			
Injection drug use	0.371 (0.066-2.079)	0.260		
Heterosexual contact	0.970 (0.461-2.040)	0.936		
Heterosexual contact & IDU	0.186 (0.015-2.225)	0.184		
Receipt of blood/blood products	-	1.000		
Other	0.796 (0.324-1.955)	0.619		
Smoking Status				
No	1.000			
Current smoker	1.409 (0.753-2.637)	0.284		
Previous Smoker	-	1.000		
Not recorded	1.753 (0.782-3.933)	0.173		
History of Syphilis				
Negative	1.000			
Positive	0.988 (0.507-1.925)	0.971		

Table 4.2 (continued)

Variable	Univariable model		Multivariable model[#]	
	OR (95% CI)	***p*-value**	**aOR (95%CI)**	***p*-value**
Not recorded	0.921 (0.373-2.278)	0.859		
ART Treatment Regimen				
NNRTI-based	1.000			
PI-based	2.544 (0.300-21.543)	0.392		
History of AIDS-defining illness				
Negative	1.000			
Positive	0.849 (0.459-1.570)	0.601		
Age	1.018 (0.989-1.049)	0.228		
hsCRP	0.957 (0.656-1.394)	0.818		
Baseline CD4 Count	1.002 (0.999-1.004)	0.134		
Baseline Viral Load (x10^5)	1 (1-1)	0.569		
Current CD4 T-cell count	1.000 (0.999-1.001)	0.517		
Current CD4/CD8 ratio	2.066 (0.935-4.565)	0.073		
Duration of ART	1.117 (1.023-1.219)	0.013	1.12 (1.02-1.22)	0.013*
sCD14 (x10^6)	1.000 (1.000-1.000)	0.520		

Abbreviations: AIDS, acquired immunodeficiency syndrome; aOR, adjusted odds ratio; ART, antiretroviral therapy; CI, confidence interval; HIV, human immunodeficiency virus; hsCRP, high sensitivity C-reactive protein; HSV-1, herpes simplex virus-1; IDU, intravenous drug user; K/T ratio, kynurenine/tryptophan ratio; NNRTI, non-nucleoside reverse-transcriptase inhibitors; OR, odds ratio; PI, protease inhibitor; sCD14, soluble CD14.

[#] Multivariate with a forward and backward stepwise logistic regression. Model was adjusted for gender, ethnicity, age, baseline CD4 count, current CD4:CD8 ratio and duration of ART, smoking status, history of syphilis and HIV transmission risk.

*Significance at $p<0.05$.

HSV-2 seropositivity was associated with being female (OR: 2.722 95% CI: 1.210-6.127), having a history of AIDS-defining illness (OR: 1.878 95% CI: 1.074-3.283) and a longer duration of ART (OR: 1.114, 95% CI: 1.030-1.205), as shown in Table 4.3 in the univariate model. Even after adjustment for gender, AIDS defining illness, ART treatment regimen, hsCRP, baseline CD4 count, duration of ART, smoking status, history of syphilis and HIV transmission risk, being female (aOR: 3.03 95% CI: 1.31-7.00), having a history of AIDS-defining illness (aOR: 2.01 95% CI: 1.12-3.60) and a longer

duration of ART (aOR: 1.12 95% CI: 1.04-1.21) remained independently associated with HSV-2 seropositivity.

Table 4.3: Risk factors associated with HSV-2 seropositivity in univariate and multivariate regression analysis.

Variable	Univariable model		Multivariable model[#]	
	OR (95% CI)	***p*-value**	**aOR (95%CI)**	***p*-value**
Gender				
Male	1.000			
Female	2.722 (1.210-6.127)	0.016	3.03 (1.31-7.00)	0.010*
Ethnicity				
Chinese	1.000			
Indian	1.111 (0.446-2.771)	0.821		
Malay	0.787 (0.381-1.627)	0.518		
HIV Transmission Risk				
Homosexual contact	1.000			
Injection drug use	1.087 (0.199-5.935)	0.923		
Heterosexual contact	1.684 (0.864-3.280)	0.126		
Heterosexual contact & IDU	2.174 (0.185-25.607)	0.537		
Receipt of blood/blood products	-	1.000		
Other	0.684 (0.298-1.570)	0.371		
Smoking Status				
No	1.000			
Current smoker	1.101 (0.613-1.976)	0.748		
Previous Smoker	-	1.000		
Not recorded	0.537 (0.261-1.105)	0.091		
History of Syphilis				
Negative	1.000			
Positive	1.105 (0.600-2.033)	0.749		
Not recorded	1.224 (0.527-2.841)	0.638		
ART Treatment Regimen				
NNRTI-based	1.000			
PI-based	5.345 (0.633-45.114)	0.124		
History of AIDS-defining illness				
Negative	1.000			
Positive	1.878 (1.074-3.283)	0.027	2.01 (1.12-3.60)	0.019*
Age	1.025 (0.998-1.053)	0.072		
hsCRP	1.417 (0.869-2.310)	0.163		
Baseline CD4 Count	0.998 (0.996-1.001)	0.137		
Baseline Viral Load (x10^5)	1.000 (1.000-1.000)	0.400		
Current CD4 T-cell count	1.000 (0.999-1.001)	0.940		

Table 4.3 (continued)

Variable	Univariable model		Multivariable model[#]	
	OR (95% CI)	***p*-value**	**aOR (95% CI)**	***p*-value**
Current CD4:CD8 ratio	0.742 (0.390-1.410)	0.362		
Duration of ART	1.114 (1.030-1.205)	0.007	1.12 (1.04-1.21)	0.005*
Soluble CD14 (x10^6)	1.000 (1.000-1.000)	0.364		

Abbreviations: AIDS, acquired immunodeficiency syndrome; aOR, adjusted odds ratio; ART, antiretroviral therapy; CI, confidence interval; HIV, human immunodeficiency virus; hsCRP, high sensitivity C-reactive protein; HSV-2, herpes simplex virus-2; IDU, intravenous drug user; K/T ratio, kynurenine/tryptophan ratio; NNRTI, non-nucleoside reverse-transcriptase inhibitors; OR, odds ratio; PI, protease inhibitor; sCD14, soluble CD14.

[#]Multivariate with a forward and backward stepwise logistic regression. Model was adjusted for gender, AIDS defining illness, ART treatment regimen, hsCRP, baseline CD4 count, duration of ART, smoking status, history of syphilis and HIV transmission risk.

*Significance at $p<0.05$.

On the other hand, Indian ethnicity had lower odds of CMV seropositivity compared to Chinese patients (OR: 0.212 95% CI: 0.049-0.920) and current CD4:CD8 ratio was also found to be associated with 78% lower odds of CMV seropositivity (OR: 0.226 95% CI: 0.073-0.698) in univariate analysis (Table 4.4). After adjustment of ethnicity, AIDS defining illness, history of syphilis, smoking status, HIV transmission risk and current CD4:CD8 ratio, Indian ethnicity (aOR: 0.17; 95% CI: 0.04-0.80) and higher current CD4:CD8 ratio (aOR: 0.21; 95% CI: 0.07-0.68) remained significantly associated with lower odds of CMV seropositivity.

Table 4.4: Risk factors associated with CMV seropositivity in univariate and multivariate regression analysis.

Variable	Univariable model		Multivariable model[#]	
	OR (95% CI)	***p*-value**	**aOR (95%CI)**	***p*-value**
Gender				
Male	1.000			
Female	0.586 (0.117-2.950)	0.517		
Ethnicity				
Chinese	1.000		1.000	
Indian	0.212 (0.049-0.920)	0.038	0.17 (0.04-0.80)	0.025*
Malay	-	0.998	-	0.998

Table 4.4 (continued)

Variable	Univariable model		Multivariable model[#]	
	OR (95% CI)	***p*-value**	**aOR (95% CI)**	***p*-value**
HIV Transmission Risk				
Homosexual contact	1.000			
Injection drug use	1.000	1.000		
Heterosexual contact	0.000	0.997		
Heterosexual contact & IDU	1.000	1.000		
Receipt of blood/blood products	1.000	1.000		
Other	0.000	0.998		
Smoking Status				
No	1.000			
Current smoker	0.365 (0.072-1.858)	0.225		
Previous Smoker	-	1.000		
Not recorded	1.095 (0.097-12.406)	0.941		
History of Syphilis				
Negative	1.000			
Positive	-	0.997		
Not recorded	1.429 (0.171-11.925)	0.742		
ART Treatment Regimen				
NNRTI-based	1.000			
PI-based	-	0.999		
History of AIDS-defining illness				
Negative	1.000			
Positive	0.257 (0.032-2.092)	0.204		
Age	1.028 (0.958-1.105)	0.441		
hsCRP	1.218 (0.330-4.494)	0.767		
Baseline CD4 Count	1.001 (0.995-1.007)	0.661		
Baseline Viral Load (x10^5)	1.000 (1.000-1.000)	0.986		
Current CD4 T-cell count	0.999 (0.998-1.001)	0.390		
Current CD4:CD8 ratio	0.226 (0.073-0.698)	0.010	0.21 (0.07-0.68)	0.009*
Duration of cART	1.067 (0.871-1.307)	0.529		
sCD14 (x10^6)	1.000 (1.000-1.000)	0.112		

Abbreviations: AIDS, acquired immunodeficiency syndrome; aOR, adjusted odds ratio; ART, antiretroviral therapy; CI, confidence interval; CMV, cytomegalovirus; HIV, human immunodeficiency virus; hsCRP, high sensitivity C-reactive protein; IDU, intravenous drug user; K/T ratio, kynurenine/tryptophan ratio; NNRTI, non-nucleoside reverse-transcriptase inhibitors; OR, odds ratio; PI, protease inhibitor; sCD14, soluble CD14.

[#]Multivariate with a forward and backward stepwise logistic regression. Model was adjusted for ethnicity, AIDS defining illness, history of syphilis, smoking status, HIV transmission risk and current CD4:CD8 ratio.

*Significance at $p<0.05$.

In assessments for VZV seropositivity, only Indian ethnicity compared to Chinese patients (OR: 0.256 95% CI: 0.092-0.711) was significant in the univariate and multivariate model, after adjustment for ethnicity, ART treatment regimen, hsCRP, smoking status, history of syphilis, HIV transmission risk (aOR: 0.26; 95% CI: 0.09-0.72) (Table 4.5).

Table 4.5: Risk factors associated with VZV seropositivity in univariate and multivariate regression analysis.

Variable	Univariable model		Multivariable model[#]	
	OR (95% CI)	***p*-value**	**aOR (95%CI)**	***p*-value**
Gender				
Male	1.000			
Female	1.184 (0.387-3.627)	0.767		
Ethnicity				
Chinese	1.000		1.000	
Indian	0.256 (0.092-0.711)	0.009	0.26 (0.09-0.72)	0.009*
Malay	0.994 (0.318-3.109)	0.991	1.00 (0.32-3.13)	1.000
HIV Transmission Risk				
Homosexual contact	1.000			
Injection drug use	-	0.999		
Heterosexual contact	0.773 (0.269-2.225)	0.633		
Heterosexual contact & IDU	0.233 (0.018-3.047)	0.267		
Receipt of blood/blood products	-	1.000		
Other	0.530 (0.157-1.741)	0.291		
Smoking Status				
No	1.000			
Current smoker	1.671 (0.722-3.866)	0.230		
Previous Smoker	-	1.000		
Not recorded	1.775 (0.602-5.234)	0.299		
History of Syphilis				
Negative	1.000			
Positive	1.433 (0.547-3.754)	0.464		
Not recorded	0.909 (0.285-2.904)	0.873		
ART Treatment Regimen				
NNRTI-based	1.000			
PI-based	0.370 (0.069-1.996)	0.248		
History of AIDS-defining illness				
Negative	1.000			
Positive	0.711 (0.302-1.676)	0.436		

Table 4.5 (continued)

Variable	Univariable model		Multivariable model[#]	
	OR (95% CI)	***p*-value**	**aOR (95% CI)**	***p*-value**
Age	1.023 (0.982-1.064)	0.274		
hsCRP	3.071 (0.852-11.074)	0.086		
Baseline CD4 Count	1.000 (0.997-1.003)	0.911		
Baseline Viral Load (x10^5)	1.000 (1.000-1.000)	0.601		
Current CD4 T-cell count	1.000 (0.999-1.001)	0.610		
Current CD4:CD8 ratio	1.036 (0.404-2.658)	0.941		
Duration of ART	1.032 (0.923-1.154)	0.582		
Soluble CD14 (x10^6)	1.000 (1.000-1.000)	0.191		

Abbreviations: AIDS, acquired immunodeficiency syndrome; aOR, adjusted odds ratio; ART, antiretroviral therapy; CI, confidence interval; HIV, human immunodeficiency virus; hsCRP, high sensitivity C-reactive protein; IDU, intravenous drug user; K/T ratio, kynurenine/tryptophan ratio; NNRTI, non-nucleoside reverse-transcriptase inhibitors; OR, odds ratio; PI, protease inhibitor; sCD14, soluble CD14; VZV, varicella zoster virus.

[#] Multivariate with a forward and backward stepwise logistic regression. Model was adjusted for ethnicity, ART treatment regimen, hsCRP, smoking status, history of syphilis, HIV transmission risk.

*Significance at $p<0.05$.

In summary, different risk factors have been found to be associated with HHV seropositivity in our study. A longer duration on ART was associated with an increased odd of both HSV-1 and 2 seropositivity. HSV-2 seropositivity was also associated with being female and a history of AIDS defining illness. Indian ethnicity compared to ethnic Chinese patients had an 83% lower odds of CMV and 74% lower odds of VZV seropositivity. CD4:CD8 ratio was also associated with 79% lower odds of CMV seropositivity.

4.3 Correlation between K/T ratio and HHV infection loads and immune activation markers

We explored the correlations between K/T ratio and HHV antibody levels, as well as markers of immune activation using Spearman rank correlation coefficients (Table 4.6). We found that K/T ratio was positively correlated with CMV antibody levels ($p=0.002$)

and VZV antibody levels (p=0.009) with a tendency of correlation with HSV-2 antibody levels (p=0.070). Among the other markers of immune activation, K/T ratio significantly correlated with IL-6 (p=0.000), neopterin (p=0.000), sCD14 (p=0.000) and hsCRP (p=0.011).

Table 4.6: Correlation of K/T ratio with HHV antibody levels and immune activation markers.

Variable	K/T ratio	
	Rho	*p*-value
HHVantibody level		
HSV-1 antibody level	-0.099	0.134
HSV-2 antibody level	0.120	0.070
CMV antibody level	0.205	0.002*
VZV antibody level	0.173	0.009*
Immune activation markers		
IL-6	0.338	0.000*
IFN-γ	0.142	0.111
Neopterin	0.628	0.000*
hsCRP	0.169	0.011*
sCD14	0.330	0.000*

Abbreviations: CMV, cytomegalovirus; HHV, human herpesvirus; HIV, human immunodeficiency virus; hsCRP, high sensitivity C-reactive protein; HSV, herpes simplex virus; IFN-γ, interferon-gamma; IL-6, interleukin-6; K/T ratio, kynurenine/tryptophan ratio; sCD14, soluble CD14; VZV, varicella zoster virus.

*Significance at p<0.05.

We further investigated whether an increasing number of HHV seropositivity in a participant had an impact on K/T ratio and the other immune activation markers (Figure 4.1). Using Poisson regression analysis and adjusting for age, gender and ethnicity, a significantly positive association was found between the number of HHV seropositivity and hsCRP (p=0.003) and IL-6 (p=0.020), with a tendency towards significance observed

with IFN-γ (p=0.051). This indicates that multiple HHV co-infections had an increasing impact on levels of systemic inflammation in virologically suppressed HIV-infected individuals. Increasing number of HHV seropositivity was however not associated with increasing K/T ratio (p=0.948).

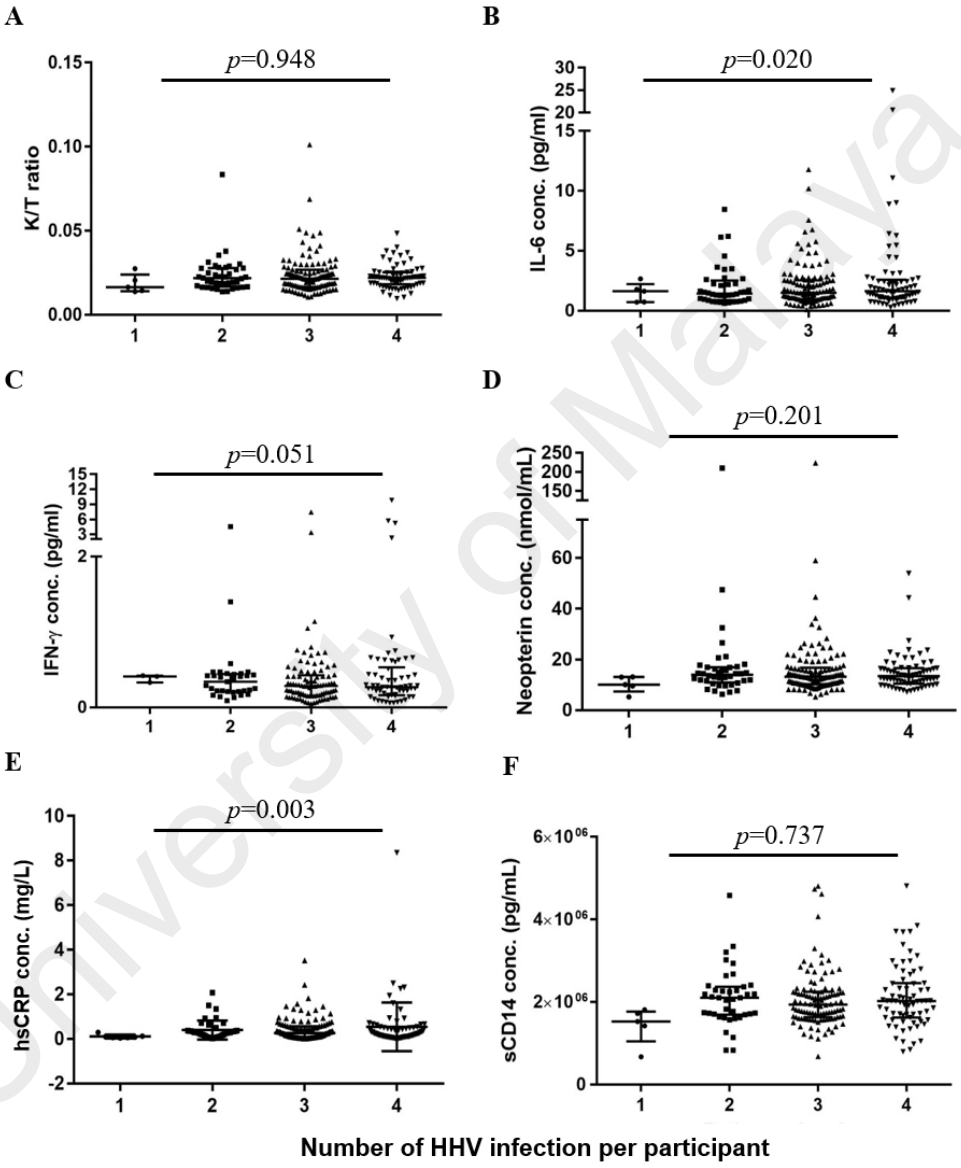

Figure 4.1: Comparisons in K/T ratio (A), IL-6 (B), IFN-γ (C), neopterin (D), hsCRP (E) and sCD14 (F) in groups with increasing number of HHV seropositivity. The *p*-values indicate statistical significance following using Poisson regression modelling, adjusted for age, gender and ethnicity. The error bars indicate the median (IQR) for each group. The numbers in the x-axis indicate the total number of HHV seropositivity per participant. *Significance at p<0.05.

4.4 The effect of CMV seropositivity on K/T ratio

We next explored which combinations of HHV infections had the greatest effect on modulating K/T ratios in an individual (Figure 4.2, 4.3 and 4.4) utilizing the SOM approach. Figure 4.2 and Figure 4.3 show the clustering of two types of HHVs, with Figure 4.2Ai indicating the clustering of HSV-1 and HSV-2, Figure 4.2Bi indicating the clustering of HSV-1 and CMV, Figure 4.2Ci indicating the clustering of HSV-1 and VZV, Figure 4.3Ai indicating the clustering of HSV-2 and CMV, Figure 4.3Bi indicating the clustering of HSV-2 and VZV, and Figure 4.3Ci indicating the clustering of CMV and VZV. These figures correspond to the areas describing the different intensities of K/T ratio in Figure 4.2Aii, Bii and Cii. This is similarly described in Figures 4.3 and 4.4, but with different combinations of HHVs. The level of K/T ratio of different combination HHVs can be studied from the density of the colour shades in the nodes network for each feature map. The darkest blue shade represents the lowest value of K/T ratio whereas the darkest red shade indicates the highest value of K/T ratio. Figure 4.2A shows that the highest intensity of K/T ratio corresponded to the HSV-1 seropositive cluster (Figure 4.2Ai, cluster 1) whereas the lowest K/T ratio levels was found in the HSV-1 and HSV-2 seronegative cluster (Figure 4.2Ai, cluster 2). When combination of HSV-1 and CMV was analyzed, the highest K/T ratio level was clustered with both HSV-1 and CMV seropositivity (Figure 4.2Bi, cluster 3) whereas the lowest K/T ratio with CMV seronegative (Figure 4.2Bi, cluster 2). On the other hand, clustering of HSV-1 and VZV illustrates that the highest and the lowest level K/T ratio is in the HSV-1 and VZV seronegative clusters, respectively (Figure 4.2Ci, cluster 2). Combinations of HSV-2 and CMV (Figure 4.3Ai, cluster 3), HSV-2 and VZV (Figure 4.3Bi, cluster 1), and CMV and VZV (Figure 4.3Ci, cluster 1) corresponded with the highest level of K/T ratio, which means these combinations have an effect on K/T ratio level.

In Figure 4.4A and B, three different types of HHV were analyzed together. We found that the highest K/T ratio is corresponded with the cluster of CMV seropositivity in the analysis of HSV-1, HSV-2 and CMV combinations (Figure 4.4A), whereas in the clustering of HSV-2, CMV and VZV, the highest K/T ratio corresponded with HSV-2 and CMV seropositive cluster. For both clusters, the lowest K/T ratio was in the areas where CMV was seronegative. Subsequently, we analyzed all possible combinations for the four HHVs tested and found that individuals seropositive for HSV-2, CMV and VZV were clustered in areas indicating the highest intensity of K/T ratio (Figure 4.4Ci, cluster 1). Additionally, the area indicating the lowest value of K/T ratio corresponded with clusters where none of the individuals were seropositive for CMV (Figure 4.4Ci, cluster 3).

Notably, there were also different intensities of K/T ratio within individual clusters of HHV combinations suggesting the role of other unmeasured factors contributing to K/T ratio modulation in these patients. These data imply that there is a complex modulation of the kynurenine-tryptophan pathway in the presence of multiple HHV infections and among the four HHVs tested, co-infections involving CMV had the strongest modulating effect on K/T ratio.

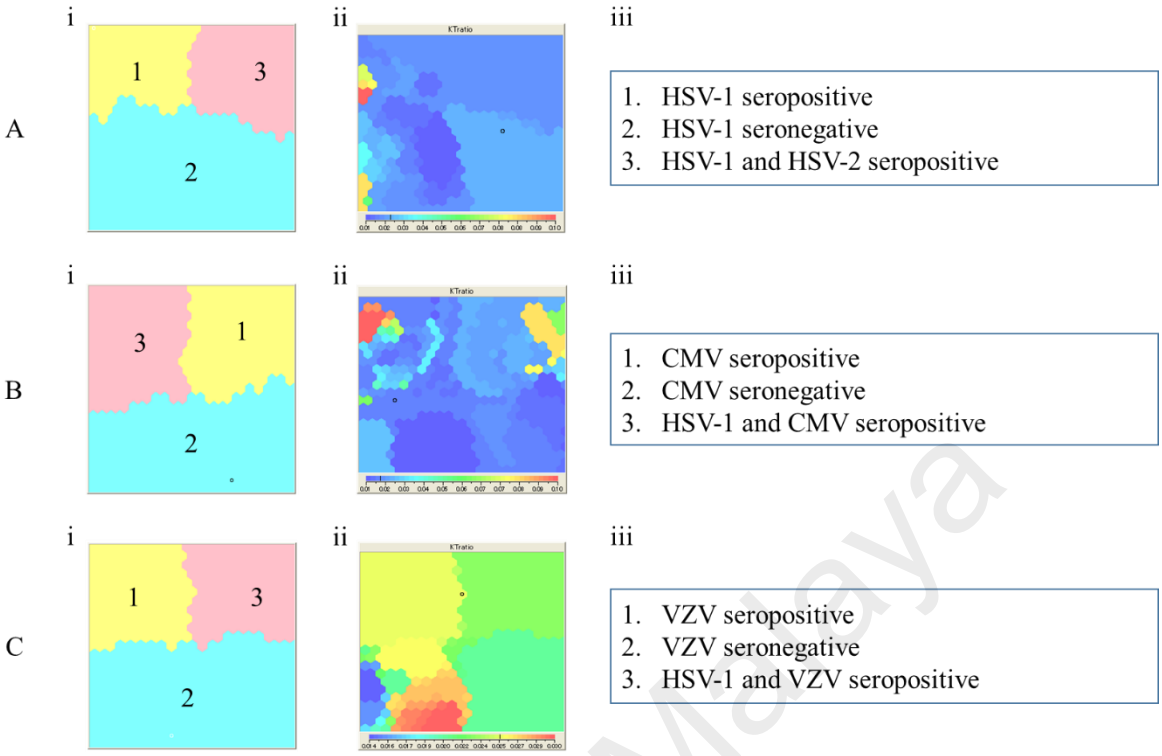

Figure 4.2: Clustering of herpes simplex virus-1 (HSV-1), herpes simplex virus-2 (HSV-2), cytomegalovirus (CMV) and varicella zoster virus (VZV) by self-organizing map (SOM) on kynurenine/ tryptophan ratio (K/T ratio). **Ai**, Clustering map of HSV-1 and HSV-2; **Bi**, Clustering map of HSV-1 and CMV; **Ci**, Clustering map of HSV-1 and VZV; **Aii, Bii, Cii,** Feature map of K/T ratio, which was associated with no weight adjustment in the SOM analysis; **Aiii, Biii, Ciii,** Combination of HHVs for the cluster **i**.

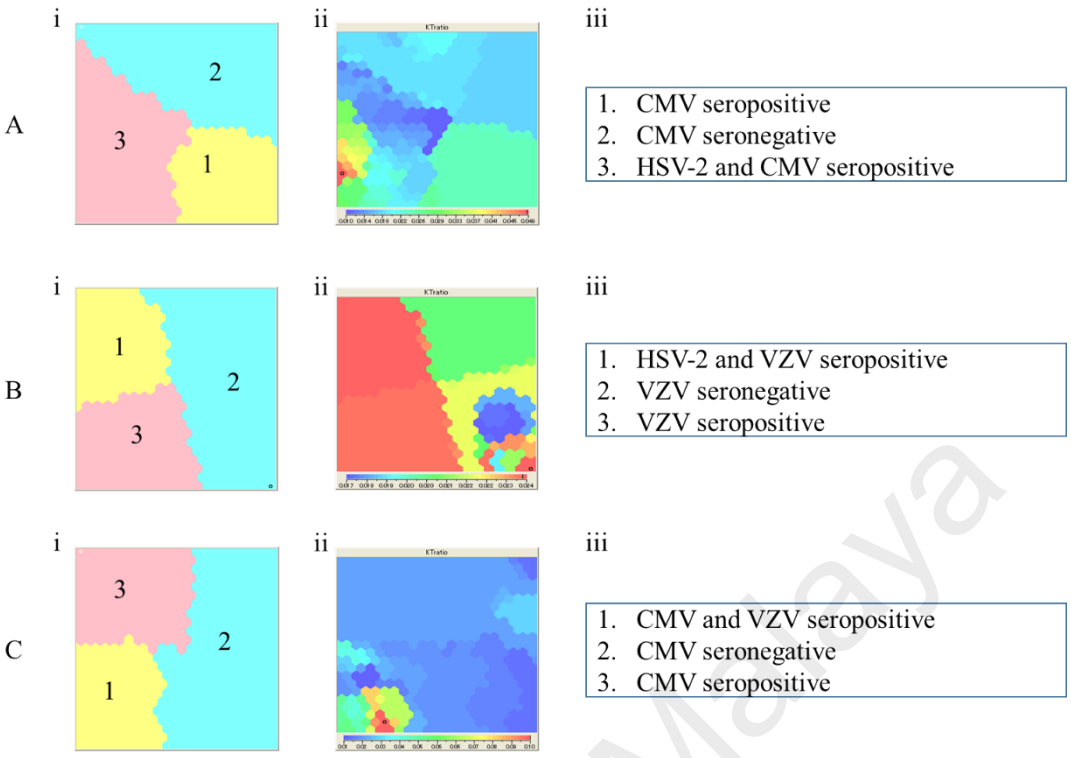

Figure 4.3: Clustering of herpes simplex virus-1 (HSV-1), herpes simplex virus-2 (HSV-2), cytomegalovirus (CMV) and varicella zoster virus (VZV) by self-organizing map (SOM) on kynurenine/ tryptophan ratio (K/T ratio). **Ai**, Clustering map of HSV-2 and CMV; **Bi**, Clustering map of HSV-2 and VZV; **Ci**, Clustering map of CMV and VZV; **Aii, Bii, Cii,** Feature map of K/T ratio, which was associated with no weight adjustment in the SOM analysis; **Aiii, Biii, Ciii,** Combination of HHVs for the cluster **i**.

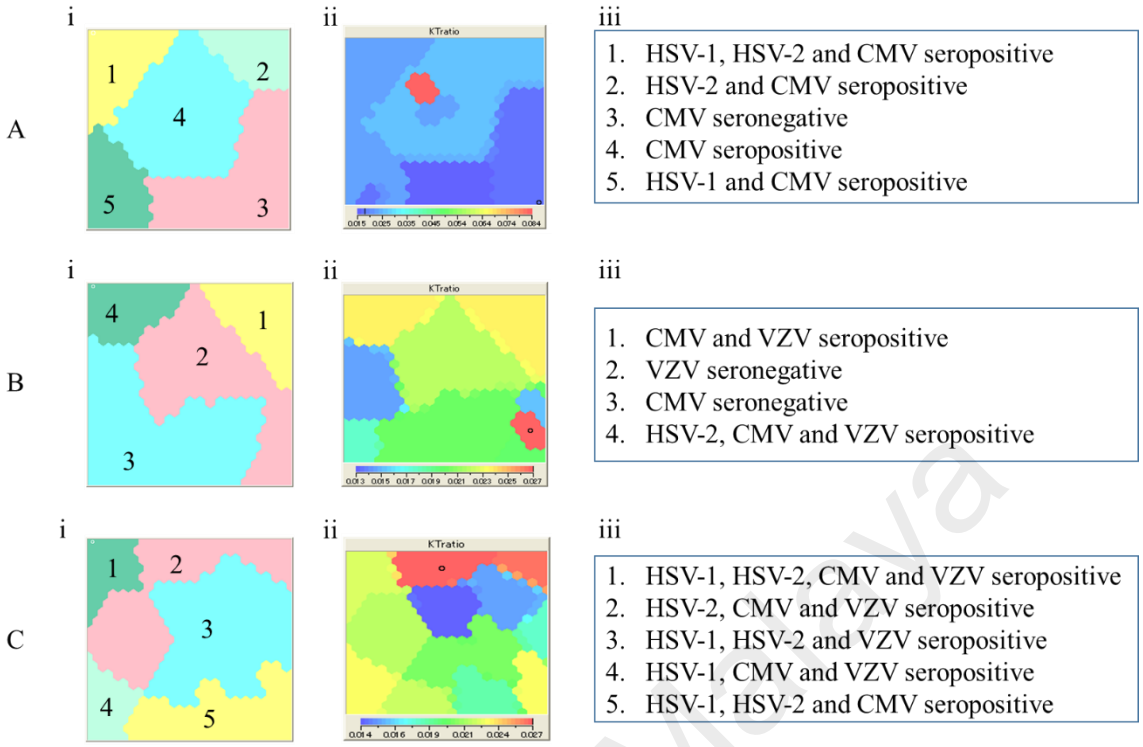

Figure 4.4: Clustering of herpes simplex virus-1 (HSV-1), herpes simplex virus-2 (HSV-2), cytomegalovirus (CMV) and varicella zoster virus (VZV) by self-organizing map (SOM) on kynurenine/ tryptophan ratio (K/T ratio). **Ai**, Clustering map of HSV-1, HSV-2 and CMV; **Bi**, Clustering map of HSV-2, CMV and VZV; **Ci**, Clustering map of all four HHVs; **Aii, Bii, Cii,** Feature map of K/T ratio, which was associated with no weight adjustment in the SOM analysis; **Aiii, Biii, Ciii,** Combination of HHVs for the cluster **i**.

4.5 Influence of HHV co-infection and K/T ratio on CD4 T-cell recovery

To assess the factors associated with early and late immune recovery, we performed generalized linear estimation on our panel data which recorded regular assessments of CD4 T-cell counts and HIV RNA that extended beyond 60 months in most cases (Table 4.7). Median follow-up duration for ART-mediated viral suppression was 189 (140-266 months). Participants contributed a median of 3 CD4 T-cell count observations/patient (IQR, 3-4) in the model for 0-12 months post ART and a median of 11 CD4 T-cell count observations/patient (IQR, 7-15) in the model assessing risk factors >12 months post ART. An effect modifying the interaction between age and the log transformed K/T ratio was found and incorporated into the model. After adjustment for age, gender, ethnicity and all other univariately significant variables and interactions, we found that a higher log transformed K/T ratio was associated with an increase in CD4 T-cell counts. This translated to an average increase of 5.38 cells/μL increase for every 10% increase in K/T ratio in the early phase of recovery from 0-12 months post-ART. Age and K/T ratio were found to have a statistically significant interaction effect. Other factors significantly associated with early CD4 T-cell recovery included a history of AIDS-defining illness, younger age, shorter duration on ART, higher log baseline and viral load (time-dependent), higher baseline CD4 and higher CD8 T-cell counts (time-dependent).

Table 4.7: Risk factors associated with CD4 T-cell recovery between the 0 to 12 month period following ART initiation.

Variable	Univariate β-coefficient	*p*-value	Multivariate β-coefficient	*p*-value
Age	-1.98 (-3.14, -0.81)	0.001*	-11.91 (-18.10, -5.72)	<0.001*
Gender		0.268		0.988
Male	Ref		Ref	
Female	19.31 (-14.84, 53.47)		-0.16 (-19.81, 19.50)	
Ethnicity		0.327		0.504
Chinese	Ref		Ref	
Indian	14.20 (-27.79, 56.19)		10.93 (-10.70, 32.55)	
Malay	23.77 (-9.04, 56.59)		6.89 (-10.41, 24.19)	
History of AIDS-defining illness		0.017*		0.011*
No	Ref		Ref	
Yes	31.79 (5.75, 57.84)		17.24 (3.93, 30.55)	
Hep C antibody	-37.71 (-91.11, 15.68)	0.166		
Hep B antigen	-19.63 (-65.94, 26.67)	0.406		
Number of HHV seropositivity		0.048*		
1	Ref			
2	-35.42 (-112.50, 41.66)			
3	13.05 (-60.40, 86.51)			
4	4.38 (-70.91, 77.86)			
hsCRP (Log)	7.56 (-2.52, 17.64)	0.142		
Duration of ART	-4.50 (-7.99, -1.01)	0.011*	-2.92 (-5.07, -0.77)	0.008*
Baseline Viral Load (log)	-28.27 (-37.04, -19.49)	<0.001*	13.21 (7.0, 18.73)	<0.001*
Viral Load (log)	-15.64 (-18.70, -12.58)	<0.001*	-13.61 (-15.25, -11.96)	<0.001*
Baseline CD4 T-cell count	1.00 (0.94, 1.06)	<0.001*	1.00 (0.94, 1.06)	<0.001*
Time-dependent CD8 T-cell Count	0.117 (0.91, 0.14)	<0.001*	0.04 (0.03, 0.06)	<0.001*
Log KTratio	-42.04 (-75.06, -8.47)	0.014*	129.95 (60.05, 199.85)	<0.001*
INTERACTION				
logKTratio X Age			-3.13 (-4.81, -1.46)	<0.001*

Abbreviations: AIDS, acquired immunodeficiency syndrome; ART, antiretroviral therapy; HHV, human herpesvirus; hsCRP, high sensitivity C-reactive protein; K/T ratio, kynurenine/tryptophan ratio.

*Significance at $p<0.05$.

In analysing factors associated with CD4 T-cell recovery beyond 12 months on ART (Table 4.8), an increase in log transformed K/T ratio was associated with a decrease in CD4 T-cell counts. Thus for every 10% increase in K/T ratio, there was a 1.05 cell/μL decline in CD4 T-cell count >12 months post-ART. Additionally, an increasing number of HHV co-infections compared to individuals with a single HHV infection was found to be associated with on an average 137-160 cells/uL decrease in CD4 T-cell counts beyond the initial 12 months of ART. Other independent factors found to be significantly associated with better CD4 T-cell recovery >12 months post-ART included younger age, female sex, Indian compared to Chinese ethnicity, higher log hsCRP, longer duration of ART, higher log baseline viral load, higher log viral load (time-dependent), higher baseline CD4 T-cell count and higher time-dependent CD8 T-cell counts (time dependent) (Table 4.8).

In brief, poorer CD4 T-cell recovery was associated with a higher number of HHV infections and higher K/T ratio in our study.

Table 4.8: Risk factors associated with CD4 T-cell recovery >12 months period following ART initiation.

Variable	Univariate β-coefficient	*p*-value	Multivariate β-coefficient	*p*-value
Age	-3.15 (-4.05, -2.25)	<0.001*	-27.88 (-34.13, -21.60)	<0.001*
Gender		<0.001*		<0.001*
Male	Ref		Ref	
Female	63.42 (37.22, 89.63)		52.87 (28.56, 77.18)	
Ethnicity		0.004*		<0.005*
Chinese	Ref		Ref	
Indian	59.59 (20.40, 98.77)		65.90 (28.52, 103.29)	
Malay	25.20 (-3.18, 53.58)		26.93 (2.08, 51.79)	
History of AIDS-defining illness		<0.001*		<0.001*
No	Ref		Ref	
Yes	37.08 (17.51, 56.66)		30.22 (13.73, 46.72)	
Hep C antibody	1.04 (-39.10, 41.17)	0.960		
Hep B antigen	-1.99 (-39.87, 35.90)	0.918		
Number of HHV seropositivity		0.003*		<0.001*
1	Ref		Ref	
2	-89.05 (-160.15, -17.94)		-160.97 (-2222.46, -99.48)	
3	-101.77 (-170.30, -32.25)		-152.27 (-211.29, -93.26)	
4	-73.70 (-142.67, -4.74)		-137.75 (-197.58, -77.92)	
hsCRP (Log)	-31.90 (24.14, 39.66)	<0.001*	18.29 (11.49, 25.09)	<0.001*
Duration of ART	1.96 (-0.76, 4.69)	0.157	10.47 (7.92, 13.02)	<0.001*
Baseline Viral Load (log)	-23.07 (-30.01, -16.12)	<0.001*	8.38 (2.12, 14.63)	<0.001*
Viral Load (log)	-29.04 (-46.50, -11.58)	0.001*	-35.76 (-49.33, -22.18)	<0.001*
Baseline CD4 T-cell count	0.88 (0.81, 0.96)	<0.001*	0.99 (0.92, 1.07)	<0.001*
Time-dependent CD8 T-cell Count	0.20 (0.18, 0.22)	<0.001*	0.16 (0.14, 0.18)	<0.001*
Log KTratio	-40.27 (65.88, -14.66)	0.002*	-25.39 (-48.85, -1.94)	0.034*
INTERACTION				
logKTratio X Age			-6.81 (-8.47 - -5.16)	<0.001*

Abbreviations: AIDS, acquired immunodeficiency syndrome; ART, antiretroviral therapy; HHV, human herpesvirus; hsCRP, high sensitivity C-reactive protein; K/T ratio, kynurenine/tryptophan ratio.

*Significance at $p<0.05$.

CHAPTER 5: DISCUSSION

5.1 Seroprevalence of HHV infections among HIV-infected individuals

This is the first study to report the seroprevalence of common HHV infections among treated HIV-infected individuals in Malaysia. This is important as it helps inform the natural history, burden and transmission risk of HHV co-infections among HIV infected individuals in our setting. We found a relatively high seroprevalence with multiple HHV occurring concurrently among treated HIV-infected individuals in our study. These findings are expected and similar to that reported in other developing country settings and in immunocompromised hosts, all of which show a high burden of HHV co-infection in HIV (Barrett et al., 2014; Gianella et al., 2015; Mehrkhani et al., 2011; Romanowski et al., 2009; Schaftenaar et al., 2014). Understanding the burden of HHV co-infections in HIV is particularly important in the context of its role in driving persistent inflammation and possibly the development of age-related complications specifically neurocognitive and cardiovascular disorders, which we have recently shown to be significantly higher among young treated HIV-infected individuals compared to age, gender and ethnically matched uninfected controls in our setting (Rajasuriar et al., 2017). It is however important to view the prevalence data obtained in our study with some caution as these rates only reflect the seroprevalence from a single health centre and may not be reflective of the true prevalence in the larger HIV population in Malaysia.

5.2 Risk factors associated with HSV-1, HSV-2, CMV and VZV seropositivity

We identified several clinical factors that were associated with a seropositive status for HSV-1, HSV-2, CMV and/or VZV in this study. However, these should be interpreted with caution as seroprevalence was very high, resulting in a relatively small group of seronegative participants, and the effect of performing multiple comparison cannot be ruled out. We found that seropositivity to HSV-1 and HSV-2 was significantly associated

with a longer duration on ART, which indicates that the longer the patients are on ART, the risk of acquiring either new HSV-1 and HSV-2 infections or reactivation increases. However, the risk of acquiring STI after ART in HIV-infected individuals warrants further research.

Moreover, we found that HSV-2 seropositivity was also significantly associated with being a female and a history of AIDS defining illness. Female sex has been found to have a higher risk of HSV-2 infection. Page et al. (2012) found that females had nearly a 4-fold higher odds for acquiring HSV-2 compared with males among HIV-uninfected US military adults. Among HIV-infected individuals in Canada, women were 2.7 times more likely to be HSV-2 seropositive compared to men (Romanowski et al., 2009). A history of AIDS-defining illness which denotes the severity of immunodeficiency prior to initiation of ART was associated with an increased risk of HSV-2 seropositivity and this is consistent with the biology of herpes viruses which causes clinical disease in states of immune suppression. Prior studies have reported socio-demographic factors such as heterosexual and participants identified as endemic, increasing age and ethnicity as risk factors for HSV-1 and HSV-2 (Romanowski et al., 2009; Schaftenaar et al., 2014) but these were not observed in our study potentially due to the small sample size and narrow age range in our cohort. Education and income have also been identified to be important risk factors for HSV-1, however, this data was not collected in our cohort and thus could not be confirmed in our setting.

CMV and VZV serostatus was associated with ethnicity in our study. Ethnic Indians had lower odds of CMV and VZV seropositivity compared to Chinese. The reason behind this is unclear, but ethnic differences could be modulated by multiple factors including differences in socio-economic background or genetic differences in immune responses towards a viral infection. This has further evident in a study, where they aimed to determine the prevalence of cervicovaginal HPV infection in multi-ethnic Malaysian

population. They found that Indian ethnicity is one of the independent risk factors associated with higher rates of HPV infection (Khoo et al., 2017). Besides, it may be due to the small number of Indian ethnicity in the cohort. These need to be explored in more detail in future studies.

CMV seropositivity was also associated with lower current CD4/CD8 ratio in our study. This is consistent with recent findings that low CD4/CD8 ratio in treated HIV-infected individuals is associated with poor immune function, SNAEs and increased mortality (De Salvador-Guillouët et al., 2015; Mussini et al., 2015; Serrano-Villar, Pérez-Elías, et al., 2014; Serrano-Villar, Sainz, et al., 2014).

5.3 K/T ratio correlates with HHV co-infection loads and markers of immune activation

We further analyzed the correlation between K/T ratio with HHV antibody levels and other immune activation markers. We found CMV and VZV IgG levels positively correlated with K/T ratio, which remained significant, even after excluding patients with chronic hepatitis B and C co-infections. This implies that CMV and VZV are important and persistent modulators of the IDO pathway in patients receiving long-term suppressive ART. Prior studies in HIV-infected individuals have found derangements in K/T ratio to be associated with the loss of T_H17 cells which promotes microbial translocation and persistent inflammation (Favre et al., 2010; Jenabian et al., 2013). We also observed a strong relationship between K/T ratio and sCD14, a surrogate marker for microbial translocation. Moreover, we found that K/T ratio and other markers of systemic inflammation (IL-6, neopterin and hsCRP) were positively correlated, which is consistent with prior studies (Bipath et al., 2015). Our data suggests that derangements in the kynurenine-tryptophan pathway may potentially be a common marker reflecting immune dysfunction driven by both viral and bacterial antigen exposure, both of which have been

shown to be associated with chronic immune activation and multiple co-morbidities in HIV (Cassol et al., 2010; Masiá et al., 2014).

We also assessed if there was a significant association between increasing seropositivity to multiple HHV pathogens and markers of immune activation as a simplistic method to explore the potential additive nature of immune modulation by HHV. We observed higher hsCRP and IL-6 levels in individuals with increasing seropositivity to multiple HHV pathogens after adjustment with age, gender and ethnicity. K/T ratio however, did not differ across increasing number of HHV seropositivity implying that the modulating effect of HHV on tryptophan metabolism was complex and not additive in nature.

5.4 CMV seropositivity had the strongest modulating effect on K/T ratio

We used an SOM approach to explore the effect of differing combinations of HHV seropositivity on K/T ratio given that all the HIV-infected participants in our cohort were seropositive to at least one of the HHVs tested. We did a stepwise analysis, where we initially analyzed combinations of 2 different types of HHVs on the modulation of K/T ratio and then analyzed combinations of 3 HHVs and subsequently all 4 HHVs. This approach helped us discern how concentrations of K/T ratio changed as each HHV infection was added to the models.

From the clustering of three and four different types of HHV, it was apparent that the lowest K/T ratio was observed in clusters where individuals were negative for CMV. This is further confirmed when the clustering of all HHVs was done. The combination of HSV-2, CMV and VZV was associated with the highest derangement of the kynurenine-tryptophan pathway as expressed by the highest K/T ratio while the lowest modulating effects of this pathway were observed in individuals seronegative for CMV. CMV has

previously been described to be among the most immunogenic chronic viral infections (Stern-Ginossar et al., 2012) as it has a complex biology that evades host recognition (Freeman et al., 2016). In the setting of HIV, T-cell repertoires have been shown to reduce in favor of CMV-specific CD8 T-cell expansion (Naeger et al., 2010) which outnumber the proportion of T-cells against other HHVs (Fletcher et al., 2005). CMV has also been shown to elicit a bystander activation effect on non CMV-specific T cells through the release of cytokine and cytokine receptor homologs (Kotenko et al., 2000). These unique effects of CMV on the host immune system may explain its strong modulating effect on K/T ratio compared to the other HHVs.

5.5 HHV co-infection and K/T ratio influence CD4 T-cell recovery

Immune recovery is highly variable despite years of suppressive ART (Kelley et al., 2009; Nakanjako et al., 2008; Onen et al., 2009) and associated with SNAEs (Baker et al., 2008, p. 4; Lau et al., 2007; Wester et al., 2011). In this study, we explored the potential role of HHV seroprevalence and persistent kynurenine-tryptophan dyregulation on CD4 T-cell recovery. HHV seroprevalence, quantified as an increasing number of HHV seropositivity in a participant, was associated with a significant decline in CD4 T-cells following 12 months of suppressive therapy, confirming findings from a prior cross-sectional study which showed reduced immune reconstitution in treated HIV-infected individuals co-infected with CMV (Barrett et al., 2014). Despite a statistically significant association in multivariate analysis, the impact of K/T ratio on changes in CD4 T-cells post-ART was relatively small and clinically insignificant. A prior study in Uganda assessing the impact of K/T ratio measured at 12 months post-ART on subsequent CD4 T-cell recovery also reported a significant but small decrease in CD4 T-cell counts (2 cells/μL/month) with each doubling of K/T ratio (Byakwaga et al., 2014). Downstream catabolites of tryptophan metabolism such as kynurenine and picolinic acid have been

shown to inhibit T-cell proliferation (Frumento et al., 2002). Kynurenine has recently been shown to inhibit IL-2 mediated signaling in memory CD4 T-cells via increased reactive oxygen species production, making them susceptible to Fas-mediated apoptosis (Dagenais-Lussier et al., 2016). Additionally, HHVs such as CMV has also been associated with bystander activation-induced cell death (McNally et al., 2001) which could further contribute to HHV-associated CD4 T-cell loss independent of its effect on tryptophan metabolism. Indeed, we found higher systemic inflammation levels with increasing number of HHV seropositivity in this cohort. Periodic subclinical reactivation of HHV during treated HIV disease may also provide increased target cells for HIV infection (Gianella et al., 2014) and promote further CD4 T-cell loss. Thus strategies to control HHV infection/re-activation rather than IDO inhibition in HIV may be beneficial in optimizing immunological responses to ART. Although a prior study with valgancyclovir (Hunt, Martin, et al., 2011) in ART-treated individuals did not show a significant benefit in improving CD4 T-cell counts despite an impact on CD8 T-cell activation, no studies have so far been conducted in a resource limited setting where background levels of immune activation and IDO activity are higher despite ART (Byakwaga et al., 2014; Hunt et al., 2011) and the interaction between HHV and HIV on clinical outcomes are likely to be more significant. Our recovery model assessed from 0-12 month post-ART showed that an increase in CD4 T-cell counts were associated with K/T ratio increase. We speculate that the associations assessed during this period may largely reflect the redistribution of CD4 T-cells from the lymph nodes rather than de novo CD4 T-cell production and thus may not reflect the true biological relationship between these parameters (Corbeau & Reynes, 2011).

5.6 Limitations of the study

There were several important limitations to this study. First, this was a cross-sectional study and hence, we cannot infer any causal relationship from the associations found in this study. Secondly, we used IgG antibody levels as a surrogate of HHV infections and did not measure HHV DNA levels as done in some studies (Conde-Glez et al., 2013; Romanowski et al., 2009). We also cannot exclude the possibility that increased HHV antibody levels found could have partially been contributed by an increase in global B cell activation as previously described in HIV (Brunt et al., 2014) and assessments of cellular proliferative index of total non-specific B cells and total IgG plasma levels would have helped discern this (Victora & Nussenzweig, 2012). Nevertheless, numerous studies assessing the modulating effect of HHV on host immune responses (Naeger et al., 2010) and clinical outcomes (Lichtner et al., 2014; Parrinello et al., 2012) have also utilized IgG as a surrogate for HHV infection and found these to reliably correlate with morbidity and mortality outcomes in HIV infected and uninfected individuals. Additionally, we only measured HHV IgG levels at a single time-point post-ART and thus acknowledge that the potential influence of the duration of HHV infection on K/T ratio and immune reconstitution could not be reliably assessed in this study. Thirdly, we only focused on HSV-1, HSV-2, CMV and VZV but not the other HHVs in our study, as these four are the common HHV infections in HIV-infected individuals (Gianella et al., 2015). Fourthly, recent studies have described the influence of genetic factors associated with K/T ratio in treated HIV (Lee et al., 2016). The influence of these host factors was not assessed in our study and thus we cannot be certain of their potential modifying effect on the associations between HHV seropositivity and K/T ratio assessed in our study. Furthermore, data from our study was from a single tertiary site with an over-representation of ethnic Chinese participants and thus our findings may not be generalizable to the HIV population in Malaysia. Finally, the number of patients who

were seronegative for specific HHV infections particularly in CMV was quite small and this could have impacted our ability to demonstrate significant differences in measured markers of immune activation between seropositive and negative individuals.

CHAPTER 6: CONCLUSION

6.1 Conclusions

In this study, we explored the effect of HHV co-infections on K/T ratio in treated HIV-infected individuals and assessed its impact on CD4 T-cell recovery in the developing country setting. We found that the seroprevalences of HSV-1, HSV-2, CMV and VZV in HIV-infected subjects were relatively high as compared to published data from other developing countries and that co-infections with multiple HHV were prevalent in our setting. Additionally, both demographic and HIV-related factors were associated with an increased risk of HHV infections in treated HIV-infected individuals in our setting. Plasma K/T ratio was found to be significantly correlated with HSV-2, CMV and VZV IgG levels. In patients with multiple HHV infections, CMV appeared to have the strongest modulating effect on K/T ratio. Increasing number of HHV seropositivity and K/T ratio was also significantly correlated with multiple markers of immune activation and systemic inflammation. An increasing number of HHV seropositivity was independently associated with poorer CD4 T-cell recovery following 12 months of ART; however, the effect of K/T ratio on CD4 T-cell recovery was only marginal. This observation has significant implications in the developing country setting where chronic HHV co-infection is highly prevalent.

6.2 Future research perspective

The following suggestions are built upon the findings of the present study and provides a better understanding on the role of HHV infections in driving morbidity and mortality among treated HIV-infected individuals in the developing country setting:

1. Investigation on the role of other HHVs, including HHV6, HHV7, HHV8 and EBV in modulating persistent immune activation and inflammation among HIV-infected individuals.
2. Comparative analysis in HHV-positive HIV-infected individuals and HHV-positive HIV-negative control group to explore the differences between their influence of HHV on K/T ratio and other markers of immune activation in the presence and absence of HIV infection.
3. Exploring the association between HHV-specific immunity and clinical comorbidities including atherosclerotic disease and neurological dysfunction.
4. Performing an interventional study with newer anti-CMV treatment such as Letermovir to explore the impact of CMV treatment on reduction in immune activation levels and potential changes in CD4 T-cell counts.

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LIST OF PUBLICATIONS AND CONFERENCE ATTENDED

Conference attended

Siew Hwei Yap, Noor Kamila Abdullah, Nuruljannah Nor Azmi, Marie Larsson, Iskandar Azwa, Adeeba Kamarulzaman, Kok Hoong Leong, Yin Ling Woo, Reena Rajasuriar. The prevalence and risk factors for human herpesvirus (HHV) in treated HIV-infected individuals in University Malaya Medical Centre, Malaysia. Poster presentation at International Scientific Symposium - Institut Pasteur International Network, 14th-16th October 2015. Paris, France.

Published Manuscript

Khoo, S. P., Bhoo-Pathy, N., **Yap, S. H.**, Anwar Shafii, M. K., Hairizan Nasir, N., Belinson, J., … Woo, Y. L. (2017). Prevalence and sociodemographic correlates of cervicovaginal human papillomavirus (HPV) carriage in a cross-sectional, multiethnic, community-based female Asian population. *Sexually Transmitted Infections*.

Ma'som M, Bhoo-Pathy N, Nasir NH, Bellinson J, Subramaniam S, Ma Y, **Yap SH**, Goh PP, Gravitt P, Woo YL. (2016). Attitudes and factors affecting acceptability of self-administered cervicovaginal sampling for human papillomavirus (HPV) genotyping as an alternative to Pap testing among multiethnic Malaysian women. *BMJ Open, 6*(8): e011022.

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