ROLE OF GBV-C AND HUMAN HERPES VIRUS COINFECTIONS IN AIDS DEVELOPMENT IN HIV-1 SEROCONVERTERS

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ABSTRACT

Background: GB virus type C (GBV-C) co-infection prolongs survival among Human Immunodeficiency Virus (HIV) infected individuals. Chronic immune activation is associated with HIV-1 disease progression.

Objective: To investigate the effect of GBV-C coinfection and herpes virus reactivation on AIDS development in HIV-1 seroconverters.

Methods: A total of 272 men HIV-1 seroconverters were included for the analysis. Cox proportional hazards (PH) regression models were employed to evaluate the effects of GBV-C and herpes viruses (CMV, EBV, HHV6, HHV8) on time from HIV-1 seroconversion to AIDS development. In addition, Gray's piecewise constant time-varying coefficient (PC-TVC) model that accounts for varying covariate effects over time was employed to estimate the effects for the variables that did not follow PH assumption.

Results: In Cox PH model analysis, GBV-C coinfection delayed AIDS development statistically significant in HIV-1 seroconverters. The $\log_{10}$ GBV-C RNA increase was associated with a 15% decrease in AIDS development, while the high HHV8 and CMV reactivation increased AIDS development respectively. The effects of HHV6 and EBV on AIDS development were not statistically significant. Using Gray PC-TVC model, GBV-C coinfection was associated with

delaying AIDS development, especially starting from year 3 of HIV-1 infection, then the hazard ratios decreased over time until 10 years, and kept in low level after 10 years of infection. HHV8 reactivation increased the chance of AIDS development, especially after 3 years of HIV-1 infection. The effect of CMV reactivation was constant with a hazard ratio of 1.38. In addition, two variables, age and baseline CD4+ T cell counts, which were not statistically significant in Cox PH regression model analysis, were statistically significant in Gray PC-TVC model. Similar to Cox PH analysis, the effects of HHV6 and EBV were not statistically significant either on AIDS development.

Conclusion: GBV-C co-infection delayed HIV-1 disease progression. HHV8 and CMV accelerated AIDS development. The effects of HHV6 and EBV were not statistically significant on AIDS development.

Public health importance: This study has important implications for investigating viral coinfections on AIDS development and providing alternative ideas to delay HIV disease progression.

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1.0 INTRODUCTION

Human immunodeficiency virus (HIV) is a lentivirus and responsible for causing acquired immunodeficiency syndrome (AIDS), a condition in which progressive failure of the immune system allows life-threatening opportunistic infections and cancers to thrive. The average survival time after infection with HIV is estimated to be 9 to 11 years. The relationship of HIV load and CD4+ T cell counts over the course of untreated HIV infection is shown in Figure 1 (1) (See Wikipedia, HIV, http:// http://en.wikipedia.org/wiki/HIV (as of Aug. 29, 2014, 20:50 GMT).).

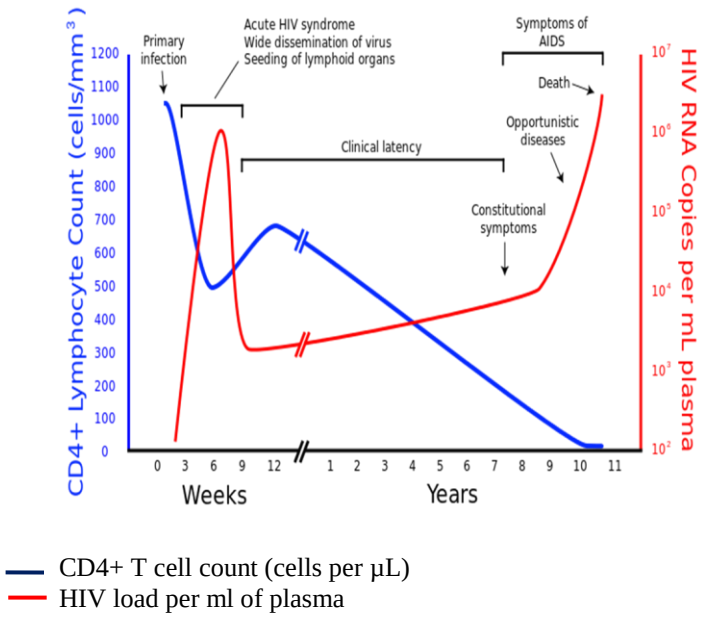


Figure 1. Relationship of HIV Load and CD4+ T Cell Counts over the Course of HIV Infection

It is estimated that over 75 million people have become infected with HIV and 36 million have died due to their infection. GBV-C coinfection could be beneficial for HIV-1 positive people (2), but the effect of dynamic changes of GBV-C RNA level on AIDS development is unclear. Herpes virus reactivation is associated with chronic inflammation (3). There is no report about herpes virus reactivation on HIV-1 disease progression in a longitudinal cohort study. In this study we investigated the effect of herpes virus reactivation and GBV-C co-infection on AIDS development in HIV seroconverters in Multicenter AIDS Cohort Study (MACS).

1.1 GB VIRUS TYPE C

GB virus type C (GBV-C) belongs to the Flavivirus family (4). It has a single stranded positive RNA genome of about 9.3 kb and contains a single open reading frame (ORF) encoding two structural (E1 and E2) and five non-structural (NS2, NS3, NS4, NS5A, and NS5B) proteins (5). It is a nonpathogenic human virus and distributed worldwide. It may establish persistent infection without clinical symptoms or disease in either immunocompromised or healthy individuals (6). GBV-C replicates in classification determinant 4 (CD4)+ T cells and in vitro infection of lymphocytes with GBV-C before HIV-1 infection reduces the replication of HIV-1, suggesting a direct inhibitory effect of GBV-C on HIV (7). A meta-analysis of studies related to HIV infected subjects found that the mortality ratio decreased 0.59 for those with GBV-C coinfection (8). Studies reported biological effects of GBV-C, which induces an HIV-inhibitory cytokine profile, decreases T-cell activation, blocks interleukin 2-mediated CD4+ T-cell proliferation, and reduces expression of the HIV entry receptors C-C chemokine receptor type 5 (CCR5) and C-X-C chemokine receptor type 4 (CXCR4) in vitro (7, 9, 10). Some studies about

HIV-1 positive people found a survival benefit of co-infection with GBV-C (2, 7, 11), but not all of them (12, 13). The interactive effect of GBV-C on HIV-1 need to be further investigated.

1.2 HUMAN HERPES VIRUSES

Herpesviridae is a large family of DNA viruses. The family name is derived from the Greek word herpein ("to creep"), referring to the latent, recurring infections typical of this group of viruses. Herpesviridae cause latent or lytic infections. Herpes viruses are known for their ability to establish lifelong infections by immune evasion by encoding a protein mimicking human interleukin 10 (hIL-10), by downregulation of pro-inflammatory cytokines IFN-γ, IL-1α, GM-CSF, IL-6 and TNF-α and the Major Histocompatibility Complex II (MHC II) in infected cells by detaining the newly formed MHC in the endoplasmic reticulum (ER). The MHC cannot reach the cell surface and therefore cannot activate the T cell response (14-16).

There are 8 herpes virus types that infect humans: herpes simplex viruses 1 (HSV-1), herpes simplex viruses 2 (HSV-2), varicella-zoster virus (VZV), human herpes virus 7 (HHV7), Epstein-Barr virus (EBV), cytomegalovirus (CMV), human herpes virus 6 (HHV6), and human herpes virus 8 (HHV8). HSV-1，HSV-2 and VZV are neuron tropic, HHV7 is detected in nearly 83% of the health volunteers (17). More than 90% of adults have been infected with at least one of the herpes viruses, and a latent form of the virus remains in most people (18, 19). Immune suppression is one of the most important factors related to herpes virus reactivation, and subsequently symptomatic infection(3). Because of the high prevalence of herpes virus infection, HIV-1 infected subjects are commonly co-infected with human herpes viruses. Due to CD4+ T cell depletion and immune deficiency resulted from HIV-1 infection, the reactivation of herpes

virus infections often occurs and contributes to the chronic immune activation and inflammation that may drive the HIV-1 disease progression (3, 20, 21).

In this study, GBV-C, EBV, CMV, HHV-6, and HHV-8 loads were tested longitudinally in plasma to study the viral coinfections on the effects of HIV-1 disease progression.

1.3 AIDS-DEFINING CLINICAL CONDITION

AIDS-defining clinical condition is the terminology given to a list of diseases published by the United States government-run Centers for Disease Control and Prevention (CDC) that are associated with AIDS. A patient has AIDS if he or she is infected with HIV and has one of the followings: a CD4+ T-cell count below 200 cells/μL; or a CD4+ T-cell percentage of total lymphocytes of less than 15%; or has one of the defining illnesses(22).

2.0 STUDY OBJECTIVES AND HYPOTHESES

2.1 STUDY POPULATION AND DESIGN

The study population was selected from the participants in MACS which is ongoing in Baltimore, Chicago, Pittsburgh, and Los Angeles. At six-month intervals, HIV-related clinical status is assessed, an interviewer administered questionnaire is completed, and blood is obtained for analysis, including tests for HIV-1 sero-positivity, HIV-1 RNA levels and CD4+ T-cell counts. In this study, the subjects were followed from April 1984 to November 2013. For GBV-C measurement, RNA was extracted from plasma and reverse transcriptase real time polymerase chain reaction (RT-PCR) assay was used to quantify GBV-C RNA load in blood plasma, which was conducted by Dr. Yue Chen's lab at the Department of Infectious Diseases and Microbiology at the University of Pittsburgh. The reactivations of HHV8, HHV-6, CMV, EBV were also tested in the plasma by real-time PCR, which was conducted by Dr. Charles Rinaldo's lab at the Department of Infectious Diseases and Microbiology at the University of Pittsburgh. The study was approved by the Institutional Review Board of the University of Pittsburgh. Plasma samples after HIV seroconversion and all subsequent available samples were tested for GBV-C RNA and herpes virus DNA in order to determine the effect of dynamic changes of viral coinfection on HIV-1 disease progression. To be included in this study, the date of HIV seronegative and the first visit at which he was seropositive) had to be known within a window

of 1.5 year. During this time period, CD4+ T cells and HIV load become relative stable after acute HIV infection(23). According to our data, herpes virus activation is not consistent and it is hard to impute the missing values. In this study, if more than half of the visiting data were missing, they were excluded for analysis. GBV-C loads were imputed as last observation carried forward (LOCF). Herpes virus reactivation ratio was presented as the ratio of herpes virus positive visits to the total visits tested per subject. The outcome was time from HIV-1 seroconversion to AIDS diagnosis or censoring because of death, highly active antiretroviral therapy (HAART) or end of follow up.

A total of 484 men in MACS who had documented HIV-1 seroconversion were tested for viral coinfections, 152 were excluded because first visit after HIV-1 seroconversion was more than 1.5 years, 60 subjects were excluded because more than half of the observations with GBV-C or Herpes virus tests are missing. Total 272 men were left for analysis (Figure 2).

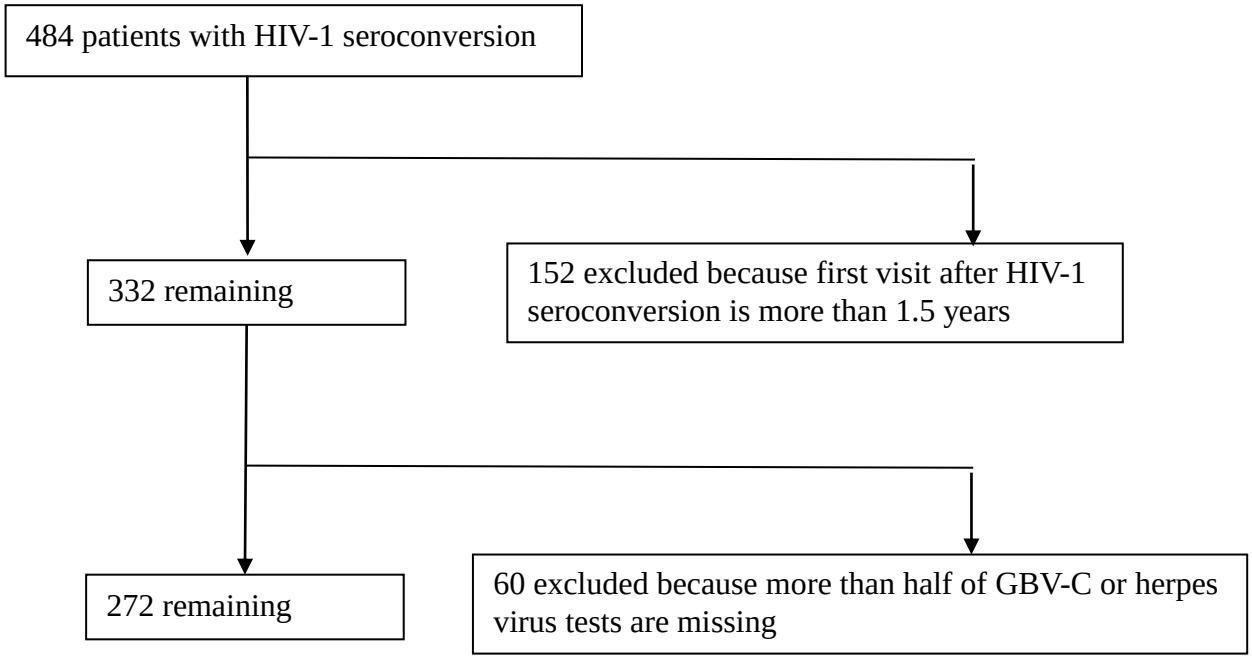


Figure 2. Selection Process of Subjects with HIV Seroconversion for the Evaluation of GBV-C and Herpes Virus Status

The dependent variable was the time from HIV-1 seroconversion to the diagnosis of AIDS or censoring. The definition of event or censoring time:

1. Patients with AIDS, not treated with HAART or HAART treatment after AIDS development: Middle time between AIDS diagnosis time and last AIDS free year – middle time of last HIV negative and first HIV positive (total 129 patients; 889 observations)
2. Patients with AIDS, treated with HAART before AIDS development:

   Time of HAART treatment- middle time point of last HIV negative and first HIV positive (total 19 patients; 158 observations)

3. Patients without AIDS, were not treated with HAART and died because of other reasons:
   Time of death - middle time point of last HIV negative and first HIV positive
   (total 12 patient; 82 observations)
4. Patients without AIDS, were not treated with HAART, and still alive:
   Last alive time- middle time point of last HIV negative and first HIV positive
   (total 12 patients; 78 observations);
5. Patients without AIDS, were treated with HAART, and still alive:
   Time of HAART treatment- middle time point of last HIV negative and first HIV positive
   (total 97 patients; 930 observations)
6. Patients without AIDS were treated with HAART and died:
   Time of HAART treatment- middle time point of last HIV negative and first HIV positive
   (total 3 patients; 43 observations)

2.2 PREDICTOR VARIABLES

The main independent variables of interest are GBV-C and human herpes virus (CMV, EBV, HHV6, HHV8) coinfections. GBV-C level was treated as continuous and time varying variable, and herpes viruses were presented as the ratio of test positive visits to the total visits per subject. The detection limits per reaction were shown in Table 1. Age at the time of seroconversion, baseline CD4+ T cell counts and HIV-1 load were analyzed as continuous variables. Race was treated as categorical form.

Table 1. Upper and Lower Detection Limits in Number of Quantities Per Reaction

	CMV	EBV	HHV6	HHV8	GBV-C
Upper DL/reaction	500,000	650,000	500,000	400,000	190,000,000
Lower DL/reaction	1	2.5	2	1.5	1,900

DL, Detection Limits

2.3 RESEARCH HYPOTHESIS

GBV-C coinfection during HIV-1 infection reduces the risk of AIDS development. Herpes virus reactivation, in contrast, is associated with an increased risk of AIDS development among HIV-1 positive persons. High CD4+ T cell counts and low HIV-1 load at initial HIV-1 infection delay HIV disease progression.

2.4 THE AIMS OF THE STUDY

To explore the impact of GBV-C co-infection and herpes virus (CMV, EBV, HHV6, HHV8) reactivation during HIV-1 infection on AIDS development

- Probability of survival from HIV-1 seroconversion to AIDS development assessed by Kaplan-Meier estimate
- The effects of univariate variables (GBV-C, CMV, EBV, HHV6, HHV8, age, baseline CD4 and HIV-1 viral load) on the duration from HIV-1 seroconversion to AIDS development

- The effects of herpes virus reactivation and dynamic changes of GBV-C load after HIV-1 seroconversion on AIDS development adjusted for age, baseline CD4 and HIV-1 load

3.0 STATISTICAL METHODS

3.1 DESCRIPTIVE STATISTICS

The patients were categorized according to the events and censoring status. The frequency and proportion of each status were shown. For categorical variables, visit and race, the proportion of each category within each variable was assessed. For continuous variables (HHV8, HHV6, CMV and EBV reactivation ratio, $\log_{10}$GBV-C load), mean and standard deviation were evaluated.

3.2 KAPLAN-MEIER SURVIVAL ESTIMATES

The Kaplan-Meier product-limit method was used to estimate the probability of progression to AIDS from HIV-1 seroconversion at any follow-up time. The assumptions used in this analysis was patients whom were censored have the same survival prospects as those whom were followed. The survival probability Pt at any particular time interval [t, t+1) can be calculated using the formula below:

$$\mathbf{P_t = (n_t - d_t)/n_t = 1 - d_t/n}$$

where n_t is the number of patients alived at the beginning of the time interval [*t*, *t*+1) and therefore were at risk of progression to AIDS during this interval; d_t is the number of patients

died in the time interval [t, t+1). Patients who have progressed to AIDS, died, dropped out, moved out; or lost in follow up during the interval [t, t+1) were considered being at risk (counted in the denominator) but being censored (not counted in the numerator) during this interval. The overall survival probability at time t then can be calculated using the product-limit formula $S(t)=S(t-1)\times P_t$. Therefore, we have

$$\mathbf{S(t)= \Pi(1- d_t/n_t)}$$

3.3 COX PROPORTIONAL HAZARDS MODEL

The Cox proportional hazards (Cox PH) model is used to estimate the hazard ratio (HR) (24). According to the model with static explanatory variables X_1, X_2, … the hazard function at time t is as follows:

$$\boldsymbol{h(t \mid X) = h_0(t)} \exp(\boldsymbol{\beta_1 X_1 + \beta_2 X_2 + \cdots})$$

where h_0(t) is the unspecified baseline hazard function at time t and $\boldsymbol{\beta}$'s are the unknown regression coefficients. Note that exp($\boldsymbol{\beta}$) is the hazard ratio when the corresponding covariate X increases by 1 unit. Univariable (unadjusted) and multivariable Cox PH models were used to determine the association between GBV-C infection and AIDS development. Hazard ratio, 95% confidence interval, and p value were estimated and calculated. The PH assumption was assessed using Gray's test(25).

When the values of variables do not change over time or when variables are collected at only one time point, these variables are static variables. In this study, the GBV-C status was tested longitudinally for each patient and treated as a time-dependent covariate in fitting a Cox model. If a covariate is collected more than once during the study follow-up, treating it as a time-

dependent covariate rather than the baseline static covariate in the model will result in a more robust estimate on covariate effect because this will utilize all information of this variable. The model described below incorporates time-dependent covariates X_α into the standard Cox PH model

$$\boldsymbol{h(t \mid X) = h_0(t)\ \exp(\alpha X_\alpha(t) + \beta_1 X_1 + \beta_2 X_2 + \ldots\ \ldots).}$$

The unknown regression parameters α and β's are still estimated by maximizing the partial likelihood function and the unspecified baseline hazard function $h_0(t)$ is estimated via the Breslow or Efron estimates. For this study, the time-dependent covariates include GBV-C load, and time static variables include GBV-C load adjusting for herpes virus reactivation, baseline CD4+ T cell count, and baseline HIV-1 viral load.

3.4 GRAY'S TIME-VARYING COEFFICIENTS MODEL

When a covariate violates the PH assumption, the corresponding regression coefficient or the hazard ratio will change significantly over time. An alternative survival model, Gray's piecewise constant time-varying coefficient (PC-TVC) model can be used to estimate time-varying covariate effect that captures the dynamic changes of hazard ratio (24). Gray's PC-TVC model(26) is more flexible in capturing the temporal dynamics of covariate effects, which allows for a departure from the PH assumption via introduction of time-varying regression coefficients. The model specifies the hazards with the following form

$$\boldsymbol{h(t \mid X) = h_0(t)\ \exp(\beta_1(t) X_1 + \beta_2(t) X_2 + \cdots),}$$

where the time interval is partitioned into nonoverlapping subintervals {j: [τj−1, τj), j = 1, 2, …, M+1} and unknown regression coefficients $\beta(t)$ remain constant within each of the subinterval

$[\tau_{j-1}, \tau_j)$ and will be estimated through maximizing the corresponding penalized partial likelihood function.

4.0 RESULTS

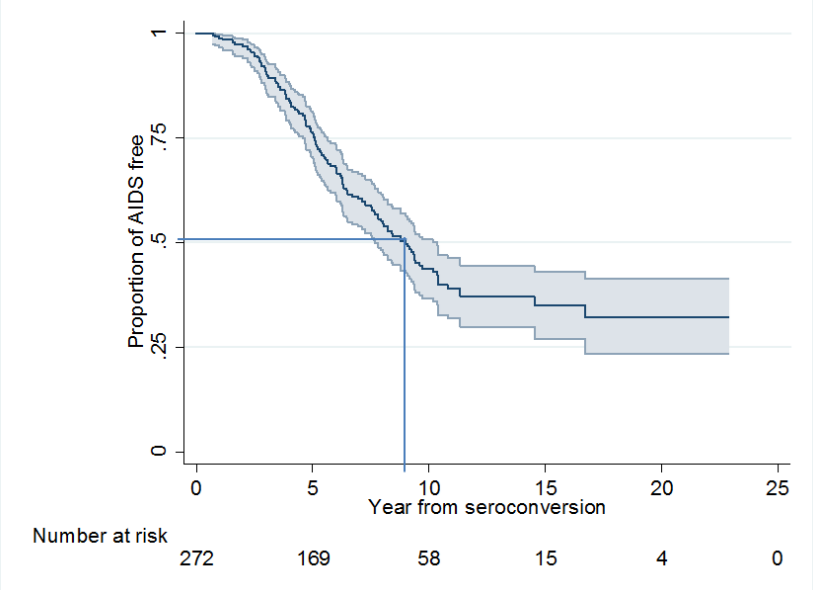
4.1 SUMMARY STATISTICS

According to the patient outcomes during the following up, they were categorized into six groups and shown in Table 2. 1) There were 129 (47.43%) patients developed AIDS without HAART (AIDS), the survival time is the difference of middle time between AIDS diagnosis time and last AIDS free year and middle time of last HIV negative and first HIV positive; 2) Nineteen patients developed AIDS and treated with HAART. HAART treatment was before AIDS (AIDS on_HAART), the survival time is the time of HAART treatment subtracting middle time point of last HIV negative and first HIV positive (total 19 patients; 158 observations); 3) Twelve subjects did not develop AIDS, were not treated with HAART, but died because of other reasons (death before AIDS), the survival time is last alive year subtracting middle time point of last HIV negative and first HIV positive (total 12 patients, 82 observations); 4) Twelve subjects who did not develop AIDS, were not treated with HAART and still alive (no event), the survival time is last alive year subtracting middle time point of last HIV negative and first HIV positive (12 patients, 78 observations); 5) Ninety-seven subjects who did not develop AIDS, but treated with HAART (no_AIDS on_HAART), the survival time is the time of HAART treatment subtracting middle time point of last HIV negative and first HIV positive (97 patients, 930 obervations); 6） Three subjects who did not develop AIDS, but treated with HAART and died (death

on_HAART). The survival time is the time of HAART treatment subtracting middle time point of last HIV negative and first HIV positive (3 patients; 43 observations). The patients in the first group (AIDS) were treated as event group and other groups were treated as censoring. The Kaplan-Meier plot showed the proportion of AIDS free subjects. The 50% time from HIV-1 seroconversion to AIDS development was 8.9 years (Figure 3).

Table 2. Categories of Events or Censoring

Events/censor	Frequency	Percent(%)
AIDS	129	47.4
AIDS on_HAART	19	7.0
death before AIDS	12	4.4
no event	12	4.4
no_AIDS on_HAART	97	35.7
death on_HAART	3	1.1
Total	272	100



All the 272 patients are included and the horizontal and vertical lines show the 50% survival time

Figure 3. Kaplan-Meier Plot of Time from HIV-1 Seroconversion to AIDS Diagnosis

According to the sample selection standards in method section, total 272 subjects were included for the analysis. The total visit distribution was shown in Table 3. There were 7 patients

who had only one visit and 2 patients with the maximum number of visit were 23. The median visit number is 6 per subject.

Table 3. Distribution of Visit per Subject

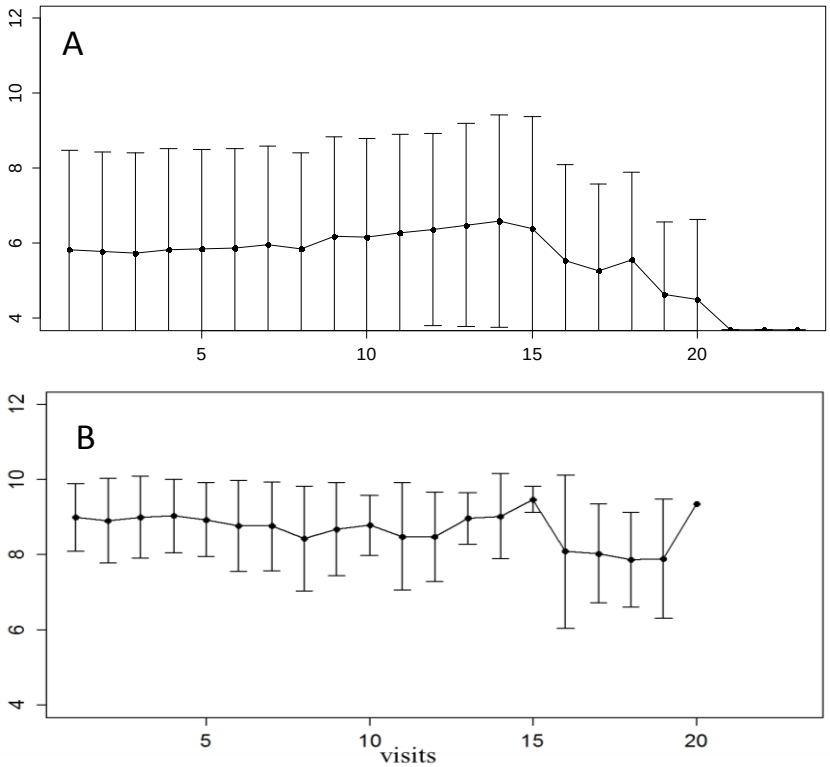
Visit	Patients	Percent(%）	Cumulative (%)
1	7	2.6	2.6
2	13	4.8	7.4
3	15	5.5	12.9
4	21	7.7	20.6
5	32	11.8	32.4
6	21	7.7	40.1
7	34	12.5	52.6
8	22	8.1	60.7
9	22	8.1	68.8
10	17	6.3	75.0
11	20	7.4	82.4
12	9	3.3	85.7
13	10	3.7	89.3
14	9	3.3	92.7
15	7	2.6	95.2
16	1	0.4	95.6
17	1	0.4	96.0
18	2	0.7	96.7
19	1	0.4	97.1
20	2	0.7	97.8
21	1	0.4	98.2
22	3	1.1	99.3
23	2	0.7	100.0
Total	**272**		**100**

There were total five different ethnic groups, 233 (85.7%) of them were white non Hispanic (Table 4). So in the analysis, white non-hispanic was treated as one category and all other ethnic, white Hispanic, black non Hispanic, Asian or Pacific Islander and other were treated as another category.

Table 4. Distribution of Patients by Race

Race	Patient number	Percent (%)
White-nonHispanic	233	85.7
White-Hispanic	13	4.8
Black-nonHispanic	24	8.8
Asian/Pacific Islander	1	0.4
Other	1	0.4
Total	272	

The changes of log_{10} transformed GBV-C RNA load over time for all the patients are shown in Figure 4A. The mean viral load range was between 5.7 and 6.3 in the first 15 visits. The log_{10} transformed GBV-C RNA load changes for GBV-C RNA positive patients were shown in Figure 4B. The mean range of was between 8.2 and 9.3. The high GBV-C loads detected in plasma is consistent with Bhattarai's report (27).



A) Including all the patients, B) Patients who were GBV-C RNA positive.

Figure 4. Changes of Mean Log_{10} GBV-C Load over Time after HIV-1 Seroconversion

Herpes virus reactivation was presented as the ratio of detectable viral load visits to the total visits per subject (Table 5). The mean reactivation ratio for EBV was 0.26, which was the highest among the four herpes viruses. The mean ratio for HHV8 and CMV was 0.12 and 0.16 respectively. HHV6 had the lowest mean reactivation rate of 0.01.

Table 5. Herpes Virus Reactivation Ratio

Variable	N	Mean	SD	RP* (%)
HHV8	272	0.12	0.2	40.4
HHV6	272	0.01	0.1	3.3
CMV	272	0.16	0.2	64.0
EBV	272	0.26	0.3	75.7

*** RP, reactivation percentage**

4.2 RESULTS OF THE COX PROPORTIONAL HAZARDS MODEL

The results of the Cox PH regression analysis were shown in Table 6. In the unadjusted analysis, the increase of GBV-C level was associated with a statistically significant reduction in AIDS development (HR = 0.83, 95% CI: .76–.90, $p<0.001$). HHV8 and CMV reactivation had a statistically significant increase in AIDS development (HR=1.18 and 1.32). The effect of HHV6 and EBV were not statistically significant ($p>0.05$). Age and race were not statistically significant either. Baseline CD4+ T cell counts were in bounder line ($p=0.05$) and HIV-1 viral load was statistically significant ($p<0.05$, HR=1.65). In Cox PH regression analysis adjusting for baseline CD4 counts, HIV-1 load, age and race, the hazard ratio of AIDS development for GBV-C RNA load was 0.80 (95% CI: .0.77–.92). Similar to unadjusted analysis, HHV8 and CMV statistically significantly increased AIDS development and HHV6 and EBV did not statistically significantly increase the risk of AIDS development. In the final model, including all the variables which were significant in the univariable analysis and also variables age and CD4

counts which were presumed to be clinically important to fit the multivariable model. By adjusting for all other variables, GBV-C still statistically significantly delayed AIDS development and HHV8, CMV statistically significantly increased AIDS development (Table 6).

Table 6. Cox PH Model Analysis to Estimate the Effect of Viral Coinfection on AIDS Development

	Unadjusted		Adjusted		Final	
Variable	**HR+**	**Unadjusted P+**	**HR***	**Adjusted P***	**HR#**	**Final p#**
GBV-C	0.83	**0.00**	0.80	**0.00**	0.85	**0.00**
HHV8	1.18	**0.00**	1.10	**0.02**	1.10	**0.04**
HHV6	1.07	0.62	1.10	0.28	1.09	0.41
CMV	1.32	**0.00**	1.30	**0.00**	1.26	**0.00**
EBV	1.05	0.16	1.00	0.56	0.97	0.45
age	1.01	0.51			1.00	0.90
RACE	0.93	0.78				
CD4	1.00	0.05			1.00	0.10
HIV load	1.65	**0.00**			1.44	**0.00**

+ total nine univariate variable models
* total five models: GBV-C, HHV8, HHV6, CMV, EBV adjusted by age, race, baseline CD4+ T cell count and baseline HIV viral load respectively
# one multiple variable model, adjusted by all the variables except for race

4.3 POWER ANALYSIS

Based on n=272, 47.47% events (developed AIDS), 80% power and type I error of 0.05, the detected standardized hazard ratio (value-mean/sd) is 1.32.

4.4 DIAGNOSIS OF COX PH MODEL

To assess the overall goodness of fit of a Cox PH regression model, the cumulative observed versus the cumulative expected number of events for subjects with observed (not censored) survival times were plotted. If the model fit is adequate, then the points should follow a 45-degree line

beginning at the origin (28). The cumulative hazard plot of the Cox-Snell residuals for the final Cox PH model was shown in Figure 5. The hazard function was reasonably straight line that has zero interception. It approximates the 45-degree line very closely except for very large values of time. The model is lack of fit especially in the later time points. The fitted final model could be used to test the variable effects on AIDS development but it is not appropriate to do prediction.

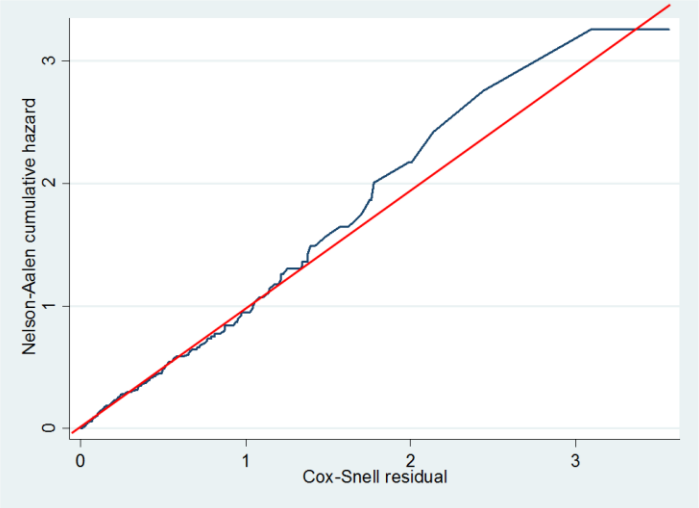
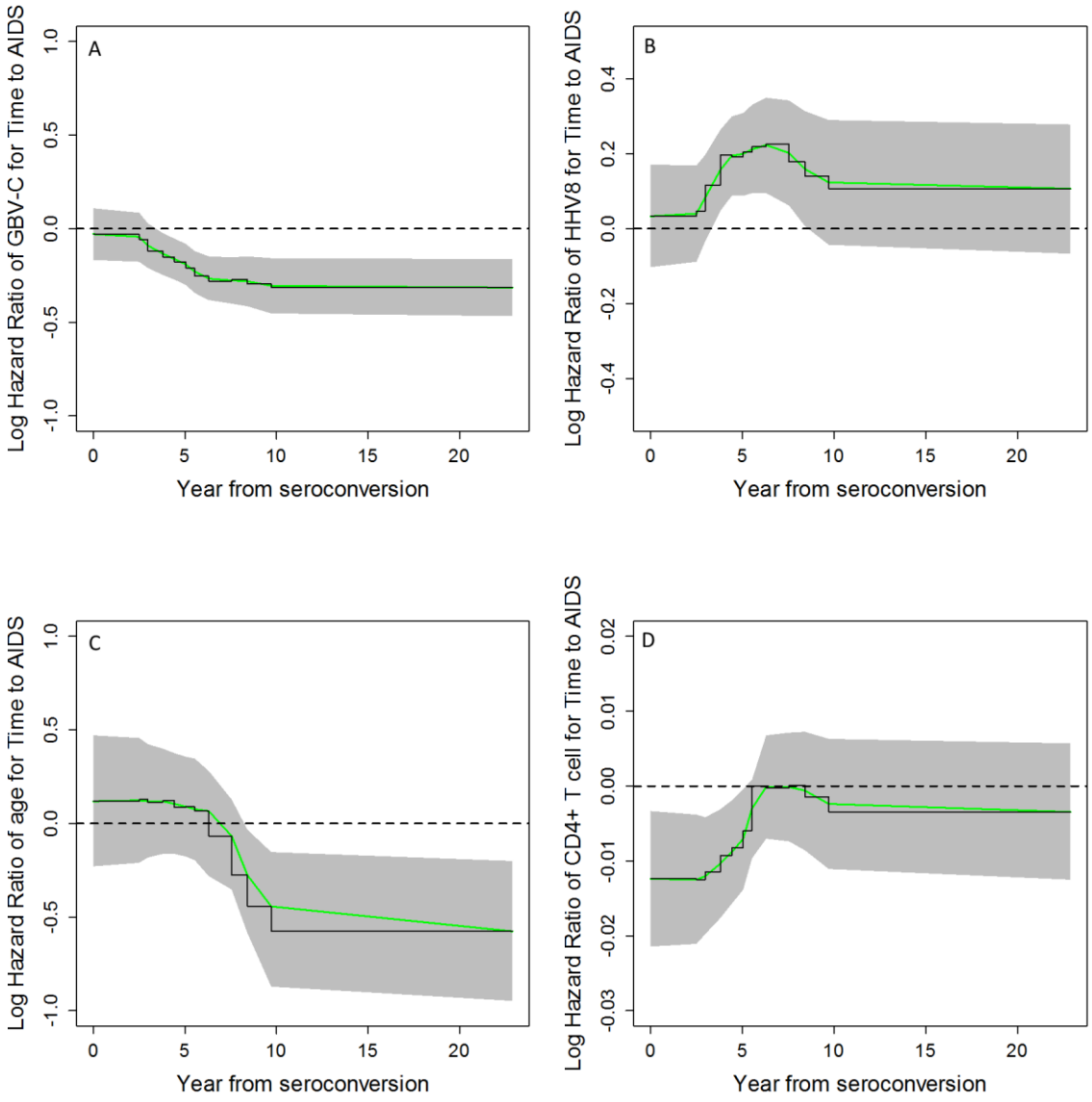


Figure 5. Coxsnell Residual Plot for Overall Goodness of Fit

4.5 GRAY'S TIME-VARYING COEFFICIENTS MODEL

Cox PH models assume that the hazard ratio is constant over time. By definition, the Cox model is constrained to follow this assumption. It is important to evaluate its validity. The tests of PH assumption were evaluated using Gray's test. In this situation, covariate effects are not constant over time, an alternative survival model that accounts for varying covariate effects should be used. We choose Gray's piecewise constant time-varying coefficient (PC-TVC) model to reanalyze the data. In this study the effect of HHV8, CD4 count and age were not constant,

also we are interested in effect of GBV-C load over time. So, we employed PC-TVC to evaluate the spline effects of these variables together with linear effect variables, CMV, EBV, HHV6, HIV RNA load on AIDS development. The influences of GBV-C load was not statistically significant in the first 3 years after HIV infection, then the hazard ratios decreased over time till year 10, and kept in low level after 10 years of infection (Figure 6A). The effects of HHV8 was not statistically significant in the first 3 years after HIV infection, then the hazard ratio increased over time till year 7, and decreased afterwards but still higher than the first 3 years of initial HIV infection (Figure 6B). Age increased the chance of AIDS development in the first 7 years after HIV seroconversion, but it decreased in the later time of HIV infection (Figure 6C). CD4+ T cell counts were associated with decreasing the possibility of AIDS development in the first 6 years after HIV seroconversion, and the effect become not distinct in later time of HIV infection (Figure 6D). The effects of the variables in the analysis were shown in Table 6 and Table 7. The effects of the four variables, GBV-C load, HHV8, age, CD4+ T cell counts on AIDS development which did not adhere to PH assumptions were statistically significant (Table 7). The effects of variables that adhered to PH were shown in Table 8. CMV reactivation ratio was associated with higher ratio of AIDS development (HR=1.38, $p<0.01$). The effect of EBV and HHV6 were not statistically significant. Baseline HIV-1 load was associate with increased AIDS development ($p<0.01$)



Log Hazard Ratio (black solid lines), 95% Confidence Intervals (shaded areas). The green line is the fitted line. The black dash line is a reference line.with a hazard ratio of 1. A) the effect of GBV-C level; B) the effect of HHV8 reactivation ratio; C) the effect of age; D) the effect of CD4+ T cell counts

Figure 6. Hazard Ratio Change for Time Varying Covariate Effects from Gray PC-TVC model

Table 7. Gray's Model Analysis for None Proportional Hazard Variables

Variable	Sig.stat*	Sig.p*	PH.stat#	PH.P#
GBV-C	20.7	**<0.01**	6.242	0.01
HHV8	14.9	**<0.01**	5.139	0.03
Age	6.9	**0.04**	6.878	0.01
CD4	9.4	**0.01**	5.469	0.02

* Significance effect on AIDS development
# proportional hazard ratio test

Table 8. Gray's Model Analysis for Proportional Hazard Variables

Variable	HR	95% CI	P
CMV	1.38	1.27-1.51	**<0.001**
EBV	1.00	0.92-1.08	0.916
HIV load	1.71	1.41-2.10	**<0.001**
HHV6	1.21	0.99-1.54	0.090

5.0 DISCUSSION

Two models, Cox PH model and Gray's PC-TVC model were employed to fit the study data. Analysis using both models got the similar results, GBV-C coinfection slowed AIDS development while HHV8 and CMV reactivation accelerated HIV disease progression. The effects of EBV and HHV6 were not statistically significant. Baseline HIV-1 level was also associated with HIV disease progression. Baseline CD4+ T cell count and age were statistically significant in acceleration of AIDS development in Gray's PC-TVC model but not significant in Cox PH model. Furthermore, the effects of CD4+ T cell count and age on AIDS development varied over time after HIV seroconversion. Cox PH model provided the average estimates of coefficients, so it failed to capture the changes during the observation period. The Gray's PC-TVC model using piecewise constant penalized splines showed more details of how those effects change over time.

This study provided strong evidence that GBV-C coinfection delays AIDS development in HIV-infected subjects. The effects were related to the length of HIV infection. This was a longitudinal designed study with controlling the duration of HIV infection and testing the dynamic change of GBV-C level. Previous studies of the influences of GBV-C coinfection on HIV disease progression that did not find a survival benefit tested only samples from patients with high CD4+ T-cell counts (13), whereas studies that did find a survival benefit involved subjects with a broad range of CD4+ T-cell counts(29, 30). This longitudinal study showed that

GBV-C level in the first three years after HIV seroconversion was not statistically significantly related to AIDS development, whereas after three years of HIV seroconversion, the ratio of AIDS development decreased, supporting that the effects of GBV-C infection were related to the length of HIV infection. The mechanisms that GBV-C effects on HIV disease progression are inhibition of HIV replication (8), inducing an HIV-inhibitory cytokine profile, decreasing T-cell activation, blocking CD4 T-cell proliferation, and reducing co-receptor expression (7, 9, 10).

Infection with herpes viruses is a lifelong condition, the viruses become permanently latent in the host. In immunocompromised individuals, such as those with HIV-1 infection, impaired immunity leads to more frequent and severe symptomatic or asymptomatic herpes virus reactivation. Some studies have shown that the shedding of herpes viruses occurs more frequently among those who are also infected with HIV-1 than herpes virus infected/HIV-1 uninfected persons (31-33). Among HIV-1-infected persons, herpes virus shedding occurs more frequently and higher quantity among those with lower CD4 counts (32, 34, 35). So the frequency of herpes virus shedding may have an effect on AIDS development. Our results showed that CMV and HHV8 reactivation ratios were significantly related to AIDS development. The higher the frequency of CMV and HHV8 reactivation, the more chance of AIDS developed in HIV-1 infected subjects. HHV8 infection was associated AIDS relate tumor Kaposi sarcoma (KS) (36, 37). CMV was a major cause of morbidity and mortality in patients with AIDS in the United States (38, 39). During HIV-1 infection, there was significant activation of CMV-specific CD8+ T cells (40). Hence, sustained antigen mediated immune activation occurs in HIV-1-infected patients. The chronic immune activation and inflammation are directly associated with HIV-1 disease progression (3). In this study, EBV, which had the highest ratio of reactivation, was not associated with AIDS development. Doisne et al. reported that EBV

specific CD8+ T cells were activated during primary HIV infection (41), but EBV level was not associated with brain lymphoma. Further study showed that a new EBV viral set point was reached early in HIV infection, high EBV load was already a normal situation early in HIV infection and was not related to a decrease in immune function over time (42). This may help to explain the lack of predictive value of EBV load for the occurrence of AIDS-related lymphoma, one of the defining illnesses of AIDS diagnosis.

This is a longitudinal study, 272 subjects were followed up, and the longest followed up time is 23 years. The study analyzed the dynamic effect GBV-C level and herpes virus reactivation status. The limitation of this investigation was that possible selection bias. 152 patients were excluded because the first visit after HIV-1 seroconversion is more than 1.5 years and 60 patients were excluded because more than half of the visit data are missing in the subjects. Another limitation was that because of the sample numbers we did not classify the AIDS diagnosis. Some of them were diagnosed with AIDS because of low CD4+ T cell counts, or opportunistic infections, and some patients were because of tumors, such as lymphoma or KS. As we know lymphoma and KS are closely related to herpes virus EBV and HHV8 infection. In addition, in order to fully determine the effects of herpes viruses and GBV-C coinfection on chronic immune activation and inflammation, biomarkers of immune activation and inflammations should be included in the analysis.

6.0 CONCLUSION

High GBV-C level was related to delay AIDS development in HIV-1 infected individuals. The effects became statistically distinct after 3 years of HIV-1 seroconversion and the HR continuous decreased until 10 years and maintained in the low level afterwards. The increase of CMV and HHV8 reactivation ratio was related to accelerate AIDS development, while the effects of HHV6 and EBV on AIDS development were not significant.

7.0 PUBLIC HEALTH SIGNIFICANCE

HIV-1 is a major contributor to the global burden of disease. In 2010, HIV-1 was the leading cause of disability adjusted life years worldwide for people aged 30–44 years, and the fifth leading cause for all ages(43). HAART has transformed HIV infection from a rapid disease into a chronic condition. The success of HAART therapy depends on patient adherence. The side effects of HAART therapy have led to many people discontinuing their therapy. GBV-C viremia is associated with delaying AIDS development. The use of GBV-C to slow HIV disease progression provides us an alternative idea without the associated difficulties with patient compliance and the side effect profile of HAART drugs. Chronic immune activation and inflammation are associated with HIV disease progression. Also, herpes virus infection is related to HIV transmission(44). So decreasing herpes virus reactivation and effective treatment could not only prevent HIV transmission, but also slow AIDS development.

BIBLIOGRAPHY

1. **Pantaleo G, Graziosi C, Fauci AS.** 1993. New concepts in the immunopathogenesis of human immunodeficiency virus infection. The New England journal of medicine **328:**327-335.
2. **Williams CF, Klinzman D, Yamashita TE, Xiang J, Polgreen PM, Rinaldo C, Liu C, Phair J, Margolick JB, Zdunek D, Hess G, Stapleton JT.** 2004. Persistent GB virus C infection and survival in HIV-infected men. The New England journal of medicine **350:**981-990.
3. **Appay V, Sauce D.** 2008. Immune activation and inflammation in HIV-1 infection: causes and consequences. The Journal of pathology **214:**231-241.
4. **Murthy BV, Muerhoff AS, Desai SM, Lund J, Schmid CH, Levey AS, Mushahwar IK, Pereira BJ.** 1997. GB hepatitis agent in cadaver organ donors and their recipients. Transplantation **63:**346-351.
5. **Stapleton JT, Foung S, Muerhoff AS, Bukh J, Simmonds P.** 2011. The GB viruses: a review and proposed classification of GBV-A, GBV-C (HGV), and GBV-D in genus Pegivirus within the family Flaviviridae. The Journal of general virology **92:**233-246.
6. **Stapleton JT.** 2003. GB virus type C/Hepatitis G virus. Seminars in liver disease **23:**137-148.
7. **Xiang J, Wunschmann S, Diekema DJ, Klinzman D, Patrick KD, George SL, Stapleton JT.** 2001. Effect of coinfection with GB virus C on survival among patients with HIV infection. The New England journal of medicine **345:**707-714.
8. **Zhang W, Chaloner K, Tillmann HL, Williams CF, Stapleton JT.** 2006. Effect of early and late GB virus C viraemia on survival of HIV-infected individuals: a meta-analysis. HIV medicine **7:**173-180.
9. **Jung S, Knauer O, Donhauser N, Eichenmuller M, Helm M, Fleckenstein B, Reil H.** 2005. Inhibition of HIV strains by GB virus C in cell culture can be mediated by CD4 and CD8 T-lymphocyte derived soluble factors. Aids **19:**1267-1272.
10. **Xiang J, George SL, Wunschmann S, Chang Q, Klinzman D, Stapleton JT.** 2004. Inhibition of HIV-1 replication by GB virus C infection through increases in RANTES, MIP-1alpha, MIP-1beta, and SDF-1. Lancet **363:**2040-2046.
11. **Nunnari G, Nigro L, Palermo F, Attanasio M, Berger A, Doerr HW, Pomerantz RJ, Cacopardo B.** 2003. Slower progression of HIV-1 infection in persons with GB virus C co-infection correlates with an intact T-helper 1 cytokine profile. Annals of internal medicine **139:**26-30.
12. **Bjorkman P, Flamholc L, Naucler A, Molnegren V, Wallmark E, Widell A.** 2004. GB virus C during the natural course of HIV-1 infection: viremia at diagnosis does not predict mortality. Aids **18:**877-886.

13. **Birk M, Lindback S, Lidman C.** 2002. No influence of GB virus C replication on the prognosis in a cohort of HIV-1-infected patients. Aids **16:**2482-2485.
14. **Spencer JV, Lockridge KM, Barry PA, Lin G, Tsang M, Penfold ME, Schall TJ.** 2002. Potent immunosuppressive activities of cytomegalovirus-encoded interleukin-10. Journal of virology **76:**1285-1292.
15. **Spencer JV.** 2007. The cytomegalovirus homolog of interleukin-10 requires phosphatidylinositol 3-kinase activity for inhibition of cytokine synthesis in monocytes. Journal of virology **81:**2083-2086.
16. **Lin A, Xu H, Yan W.** 2007. Modulation of HLA expression in human cytomegalovirus immune evasion. Cellular & molecular immunology **4:**91-98.
17. **Kidd IM, Clark DA, Ait-Khaled M, Griffiths PD, Emery VC.** 1996. Measurement of human herpesvirus 7 load in peripheral blood and saliva of healthy subjects by quantitative polymerase chain reaction. The Journal of infectious diseases **174:**396-401.
18. **Staras SA, Flanders WD, Dollard SC, Pass RF, McGowan JE, Jr., Cannon MJ.** 2008. Cytomegalovirus seroprevalence and childhood sources of infection: A population-based study among pre-adolescents in the United States. Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology **43:**266-271.
19. **Staras SA, Dollard SC, Radford KW, Flanders WD, Pass RF, Cannon MJ.** 2006. Seroprevalence of cytomegalovirus infection in the United States, 1988-1994. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America **43:**1143-1151.
20. **Roxby AC, Drake AL, John-Stewart G, Brown ER, Matemo D, Otieno PA, Farquhar C.** 2011. Herpes simplex virus type 2, genital ulcers and HIV-1 disease progression in postpartum women. PloS one **6:**e19947.
21. **Schacker T.** 2001. The role of HSV in the transmission and progression of HIV. Herpes : the journal of the IHMF **8:**46-49.
22. **Centers for Disease C.** 1987. Revision of the CDC surveillance case definition for acquired immunodeficiency syndrome. Council of State and Territorial Epidemiologists; AIDS Program, Center for Infectious Diseases. MMWR. Morbidity and mortality weekly report **36 Suppl 1:**1S-15S.
23. **Mellors JW, Rinaldo CR, Jr., Gupta P, White RM, Todd JA, Kingsley LA.** 1996. Prognosis in HIV-1 infection predicted by the quantity of virus in plasma. Science **272:**1167-1170.
24. **Cox DR.** 1972. Regression models and life-tables. Journal of the Royal Statistical Society**:**187–220.
25. **Gray RJ.** 1988. A Class of K-Sample Tests for Comparing the Cumulative Incidence of a Competing Risk. The Annals of Statistics **16:**1141-1154
26. **RJ G.** 1992. Flexible methods for analyzing survival data using splines, with applications to breast cancer prognosis. Journal of the American Statistical Association **87:**942–951.
27. **Bhattarai N, Rydze RT, Chivero ET, Stapleton JT.** 2012. GB virus C viremia is associated with higher levels of double-negative T cells and lower T-cell activation in HIV-infected individuals receiving antiretroviral therapy. The Journal of infectious diseases **206:**1469-1472.
28. **Arjas E.** 1988. A graphical method for assessing goodness of fit in Cox's proportional Hazards model. Journal of the American Statistical Association **83:**204–212.

29. **Yeo AE, Matsumoto A, Hisada M, Shih JW, Alter HJ, Goedert JJ.** 2000. Effect of hepatitis G virus infection on progression of HIV infection in patients with hemophilia. Multicenter Hemophilia Cohort Study. Annals of internal medicine **132:**959-963.
30. **Heringlake S, Ockenga J, Tillmann HL, Trautwein C, Meissner D, Stoll M, Hunt J, Jou C, Solomon N, Schmidt RE, Manns MP.** 1998. GB virus C/hepatitis G virus infection: a favorable prognostic factor in human immunodeficiency virus-infected patients? The Journal of infectious diseases **177:**1723-1726.
31. **Schacker T, Zeh J, Hu HL, Hill E, Corey L.** 1998. Frequency of symptomatic and asymptomatic herpes simplex virus type 2 reactivations among human immunodeficiency virus-infected men. The Journal of infectious diseases **178:**1616-1622.
32. **Augenbraun M, Feldman J, Chirgwin K, Zenilman J, Clarke L, DeHovitz J, Landesman S, Minkoff H.** 1995. Increased genital shedding of herpes simplex virus type 2 in HIV-seropositive women. Annals of internal medicine **123:**845-847.
33. **Kim HN, Meier A, Huang ML, Kuntz S, Selke S, Celum C, Corey L, Wald A.** 2006. Oral herpes simplex virus type 2 reactivation in HIV-positive and -negative men. The Journal of infectious diseases **194:**420-427.
34. **Wright PW, Hoesley CJ, Squires KE, Croom-Rivers A, Weiss HL, Gnann JW, Jr.** 2003. A prospective study of genital herpes simplex virus type 2 infection in human immunodeficiency virus type 1 (HIV-1)-seropositive women: correlations with CD4 cell count and plasma HIV-1 RNA level. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America **36:**207-211.
35. **Corey L, Wald A, Celum CL, Quinn TC.** 2004. The effects of herpes simplex virus-2 on HIV-1 acquisition and transmission: a review of two overlapping epidemics. Journal of acquired immune deficiency syndromes **35:**435-445.
36. **Chang Y, Cesarman E, Pessin MS, Lee F, Culpepper J, Knowles DM, Moore PS.** 1994. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. Science **266:**1865-1869.
37. **Schalling M, Ekman M, Kaaya EE, Linde A, Biberfeld P.** 1995. A role for a new herpes virus (KSHV) in different forms of Kaposi's sarcoma. Nature medicine **1:**707-708.
38. **Lerner CW, Tapper ML.** 1984. Opportunistic infection complicating acquired immune deficiency syndrome. Clinical features of 25 cases. Medicine **63:**155-164.
39. **Hoover DR, Saah AJ, Bacellar H, Phair J, Detels R, Anderson R, Kaslow RA.** 1993. Clinical manifestations of AIDS in the era of pneumocystis prophylaxis. Multicenter AIDS Cohort Study. The New England journal of medicine **329:**1922-1926.
40. **Papagno L, Spina CA, Marchant A, Salio M, Rufer N, Little S, Dong T, Chesney G, Waters A, Easterbrook P, Dunbar PR, Shepherd D, Cerundolo V, Emery V, Griffiths P, Conlon C, McMichael AJ, Richman DD, Rowland-Jones SL, Appay V.** 2004. Immune activation and CD8+ T-cell differentiation towards senescence in HIV-1 infection. PLoS biology **2:**E20.
41. **Doisne JM, Urrutia A, Lacabaratz-Porret C, Goujard C, Meyer L, Chaix ML, Sinet M, Venet A.** 2004. CD8+ T cells specific for EBV, cytomegalovirus, and influenza virus are activated during primary HIV infection. Journal of immunology **173:**2410-2418.
42. **Piriou ER, van Dort K, Nanlohy NM, Miedema F, van Oers MH, van Baarle D.** 2004. Altered EBV viral load setpoint after HIV seroconversion is in accordance with lack of predictive value of EBV load for the occurrence of AIDS-related non-Hodgkin lymphoma. Journal of immunology **172:**6931-6937.

43. **Maartens G, Celum C, Lewin SR.** 2014. HIV infection: epidemiology, pathogenesis, treatment, and prevention. Lancet **384:**258-271.
44. **Des Jarlais DC, Arasteh K, McKnight C, Perlman DC, Feelemyer J, Hagan H, Cooper HL.** 2014. HSV-2 co-infection as a driver of HIV transmission among heterosexual non-injecting drug users in New York City. PloS one **9:**e87993.