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GB Virus Type C (HGV) and Human Immunodeficiency Virus (HIV) Co-Infection: Incidence and Impact on Survival in a Cohort of HIV-infected Transfusion Recipients

By

Farnaz Vahidnia

A dissertation submitted in partial satisfaction of the requirement for the degree of Doctor of Philosophy

in

Epidemiology

in the Graduate Division

of the

University of California, Berkeley

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Abstract

GB Virus Type C (HGV) and Human Immunodeficiency Virus (HIV) Co-Infection: Incidence and Impact on Survival in a Cohort of HIV-infected Transfusion Recipients

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Doctor of Philosophy in Epidemiology

University of California, Berkeley

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GB virus C (GBV-C), an RNA virus closely related to hepatitis C virus (HCV), is transmitted through sexual, parenteral, and vertical routes. GBV-C is highly prevalent among patients receiving blood products and those at high risk of sexual or parenteral exposure. Unlike HCV, GBV-C replicates mainly in lymphocytes; many attempts to find an association between GBV-C infection and human disease have been unsuccessful. Therefore, donated blood is not routinely screened for GBV-C infection. *In vitro* and clinical studies have suggested that GBV-C co-infection may inhibit human immunodeficiency virus (HIV) replication by several different biological mechanisms. Some previous studies, but not all, have shown an association between GBV-C infection and both lower HIV viral load (VL) and better survival among HIV-infected patients. Few studies describe predictors of acute GBV-C infection following transfusion in HIV-infected patients. Reports on survival benefits associated with co-infection after advent of highly active retroviral therapy (HAART) are inconclusive. An open question in many previous reports is the temporal relationship between GBV-C infection and HIV disease markers.

To address some of the currently unanswered questions concerning GBV-C and HIV co-infection, we used a limited access database obtained from the National Heart, Lung, and Blood Institute. The Viral Activation Transfusion Study (VATS) was a randomized controlled trial comparing leukoreduced (LR) vs. non-LR transfusions given to anemic HIV-infected transfusion-naïve patients. Pre- and post-transfusion samples from 489 subjects were tested for GBV-C markers. We used the VATS dataset and the results of GBV-C testing to examine two hypotheses.

First, we tested the hypothesis that GBV-C is transmitted to HIV-infected VATS subjects (n=294) via transfusion. We estimated the risk of acquiring GBV-C RNA per unit of blood transfused and examined the predictors of GBV-C acquisition. We found an incidence of 39 GBV-C infections per 100 person-years during follow-up in this population and an 8% increased risk of acquiring GBV-C associated with each additional unit of blood transfused, controlling for HAART status and baseline HIV VL. A lower HIV VL, use of HAART and white race were associated with an increased risk of subsequent GBV-C acquisition.

Second, we examined the hypothesis that GBV-C co-infection is associated with lower mortality and lower HIV VL in 489 HIV-infected VATS subjects and in two VATS sub-cohorts. GBV-C viremia was associated with significantly lower mortality and HIV VL in unadjusted analyses. We found a non-significant trend towards lower mortality and lower HIV VL among HIV-infected VATS subjects, after adjusting for HIV risk behavior and time-updated E2 antibody, HAART status, HIV VL, and CD4 cell count. Acquisition of GBV-C was associated with lower mortality in the sub-cohort of individuals who were GBV-C RNA and antibody negative at baseline (n=294), adjusting for time-updated covariates (HR= 0.31, 95% CI 0.11, 0.86).

Our results suggest high rates of GBV-C transmission by transfusion among HIV-infected subjects and an increased hazard of GBV-C acquisition with lower pre-transfusion HIV VL and current use of HAART. Our results also indicate that GBV-C viremia is associated with a trend towards lower mortality and lower HIV VL, and GBV-C acquisition via transfusion is associated with a significant reduction in mortality in HIV-infected individuals, after adjusting for HIV disease markers. These findings confirm previous reports that GBV-C infection inhibits HIV replication *in vitro* and *in vivo*.

This work is dedicated to:

my father, Firouz, for his strong faith in women's rights and education,
my mother, Manijeh, for her endless loving care and support,
my husband, Massood, for his encouraging words and understanding,
and finally, my beloved children, Alireza and Melika, for sharing their childhood with my academic challenges and making it meaningful.

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1. What We Know About GB Virus Type C (Hepatitis G Virus)

1.1. Molecular Structure

GB virus type C (GBV-C), also called hepatitis G virus (HGV) is a common non-pathogenic human virus. GB viruses, along with hepatitis C virus (HCV), bovine viral diarrhea virus, and yellow fever, dengue, and West Nile viruses belong to flaviridae family in the *Hepacivirus* genus, based on genome structure: a positive single stranded RNA[1](**Figure 1**). Three isolates of GB virus have been identified, GB virus type A (GBV-A), GB virus type B (GBV-B) and a third virus, GBV-C, which was initially named HGV.

Biophysical and electron microscopic findings of GBV-C particles demonstrate enveloped particles with a nucleocapsid structure assumed to be "core" protein[3](**Figure 2).** The genomic structure of GBV-C is best understood in analogy to HCV; however, because of phylogenetic differences, it is not classified with HCV. GBV-C and HCV share 25% amino-acid sequences, with 9600 nucleotides in HCV and 9400 in GBV-C (reviewed in:[4]). Like HCV, the GBV-C genome encodes a single long open reading frame, translated into polyproteins, which are then cleaved into two envelope glycoproteins (E1, and E2). Unlike HCV, the GBV-C genome does not encode a capsid-like protein, and the coding sequence for its nucleocapsid in unknown (reviewed in[4]). The GBV-C genome also encodes a number of non-structural proteins, NS2, NS3, NS4, NS5A and NS5B **(Figure 3).**

It is presumed, by analogy to HCV, that GBV-C enters host cells by interaction between E1/E2 and unknown cellular receptors, possibly low density lipoprotein receptors. The process is assumed to be followed by endocytosis, de-encapsidation, protein translation, synthesis of negative strand replication, and transcription of positive strand viral genome.

1.2. History and Distribution

GBV-A and GBV-B were originally identified in tamarins inoculated with serum from a surgeon whose initials were GB[6]. Investigators were never able to detect GBV-A and GBV-B in human samples[7]. GBV-C has also been detected in wild chimpanzees, but it was first identified in samples from patients with cryptogenic hepatitis and non-A non-E hepatitis in 1995; as a result, it was originally named hepatitis G virus (HGV)[8]. Independently but simultaneously, researchers who identified GBV-A and GBV-B, isolated another virus in human samples called GBV-C[1]. Further studies showed that HGV and GBV-C are actually two strains of the same virus[9]. From this point on, I will refer to the virus as GBV-C to avoid confusion.

GBV-C is genetically less diverse than HCV (six genotypes and multiple sub-genotypes), with only four identified GBV-C genotypes (reviewed in[4]). GBV-C genotype 1 is distributed in West Africa, genotypes 2a and 2b in Europe and North America, genotype 3 in Japan, North Asia and native populations of South America, and genotype 4 in South Asia (reviewed in[2, 4]). GBV-C is detectable in all ethnic groups. Infection with GBV-C is

even found in isolated populations, such as indigenous tribes in Papua New Guinea, sub-Saharan Africa, and Central and South America[10, 11]. The geographic distribution of GBV-C genotypes suggests that GBV-C is an ancient virus that infected human ancestors in Africa and evolved as the human population migrated to other parts of the world[12]. GBV-C genotypes differ in[10, 11] their coding sequence by only 2-14%, while there are up to 30% differences between HCV types featuring different clinical significance. Whether or not different GBV-C genotypes have different biological and clinical significance is not known (reviewed in[2]).

1.3. Routes of Transmission

GBV-C can be transmitted through sexual, parenteral, and vertical (mother to child transmission-, MTCT) routes[13-16]. Transmission of GBV-C RNA by transfusion and transient viremia as a result of blood transfusion (between day 6 and 14 post-transfusion) have been documented by 100% sequence similarity of the donor and recipient's post-transfusion samples[17]. A positive cross-sectional relationship ($p\text{-value} < 0.001$) between the number of blood units received and GBV-C infection (GBV-C RNA and/or anti-E2 antibody) has been reported in immuno-competent blood recipients[14]. However, other studies have not confirmed a significant association between GBV-C acquisition and number of units of blood transfused[18, 19]. A positive association between exposure to blood products and detection of GBV-C markers has been reported in health care workers[20]. GBV-C appears to be transmitted via infected blood products to transfusion recipients independent of other blood borne viruses[21-23].

Sexual transmission of GBV-C has been suggested as a possible route of transmission in some previous studies[22, 24, 25]. Yeo et al. studied risk of sexual transmission in 161 hemophilic men. They found GBV-C infection in 27% of the women in sexual contact with a GBV-C infected hemophilic man (odds ratio=2.77, p-value=0.03)[25].

Transmission of GBV-C from infected mother to infant has been documented multiple times[262716, 28] with one study finding that 41% of GBV-C infected mothers transmitted the virus to their infants[16]. Mother to child transmission can occur during either the prenatal[28] or postnatal periods[27]; is increased with vaginal delivery in HIV-infected women or use of anti-retroviral therapy (ART) by the mother; and is decreased when the infant has HIV infection[16]. It is assumed that mother to child transmission and sexual transmission are the main routes of GBV-C infection among low risk, otherwise healthy adults, and sexual transmission of GBV-C is more efficient than HCV (reviewed in[29]).

1.4. Prevalence of GBV-C infection

1.4.1. Healthy blood donors

GBV-C infection is relatively common throughout the world. GBV-C viremia is 5 to 10 times more prevalent among healthy individuals than HCV[8, 30]. The prevalence of GBV-C viremia ranges between 1and 5% among healthy blood donors from developed countries, and can reach much higher levels (up to 16%) in developing countries[14, 30-34]. Analysis of the results of 30 reports examining more than 13,000 healthy blood donors

showed GBV-C RNA in 649 (4.8%), with a higher prevalence among African-Americans (17.2%) as compared with Caucasians and Asians (3.4% and 4.5%, respectively)[34]. In the United States, approximately two percent of healthy blood donors have GBV-C viremia, and 13% have GBV-C E2 antibody, indicating prior infection[29]. The prevalence of GBV-C viremia increases with age. An investigation of serum samples from South African blacks showed 9% of children ≤ 15 years of age had GBV-C viremia, compared to 27% of those 16-35 years of age ($p=0.001$)[35].

1.4.2. High risk populations

GBV-C infection is more prevalent among individuals at high risk for sexual or parenteral exposure, i.e. injection drug users; HIV-infected individuals; men who have sex with men (MSM); multi-transfused patients; and hemophiliacs[14]. Previous findings have shown a higher prevalence of GBV-C viremia (10-35%) among patients with HCV infection (11-35%) [35, 36] or hepatitis B virus (HBV) infection (10%)[37].

The high prevalence of GBV-C RNA among men who have sex with men (13-63%)[14, 24, 38], female sex workers (14-25%)[24, 39], and female partners of GBV-C infected men (27%)[25] is suggestive of sexual transmission of GBV-C infection.

Parenteral transmission of GBV-C as a result of exposure to blood products results in a high prevalence of GBV- C infection among multi-transfused patients[40, 41 221420, 4243-45]. Eighteen percent (16/90) of multi-transfused patients participating in a study in New Delhi, India were GBV-C RNA positive[44]. In a study of 201 German hemophiliacs, 13% of those receiving non-virus-inactivated clotting factor were infected with GBV-C RNA[43]. Yeo et al. found evidence of active or past GBV-C infection in 48% (14% RNA only, 32% anti-E2 antibody only, 2% both) of 161 patients with hemophilia from 11 medical centers[46]. The prevalence of GBV-C viremia has been reported to be significantly higher in immuno-suppressed multi-transfused subjects (not including HIV-infected subjects) than immuno-competent ones[14, 47].

Because of similar routes of transmission, GBV-C infection is also highly prevalent among HIV-infected individuals. Investigators have detected GBV-C RNA using RT-PCR methods in 17-43% of HIV-1-infected individuals[14, 22, 48]. Between 30 and 65% of HIV-infected individuals have anti-E2 antibody, consistent with prior infection with GBV-C[49, 50]. Eighty-six percent of HIV-infected men in the Multicenter AIDS Cohort Study (MACS) were reported to have active (40%) or prior infection (46%) with GBV-C[51]. No significant differences have been reported in the prevalence of GBV-C according to HIV acquisition risk factors[22, 52], although it has been suggested that individuals who acquired HIV through parenteral routes may have increased GBV-C clearance[4].

1.5. GBV-C Detection Methods

Efforts to detect GBV-C infection have relied on serologic tests and tests of viral RNA. Efforts to detect anti-GBV-C antibodies using ELISA and Western Blot have not been successful. Only 25% of individuals infected with GBV-C go on to develop antibody to E2 glycoprotein. E2 antibody response to GBV-C infection is highly variable and wanes

over time, making E2 antibody results difficult to interpret. Antibody response to GBV-C E2 usually appears after clearance of GBV-C RNA, and it is uncommon to detect both GBV-C RNA and E2 antibody in serum at the same time[14, 22, 31, 53]. Thus, detection of GBV-C infection relies mainly on detection of viral RNA using nucleic acid assays which indicates active infection, i.e. RT-PCR (reverse transcriptase polymerase chain reaction) or nested RT-PCR. The nucleic acid assay tests are highly sensitive and specific[2].

1.6. Natural History of GBV-C Infection in Humans

GBV-C RNA levels can reach to 10^2- 10^7 copies per milliliter of serum in immunocompetent individuals, 10^4- 10^7 copies/ml in immunosuppresed individuals and up to 10^8 copies/ml in HIV-infected individuals. It has been suggested that the higher the level of viremia, the greater is the chance of viral transmission[28].

Although GBV-C is genetically very similar to HCV, there are important differences in some characteristics. 1) GBV- is primarily a lymphotropic virus, replicating mostly extra-hepatically in T and B lymphocytes; 2) anti-GBV-C glycoprotein (anti- E2) antibody usually appears after clearance of viremia; and 3) the majority of immunocompetent individuals infected with GBV-C clear the infection spontaneously[1, 29].

GBV-C infection can lead to three outcomes: 1) persistent viremia over decades (25-50% of infections in healthy individuals)[54]; 2) clearance of viral RNA and development of anti-E2 antibody (anti-E2), indicating prior infection (33-58% of infections in transfusion recipients)[31, 54]; and 3) clearance of viral RNA without development of anti-E2. Chronic infection requires persistent evasion of the immune system by GBV-C through a mechanism that is not well understood[55]. Unlike HCV infection, GBV-C infection in healthy individuals usually results in clearance of viremia and development of anti-E2 antibody[31]. It appears that clearance of GBV-C requires both a humoral and a cellular immune response[4]. It is uncommon to detect both GBV-C RNA and E2 antibody in serum at the same time. Anti- E2 antibody appears to offer some protection from re-infection[1, 32, 41, 56] even in the face of repeated exposures to GBV-C contaminated blood products or long-term injection drug use[4]. Although GBV-C infection may persist, most immuno-competent individuals infected with GBV-C will clear viremia spontaneously within two years[19, 41, 53].

GBV-C was initially thought to cause hepatitis in humans, but several studies failed to demonstrate an association between GBV-C infection and hepatitis (or any human disease). As a result, the term GB virus C is preferred to HGV[2, 14, 54, 57], and blood products are not routinely screened for the presence of GBV-C RNA [54]. However, due to high prevalence of GBV-C infection and risk of transmission via transfusion, some investigators have proposed routine testing of donor blood samples[34].

1.6.1. GBV-C and HIV-1 co-infection

After initital interest in GBV-C infection declined due to lack of an association between GBV-C and human disease, in 1998 reports showed decreased mortality[58] or lower HIV viral load[59] in HIV-infected individuals who were co-infected with GBV-C. These early

reports were followed by several studies confirming an association between GBV-C infection and better survival or clinical course in HIV-infected populations [46, 50, 51, 58, 60-63]. A study analyzing data from the multicenter AIDS cohort study (MACS) found individuals who lacked persistent GBV-C viremia had a 2.8 times greater hazard of mortality compared to GBV-C negative individuals (p=0.006)[51].

However, not all of the early studies in the pre-highly active antiretroviral therapy (HAART) era showed a significant positive effect of GBV-C co-infection on survival in HIV-infected populations[64-69]. Some studies found an accelerated course of HIV disease associated with loss of detectable GBV-C viremia. Differences in HIV disease progression between individuals with persistent GBV-C viremia and those who lost GBV-C RNA showed a negative association i.e. increased mortality or worse prognosis following GBV-C clearance without the development of anti-E2 antibodies[51, 65]. In a longitudinal study of 326 HIV-infected men who have sex with men, patients who remained consistently GBV-C negative had a better survival as compared with those who lost GBV-C RNA during eight years of follow-up[68]. However, persistent GBV-C viremia between the first and the last sample was associated with a lower hazard of mortality, although this difference became statistically non-significant after adjusting for time updated CD4 cell counts. The authors suggested that persistence of GBV-C viremia is a consequence of having a sufficient level of CD4 cells rather than the cause. In other words, they hypothesized that lower CD4 cell counts lead to GBV-C loss and also higher HIV-associated mortality.

A meta-analysis of 11 studies (n=912) investigating HIV-infected populations during the pre-HAART era showed no evidence of a positive effect of GBV-C viremia on HIV survival when GBV-C was measured within two years of HIV seroconversion[60]. However, the meta-analysis found a significant reduction in all-cause mortality when GBV-C infection was present more than two years after HIV seroconversion (combined HR= 0.41, 95% CI=0.23, 0.69; n=1,294). The authors concluded that the timing of GBV-C infection in relation to HIV seroconversion may be a key factor in understanding the relationship between survival among HIV-infected individuals and GBV-C co-infection. Another key variable in examining the relationship between GBV-C and HIV could be active versus prior GBV-C infection.

Studies on the effect of GBV-C co-infection on survival after the advent of HAART have yielded conflicting results. Recent analyses have sought to tease apart the relationship among HIV, GBV-C, and HAART. Several, though not all, studies found better survival, less treatment failure, and greater increase in CD4 cell count in those co-infected with GBV-C[63, 70-73], compared with GBV-C RNA negative subjects. However, other studies showed no significant difference in response to HAART among GBV-C infected subjects compared to GBV-C RNA negative ones[51, 74-76].

1.6.2 Biological Mechanism of GBV-C and HIV Interaction

Whether GBV-C has a direct beneficial effect on HIV disease progression or an accelerated course of HIV infection causes loss of GBV-C viremia remains unclear

NEJM, 2004]. Several research groups are currently investigating the interaction between GBV-C and HIV replication by conducting *in vitro* and *in vivo* studies.

There are several potential mechanisms by which GBV-C infection might confer a survival advantage. GBV-C has been shown in both *in vivo* and *in vitro* studies to interfere with HIV replication[77, 78]; modulate HIV entry receptors CCR5 and CXCR4, leading to inhibition of HIV cell entry[77-80]; alter Th1-Th2 cytokine profiles and thus enhancing anti-HIV chemokine synthesis[63]; decrease T cell activation[81, 82]; and block IL-2 mediated CD4 T cell proliferation[83]. Another suggested mechanism of interaction is inhibition of host cell apoptosis (controlled cell death) - similar to HCV- to prolong GBV-C viral replication, resulting in increased CD4 cell count; while HIV infection tends to accelerate apoptosis to increase release and spread of viral particles which leads to CD4 cell depletion[4].

1.7. Summary

In summary, GBV-C causes asymptomatic viral infection in humans and infects healthy individuals and high-risk populations through blood, sexual, and vertical exposure. Blood transfusion remains a major route of GBV-C infection, especially among multi-transfused individuals. However, the incidence of GBV-C viremia in HIV-infected individuals following transfusion is unknown. Investigators are trying to identify the mechanism by which GBV-C affects HIV disease progression, with the hope to find new therapies against HIV disease[33]. A common finding in all previous studies is that GBV-C viremia during late stages of HIV infection (as opposed to GBV-C viremia early after HIV seroconversion) is associated with improved survival and that loss of GBV-C RNA is associated with a worse prognosis[60, 68, 84]. None of the previous studies has described the role of acute GBV-C acquisition when HIV infection precedes GBV-C infection, and in most prior studies the time sequence of the events in the relationship between GBV-C viremia and markers of HIV disease progression has not been well defined. An analysis that can account for both acquisition of GBV-C infection and also time-updated CD4 cell count, HIV viral load, and ART would be valuable to help clarify the relationship between GBV-C and HIV. In the next three chapters, I describe the Viral Activation Transfusion Study dataset, which I used to address gaps in the current understanding of GBV-C and HIV co-infection, and then present the following two papers:

1) Transmission of GB virus type C via transfusion in a cohort of HIV-infected patients;
2) Acquisition of GB virus type C and lower mortality in HIV-infected patients.

Figure 1: Phylogenic relationship of the NS3 RNA helicase domains of the GBVs and other members of the Flaviviridae. Flaviviruses: Japanese encephalitis virus (JEV), West Nile virus (WNV), yellow fever virus (YFV) and dengue virus types 1 and 2; pestiviruses: hog cholera virus (HCHV) and bovine viral diarrhea virus (BVDV); HCV group: genotypes 1a, 1b, 2a, and 3a[2].

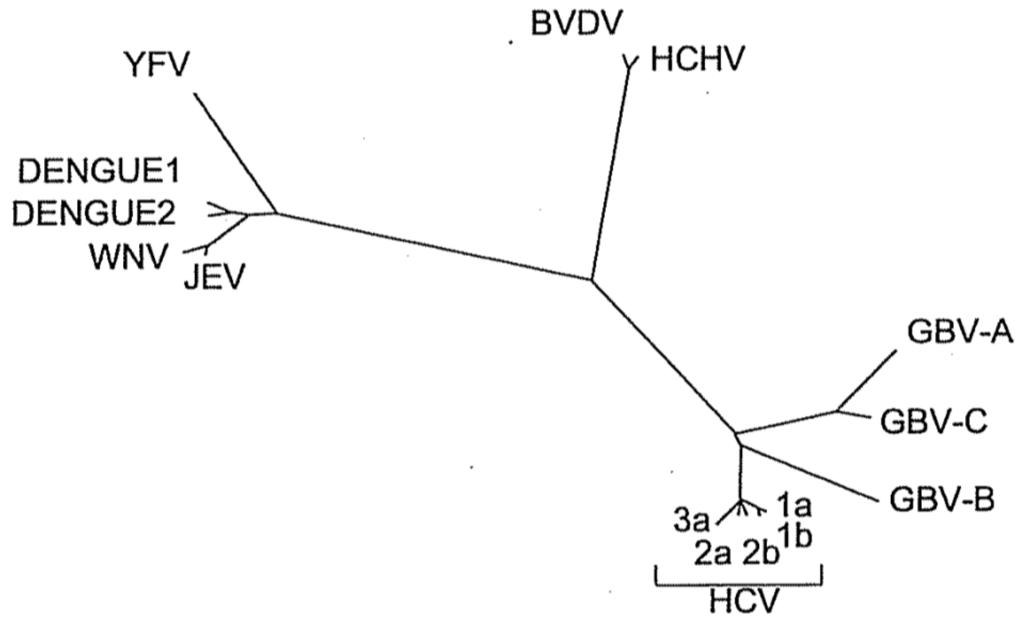


Figure 2: GBV virus C, three-dimensional model with cut-away.

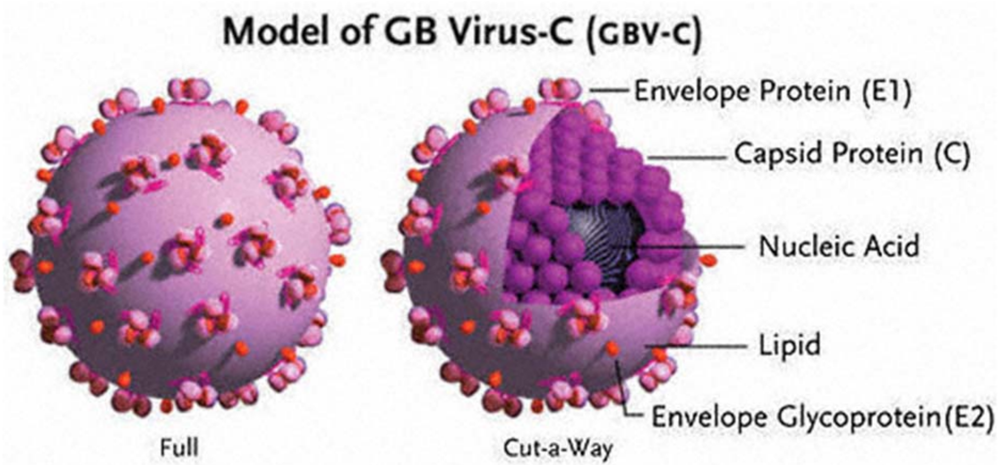
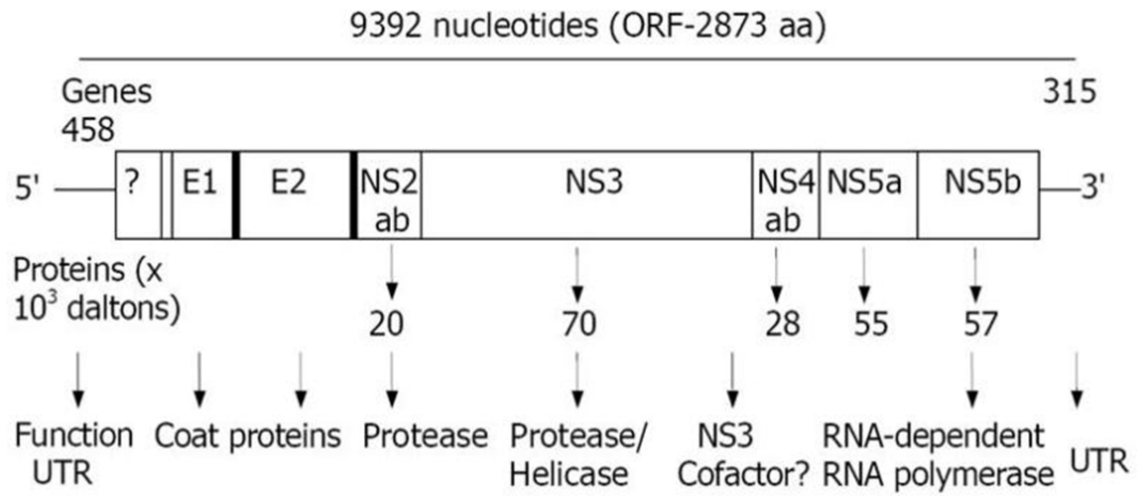


Figure 3: Schematic representation of GBV-C RNA structure, coded proteins, and their functions[5].



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2. Viral Activation Transfusion Study (VATS)

The Viral Activation Transfusion Study (VATS), a double-blind randomized clinical trial, was initiated in July 1995 in order to evaluate the effects of transfusion on patients infected with HIV and cytomegalovirus (CMV)[1, 2]. The patients were followed through their quarterly visit scheduled until June 1999 with a median follow-up of 12 months among all patients and 24 months among those who survived[2]. The VATS Study Group consisted of 11 participating transfusion and clinical sites across the U.S., a coordinating center, and a central laboratory. Blood Centers of the Pacific served as the central laboratory, storing and testing samples collected from transfusion units and serial samples collected from transfusion recipients. The study was sponsored by the National Heart, Lung and Blood Institute (NHLBI).

There is evidence showing transfusions of red blood cells (RBCs) containing leukocytes may result in immune activation i.e. increasing p24 antigen, and adversely affect the course of HIV-1 infection by increasing HIV RNA levels[1, 3, 4]. In the VATS study, patients were randomized to receive either leukoreduced (WBC- reduced) or non-leukoreduced blood units. To be eligible for the study, participants had to be transfusion-naïve at baseline and sufficiently anemic that red cell transfusion was indicated. The patients all had advanced HIV disease, with 78% having an AIDS defining illness. The mean HIV viral load was 4.5 $\log_{10}$ copies/ml (SD= 1.1), the median baseline CD4 count was 15 cells/µl (IQR= 3-73), the subjects received on average eight transfusions during study, and had a median survival of 8.4 months (IQR=2.0-21.8). Demographic information, including gender, date of birth, and race; HIV risk behavior; and clinical data (CD4 cell count, total WBC and CMV end organ disease) were collected at baseline. Other clinical information collected at baseline and at quarterly evaluations included findings on physical examination; medications used in the last 30 days; antiretroviral medication history; vaccination history; a measure of quality of life; and onset of serious HIV complication or death.

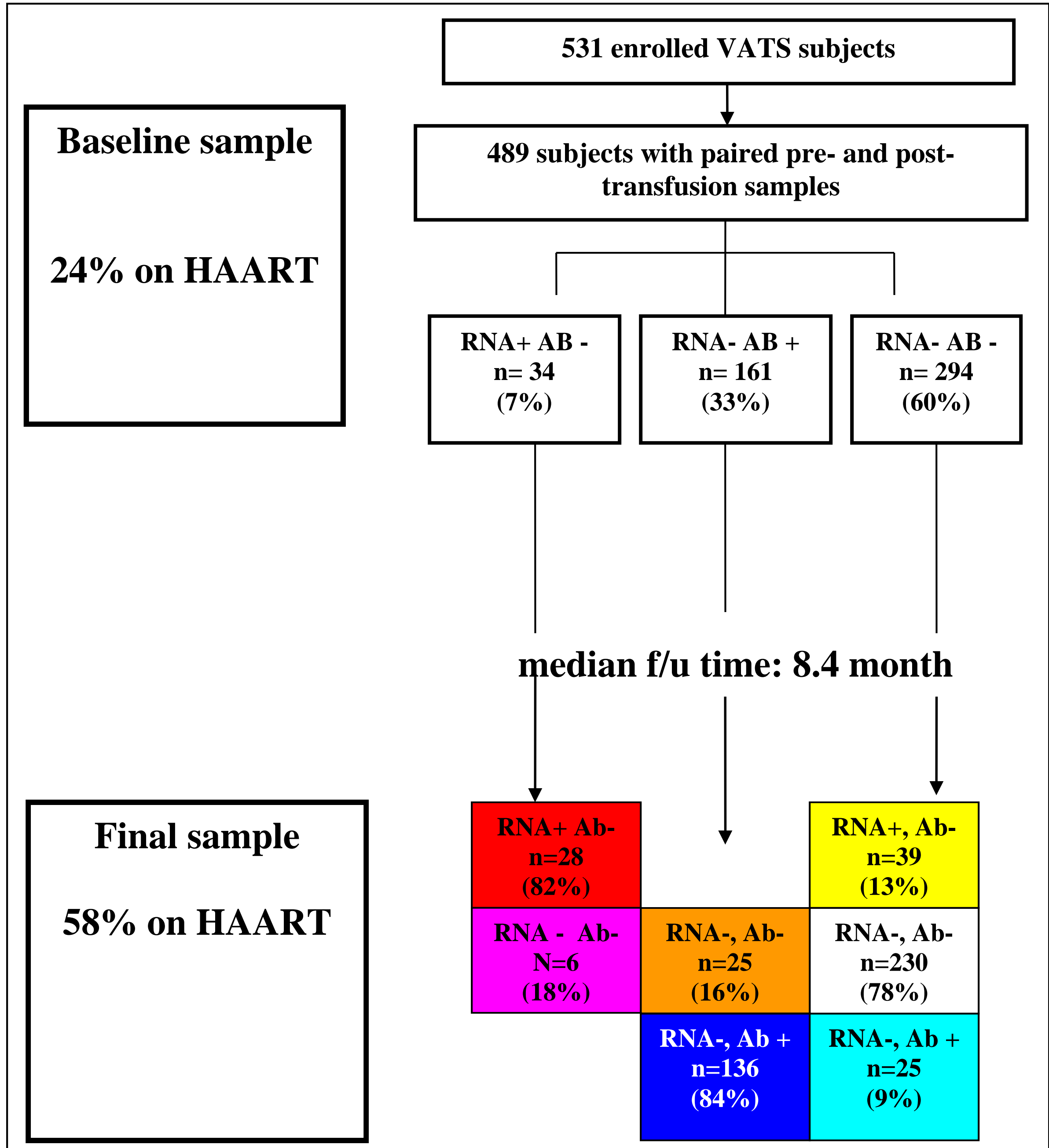
Samples were collected and stored at -70ºC at baseline (pre-transfusion), weekly after transfusion for one month, and quarterly thereafter. Pre-transfusion and weekly post-transfusion samples were collected for any additional transfusion during the study. Red blood cell (RBC) and CD4 cell count and HIV RNA and CMV DNA assays were performed at baseline and every 3 months. In June 1996, HAART was introduced into HIV treatment and was available to some but not all study subjects; thus the VATS study also collected information on HAART use. Forty-two percent of subjects never received HAART while participating in VATS (3 months post-transfusion data). During the course of the study, assays on several biological markers of HIV infection, markers of other infections and immunologic responses were conducted. The dataset also includes known survival status up to the end of the study. The original VATS study results showed no evidence of clinical benefit as a result of leukoreduced transfusion in HIV infected patients (HR for mortality= 1.35, 95% CI: 1.06, 1.72)[2].

Of the 531 subjects, 489 (92%) had paired pre-transfusion and final samples available for GBV-C evaluation at Blood Systems Research Institute (BSRI, the successor

organization to Blood Systems of the Pacific, San Francisco, CA). All available paired plasma samples were tested for GBV E2 antibody, using anti-GBenv μplate EIA assay and for RNA using the quantitative GBV-RNA RT-PCR assay (both assays provided by Roche Diagnostics, Penzberg, Germany). All interim blood samples from individuals with evidence of incident viremia or acquisition of antibody between the pre-transfusion and final samples were tested by both antibody and RNA assays. The final sample was retested following interim testing to confirm RNA positivity if it was the only RNA-positive sample. Figure 1 demonstrates GBV-C infection status of VATS subjects at baseline and final samples.

For the current analysis, we used a limited-access VATS public use dataset that provided demographic and clinical information on blood recipients in combination with GBV-C results provided by BSRI. Personal identifiers and data elements identifying paired donor and recipients were removed from the limited-access VATS dataset provided by NHLBI. Written informed consent was obtained from all VATS study subjects[1] and this supplemental study protocol was approved by the Committee for the Protection of Human Subjects at the University of California, Berkeley, USA.

Figure 1: Patterns of GBV-C infection status at baseline and final sample – VATS (1995- 1999)



Persistent viremia	(red)	Incident viremia	(yellow)
Cleared viremia	(magenta)	Loss of measurable Ab	(orange)
Never infected	(white)	Acquisition of Ab	(cyan)
Persistent Ab	(blue)		

2.1. References

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3. Transmission of GB Virus type C (HGV) via transfusion in a cohort of HIV-infected patients

3.1. Introduction

GB virus type C (GBV-C), also called hepatitis virus G (HGV), is a human flavivirus that is phylogenetically related to hepatitis C virus[1-3]. GBV-C is primarily a lymphotropic virus that appears to replicate in T and B lymphocytes[4]. Although infection may persist, most immunocompetent individuals infected with GBV-C spontaneously clear viremia within two years[5, 6]. Anti-GBV-C envelope glycoprotein (E2) antibody usually appears after clearance of viremia and may offer some protection from re-infection[6, 7]. However, E2 antibody response to GBV-C infection is highly variable and wanes over time, making data on E2 antibody difficult to interpret. It is uncommon to detect both GBV-C RNA and E2 antibody in serum at the same time, and detection of GBV-C RNA in serum indicates active infection.

GBV-C is transmitted through sexual, parenteral, and vertical (mother-to-child) routes[8-11]. Transmission of GBV-C via transfusion and transient viremia following blood transfusion have been documented by 100% sequence similarity of donor and recipient post-transfusion samples[12]. The prevalence of GBV-C viremia is between 1-5% among healthy blood donors from developed countries and is much higher in developing countries (reviewed in:[13-15]. In the United States, approximately two percent of healthy blood donors have GBV-C viremia, and 13% have E2 antibody, indicative of prior infection (reviewed in:[15]).

GBV-C was initially thought to cause hepatitis in humans, but several studies have failed to demonstrate an association between GBV-C infection and any human disease[13, 16, 17]. Hence, blood products are not routinely screened for the presence of GBV-C RNA[13]. Because of the routes by which it is transmitted, GBV-C infection is more prevalent among HIV-infected individuals, with up to 43% of HIV-infected individuals exhibiting GBV-C viremia in cross-sectional studies[9, 18]. Several, though not all studies have observed an association between GBV-C infection and prolonged survival among HIV-infected individuals (reviewed in:[15], and a meta-analysis of studies including 1,294 HIV-infected subjects confirmed an association between GBV-C infection and prolonged survival[19]. There are several potential mechanisms by which GBV-C infection might confer a survival advantage. GBV-C has been shown (*in vivo* and *in vitro* studies) to interfere with HIV replication, modulate HIV entry receptors CCR5 and CXCR4*,* alter Th1-Th2 cytokine profiles, decrease T cell activation, and block IL-2 mediated CD4 T cell proliferation[20-27].

In the present study, we quantified the risk of GBV-C acquisition per unit of blood transfused in 294 HIV-infected transfusion naïve subjects documented to be GBV-C RNA and E2 antibody negative pre-transfusion. To our knowledge, this is the first study to report the incidence of GBV-C viremia following transfusion in HIV-infected patients. In addition, to address the temporal ordering in the relationship between HIV and GBV-C RNA, we examined pre-transfusion HIV viral load and CD4 cell count as predictors of subsequent GBV-C acquisition up to 120 days post-transfusion.

3.2. Materials and Methods

Plasma samples were selected for GBV-C testing from subjects enrolled in the prospective, multi-center Viral Activation Transfusion clinical trial (VATS), a clinical trial sponsored by the National Heart, Lung and Blood Institute (NHLBI). Details of subject selection and findings of the clinical trial have been previously described[28]. Briefly, VATS evaluated the effects of leukoreduction of units for transfusion into transfusion-naïve HIV-infected patients. In VATS, patients were randomized to receive either a filtered, leukoreduced or standard non-leukoreduced blood unit[28, 29]. Study participants had advanced HIV disease and symptomatic anemia sufficient to require transfusion, with a mean HIV viral load of 4.5 $\log_{10}$ copies/ml (SD= 1.1), a median baseline CD4 count of 15 cells/µl (IQR= 3-73), and a median survival time of 8.4 month (IQR=2-21.8). At last observation, the mean HIV viral load among those who survived was 4.3 $\log_{10}$ copies/ml (SD= 1.3), and the median CD4 count was 22 cells/µl (IQR= 3-129.5).

Study samples were collected and stored at -70º C at baseline (pre-transfusion), weekly post-transfusion for one month and quarterly thereafter; pre-transfusion and weekly post-transfusion samples were collected for any second transfusion episode during the study. Of the 531 subjects, 489 (92%) had paired pre-transfusion and final samples available for GBV-C evaluation.

All available paired plasma samples were tested for GBV E2 antibody, using the anti-GBenv µplate EIA assay and for RNA using the quantitative GBV-RNA RT-PCR assay (both assays provided by Roche Diagnostics, Penzberg, Germany). All interim blood samples from individuals with evidence of incident GBV-C viremia or acquisition of GBV-C antibody between the pre-transfusion and final samples were tested by both antibody and RNA assays. The final sample was retested following interim testing to confirm RNA positivity if it was the only RNA-positive sample.

To be eligible for this analysis, subjects must have had GBV-C testing results and be GBV-C RNA and E2 antibody negative in the baseline (pre-transfusion) sample (n=294). Written informed consent was obtained from all VATS study subjects[28], and this supplemental study protocol was approved by the Committee for the Protection of Human Subjects at the University of California, Berkeley, USA.

For the current analysis, we used a limited-access VATS public use dataset that provided demographic and clinical information on blood recipients in combination with GBV-C results provided by the Blood Systems Research Institute. Personal identifiers and data elements identifying paired donor and recipients were removed from the limited-access VATS dataset provided by NHLBI. Thus, matching donor-recipient samples and performing additional laboratory testing was not possible.

3.3. Statistical Analysis

GBV-C RNA status was categorized as a binary time-varying variable (positive/ negative). Missing GBV-C RNA status for interim or final samples was imputed by carrying the most recent measurement forward. Cumulative units of RBC or platelets transfused up to 120 days after the first transfusion were described as a time-varying continuous variable. Categorical variables were compared by the χ^2 test or Fisher's exact test, as appropriate. Continuous variables were compared by Student's t test when normally distributed or alternatively by the Mann-Whitney U test.

Risk factors for GBV-C RNA acquisition were evaluated among subjects who were GBV-C antibody and RNA-negative at study entry. Evaluation of baseline HIV viral load and CD4 cell counts as predictors of GBV-C RNA acquisition in subjects known to be GBV-C RNA and antibody negative at baseline allowed us to establish a clear temporal sequence between these HIV biomarkers and GBV-C RNA. Pooled logistic regression models were used to estimate the log odds of GBV-C RNA acquisition up to 120 days after first transfusion among subjects who remained GBV-C RNA negative as a function of cumulative units transfused and other covariates. The odds ratio estimated in such a model provides an approximation of the relative hazard[30]. For each visit *t* following entry into study, the probability of acquiring GBV RNA by visit *t* was estimated among subjects who remained GBV-C RNA negative prior to visit *t* as a function of cumulative units transfused up until visit *t*. We further examined baseline $\log_{10}$ HIV viral load, current highly active antiretroviral therapy (HAART) status, baseline CD4+ T lymphocyte count (square root transformed), and race (non-white vs. white) as predictors of GBV-C acquisition, adjusting for cumulative units transfused. To ensure HIV biomarkers were measured prior to GBV-C RNA, we used baseline HIV viral load and CD4+ lymphocyte count in the adjusted models. HAART status was updated at each visit if the subject has started HAART between previous visit and visit *t*.

Repeated measures per subject were accounted for by using the robust standard errors in the form of the "sandwich" estimator. We did not include E2 antibody results in our analysis to define new infection, because GBV-C RNA is a measure of active infection, and E2 antibody levels may occur transiently following transfusion due to passive antibody and are not constant over time. Data analysis was performed using STATA 10.0 (StataCorp, College Station, Texas, United States).

3.4. Results

Of the 489 subjects screened for GBV antibody and RNA, 294 (60%) were negative for both E2 antibody and RNA at baseline and thus eligible to be included in the current analysis (Figure 1). Twenty-two (7.5%) study subjects showed evidence of GBV-C RNA acquisition within 120 days following first transfusion. These 22 incident GBV-C infections are presumed to have resulted from transfusion transmission of GBV-C. The incidence of GBV-C acquisition following transfusion in the study sample was estimated as 39 per 100 person-years of follow-up. Viremia was detected within the first 30 days in 12 (4.1%) subjects and between 31 and 120 days in 10 (3.4%) subjects. In no case was a

subject simultaneously RNA and E2-antibody positive and no subject cleared viremia within the first 120 days post-transfusion.

The median follow-up time for the current analysis was 80 days (IQR = 33-101 days), with slightly longer follow-up time for the group that acquired GBV-C (**Table 1**). This group had lower baseline pre-transfusion HIV VLs and a trend toward higher baseline CD4 cell counts compared to the GBV-C negative group (p=0.02 and 0.2, respectively). Sixty- four (21.8%) subjects were on HAART at baseline, and an additional 39 subjects initiated HAART during follow up. A larger percentage of patients who acquired GBV-C used antiretroviral drugs at some point during the study (59% vs. 33%, p=0.01). Those who acquired GBV-C were mostly white (82%), in contrast to the GBV-C negative group, of which 48% of subjects were white (p=0.01). There was no significant difference between the two groups in the type of blood components transfused (platelet vs. red blood cells) or leukoreduced vs. non-leukoreduced components.

Mean follow-up time from baseline to first detection of GBV-C RNA was 49 days (SD=36.0 days) for those who acquired GBV-C RNA. Of the patients who acquired GBV-C RNA during follow up, evidence of acquisition occurred during the 30 days following first transfusion (early) for 12 (54.5%), while for 10 other cases acquisition occurred between 31 and 120 days post-transfusion (late). These acquisition groups were similar, except for longer follow-up time for the group with later acquisition of GBV-C RNA (data not shown).

Table 2 presents results of pooled logistic regression models in which the log odds of GBV-C RNA acquisition among subjects who remained GBV-C negative was estimated as a function of exposure to cumulative blood units transfused and other covariates. Each additional unit of blood transfused was associated with a 9% increase in the odds of GBV-C acquisition (95% CI= 1.06, 1.11). Adjusting for HAART use, baseline HIV viral load and race, the estimated relative odds of GBV-C acquisition was 1.08 per unit transfused (95% CI= 1.05, 1.11). We also investigated the hazard of GBV-C acquisition as a function of baseline HIV viral load, baseline CD4 count, and current HAART exposure, controlling for cumulative units transfused. There was a reduced hazard of GBV-C acquisition with increased baseline HIV viral load (OR= 0.62 per $\log_{10}$ copies/ml, 95% CI = 0.40, 0.96), and an increased hazard of GBV-C acquisition with current HAART use (OR= 4.03, 95% CI= 1.79, 9.11), after controlling for cumulative units transfused. An increased baseline CD4 cell count was associated with a slight, non-significant increased hazard of GBV-C acquisition (OR=1.05, 95% CI=0.98, 1.12). Leukoreduction status and receiving platelet units were not associated with GBV-C acquisition in regression models controlling for cumulative units transfused.

3.5. Discussion

In the present study of a transfusion-naïve, GBV-C RNA and E2 antibody-negative, HIV-infected cohort, we found an incidence of GBV-C infection of 39 per 100 person-years following transfusion. The probability of developing GBV-C viremia was associated with the number of units of blood transfused; this association remained after controlling for

HAART use and baseline HIV VL. Higher baseline HIV VL, use of HAART, and race were all associated with subsequent GBV-C RNA acquisition, after controlling for units of blood transfused. Previous studies have found an inverse relationship between GBV-C VL and HIV VL[31, 32], and *in vitro* studies have demonstrated antagonism between the two viruses in cell culture systems[22, 23, 33]. These previous findings are consistent with our finding of an inverse relationship between GBV-C acquisition and higher HIV VL. In addition, there was a trend towards higher baseline CD4 counts in those who acquired GBV-C, which may reflect an increase in GBV-C target cells.

We found a positive relationship between the number of units transfused and the probability of GBV-C acquisition. A positive cross-sectional relationship between the number of blood units received and GBV-C exposure (GBV-C RNA and/or E2 antibody-positive) has been previously reported in immune competent blood recipients (p-value < 0.001)[9]. However, several other studies did not find a significant association between GBV-C acquisition and number of blood transfusions [34, 35].

Blood transfusion is associated with a substantial risk of GBV-C acquisition in HIV infected patients. Prior studies using RT-PCR detection methods have reported finding GBV-C RNA in 17-43% of HIV-infected individuals[18, 36]. Including the 7% of subjects who were GBV-C RNA positive pre-transfusion, prevalence of GBV-C viremia in VATS cohort members was estimated to be 14% at 120 days post-transfusion. Advanced HIV disease, high HIV VL, and low CD4 cell count at baseline are among possible explanations for the low GBV-C viremia prevalence in VATS cohort. Previous studies have reported a higher prevalence of GBV-C RNA in immunosuppressed multi-transfused subjects. However, their study populations did not include HIV-infected subjects[9, 37]. Possible explanations for higher prevalence of GBV-C infection found in previous studies could be geographic differences in the prevalence or differences in the laboratory methods used to detect GBV-C RNA, as many prior studies used nested RT-PCR methods, which have a greater sensitivity then the method we used[9, 18, 33, 38]. On the other hand, our estimate of the incidence of new GBV-C infection may be an underestimate, due to classification of E2 antibody-positive subjects as GBV-C RNA negative. Twenty-one out of 272 GBV-C RNA negative subjects had at least one post-transfusion E2 antibody positive sample, suggesting either undetected transient GBV-C viremia with subsequent E2 antibody formation or passive transfusion of donor E2 antibody to recipients. We cannot distinguish between the two possibilities.

Our findings show a significantly higher incidence rate of GBV-C infection among white subjects compared to non-whites, after adjusting for cumulative units transfused, baseline HIV viral load and current use of HAART. In the original VATS cohort (n=489), the pre-transfusion prevalence of GBV-C viremia was not significantly different between whites and non-whites (data not shown). Studies have reported a higher prevalence of GBV-C viremia among healthy blood donorsin Africa[39]. In addition, HIV-infected African-American women participating in the Women's Interagency HIV Study had a slightly higher prevalence of GBV-C viremia than HIV-infected women from other racial groups[40]. These findings may suggest that there are different predisposing factors for GBV-C infection among various populations.

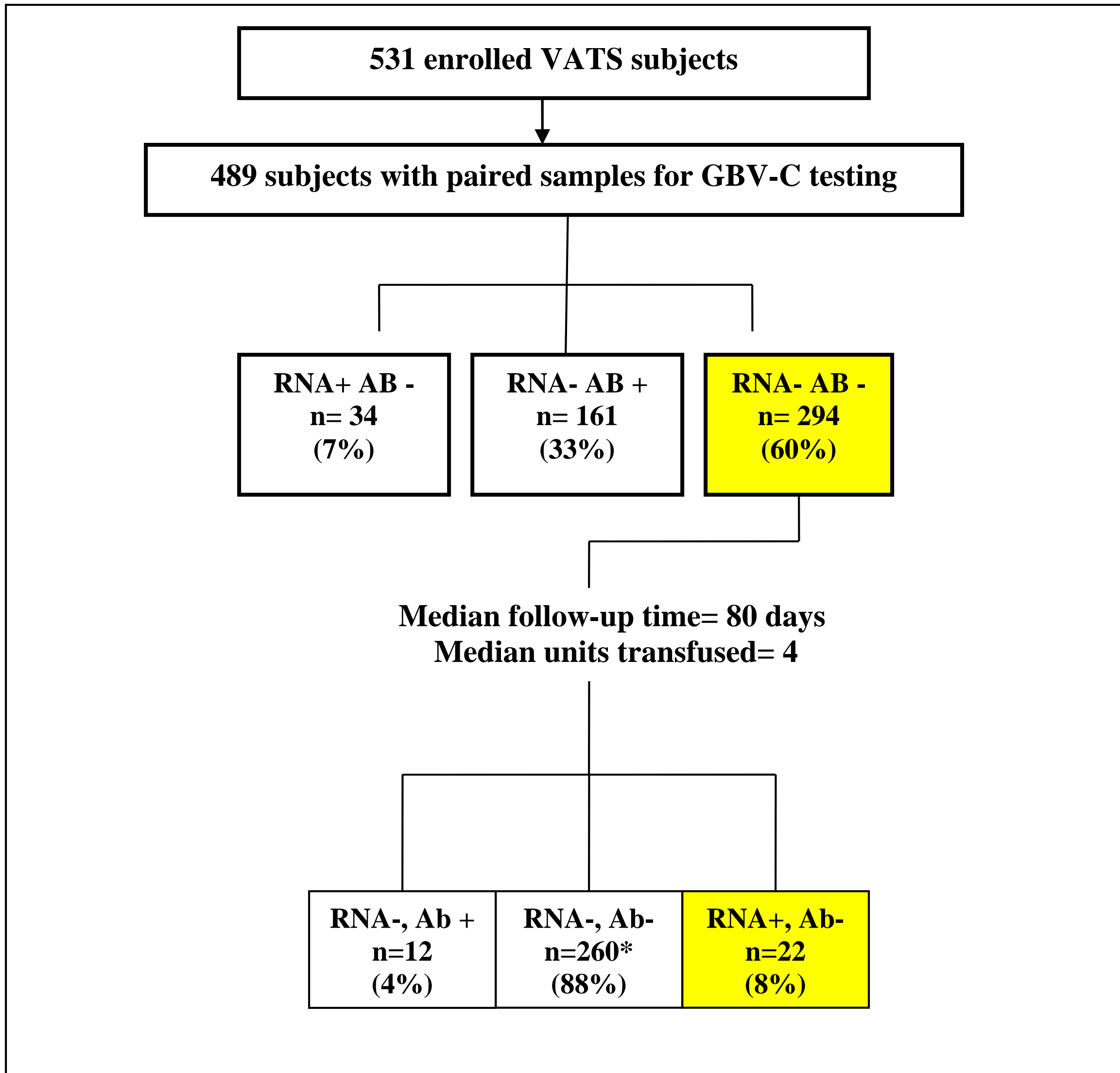
Previous studies of transfusion-associated GBV-C infection analyzed cross-sectional samples of prevalent GBV-C infection among various groups at high risk for parenteral transmission; i.e. hemophiliacs, multi-transfused individuals, injecting drug users, and transplant recipients[41-43] or patients infected with HIV[44, 45]. Acquisition of GBV-C RNA following transfusion has been studied longitudinally in multi-transfused individuals[6, 14, 34, 35, 466, 14, 34, 35, 46]. GBV-C infection has also been studied longitudinally in populations with known dates of HIV infection, to examine the impact of prevalent GBV-C viremia on the risk of HIV acquisition[11, 47] or HIV disease progression[38, 47, 48]. Bisson et al. documented a 22% higher prevalence of GBV-C viremia among those with recent HIV infection[47]. Also, Supapol et al. showed that acquisition of GBV-C by infants was associated with decreased mother to child transmission of HIV[1111]. To our knowledge, our study is the first to report the incidence of GBV-C viremia in HIV-infected patients receiving a transfusion and to examine pre-transfusion HIV status as a predictor of subsequent GBV-C acquisition in this setting.

Our study has a number of limitations. First, while GBV-C RNA measurements were performed on pre-transfusion and final samples for all subjects, while interim samples were tested only for subjects with evidence of incident viremia or acquisition of antibody. Imputation of negative results for the remaining samples could have resulted in the underestimation of GBV-C incidence if transient viremia occurred and was missed. Second, our sample size was small, and only 22 incident GBV-C RNA infections were observed; however, this study contains the largest number of documented post-transfusion incident GBV-C infections reported to date. Third, the VATS public use dataset did not include data on hepatitis B or C virus infection (HCV, HBV) or liver enzyme values. Previous reports have documented a higher prevalence of GBV-C RNA among patients with active or chronic HCV infection[49, 50]. There is very limited evidence concerning interactions among HIV, HCV and GBV-C[51]. Fourth, our binary classification of HAART use included subjects who were enrolled into the study before HAART era, as well as those who were enrolled or followed after June 1996. Thus, the "no HAART" group included subjects who never received any antiretroviral therapy as well as those who were receiving some combination of drugs that did not qualify as HAART. Fifth, we cannot exclude GBV-C acquisition by routes other than transfusion (i.e. sexual or nosocomial parenteral exposures) in our study population. However, given the health status of the VATS cohort, it is reasonable to assume that transfusion was the most likely route of acquisition in this cohort. To confirm transmission of GBV-C via transfusion, detailed RNA sequence analysis of the donor and recipient's post-transfusion samples would be required.

Our study provides evidence of high rates of GBV-C transmission by transfusion among HIV-infected subjects, and an increased hazard of GBV-C acquisition with lower pre-transfusion HIV VL and current use of HAART, controlling for the cumulative numbers of units of blood transfused. Our findings thus confirm previous reports showing an inverse relationship between HIV VL and GBV-C virmeia[21, 31, 52, 539] and *in vitro* interference between HIV and GBV-C[22, 23]. The present study provides support for the theory that inhibition of GBV-C acquisition is a partial explanation for the higher HIV viral loads among GBV-C negative patients reported in previous studies. Further

investigation to clarify the mechanisms by which GBV-C and HIV interact is warranted. Establishing evidence for transfusion transmission of GBV-C will allow additional studies of the impact of acute GBV-C acquisition on the course of HIV infection in co-infected patients.

Figure 1: Patterns of GBV-C infection status at baseline and 120 days post-transfusion, VATS (1995-1999)



* Antibody status for 171 subjects was determined later than 120 days post transfusion.

Table 1: Subject characteristics by GBV-C acquisition status, among initially GBV-C RNA/Ab negative VATS cohort members (n=294)			
Characteristic	**GBV-C RNA acquisition n=22**	**GBV-C RNA negative n= 272**	**p-value***
Follow-up time in days			
Mean (SD)	85.0 (28.9)	71.2 (34.4)	0.06
Median (IQR)	88.5 (80-108)	77.5 (32-100)	0.08
Baseline HIV viral load (**log_{10}**per ml)			
Mean (SD)	4.1 (1.4)	4.7 (1.1)	0.02
Baseline CD4 cells per µl			
Mean (SD)	70.5 (86.7)	55.5 (105.5)	0.54
Median (IQR)	30.5 (7.5-95.5)	14 (3-56)	0.08
HAART exposure			
Baseline, n (%)	6 (27.3)	58 (21.3)	0.51
By the last day of follow-up, n (%)	13 (59.1)	90 (33.1)	0.01
Cumulative units transfused			
Mean (SD)	7.2 (9.3)	5.2 (4.5)	0.08
Median (IQR)	4 (2-7)	4 (2-6)	0.41
Gender			
Male, n (%)	16 (72.7)	218 (80.2)	0.41
Age at baseline			
Mean (SD)	37.2 (1.5)	37.6 (0.5)	0.78
Race, n (%)			
White, non-Hispanic	18 (81.8)	131 (48.2)	0.01
Black, non-Hispanic	2 (9.1)	96 (35.3)	
Other	2 (9.1)	45 (16.5)	
HIV risk behavior, n (%)†			
Heterosexual	10 (45.5)	89 (32.7)	0.22
MSM	11 (50.0)	108 (58.8)	0.42
IV drug use	6 (27.3)	57 (21.0)	0.49
Other	1 (4.6)	13 (4.8)	0.96
Leukoreduced units transfused, n (%)	7 (31.8)	128 (47.1)	0.17
Received platelet units, n (%)	4 (18.2)	41 (15.1)	0.70

*** Based on t-test for equality of means, Wilcoxon rank-sum test for equality of medians, and chi-square test for categorical variables.**
† HIV risk behavior groups are not mutually exclusive.

Table 2. Odds Ratio (OR) for GBV-C RNA acquisition in HIV-infected patients estimated using pooled logistic regression models*, VATS (n=294)			
Variable	**Unadjusted OR (95% CI)**	**Adjusted for cumulative units transfused (95% CI)**	**Adjusted for all covariates† (95% CI)**
Cumulative units of blood transfused (per unit)	1.09 (1.06, 1.11)	--	1.08 (1.05, 1.11)
Baseline HIV viral load (per $\log_{10}$ copies/ml)	0.61 (0.40, 0.92)	0.62 (0.40, 0.96)	0.87 (0.54, 1.41)
Current use of HAART	4.78 (2.01, 11.3)	4.03 (1.79, 9.11)	4.75 (2.02, 11.18)
Baseline CD4 cells per μl (sqrt)	1.04 (0.98, 1.11)	1.05 (0.98, 1.12)	--
Non-White race (vs. White)	0.21 (0.06, 0.81)	0.15 (0.04, 0.60)	0.15 (0.04, 0.64)

* OR estimates discrete relative hazard of GBV-C RNA acquisition.
† Model is adjusted for cumulative units transfused, HIV viral load at baseline (pre-transfusion), time-varying HAART use and race.

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4. Acquisition of GB virus type C and lower mortality in patients with advanced HIV disease

4.1. Background

GB virus type C (GBV-C) is a common non-pathogenic human virus that is transmitted parentally, sexually and vertically (mother-to-child) and is highly prevalent among individuals infected with HIV[1-7]. Investigators have observed an association between GBV-C infection and prolonged survival among HIV-infected individuals[6, 8-14]. A meta-analysis of studies collectively including 1,294 HIV-infected subjects found a relative risk of mortality of 0.41 (95% CI 0.23, 0.69) for those with active GBV-C co-infection[15]. These findings are consistent with reported biological effects of GBV-C, which induces HIV-inhibitory cytokine profile, decreases T cell activation, blocks IL-2 mediated CD4 T cell proliferation, and reduces expression of the HIV entry receptors CCR5 and CXCR4 *in vitro*[11, 16, 17]. However, not all studies have found better survival in HIV-infected individuals co-infected with GBV-C; several studies have found no evidence of better survival or improvements in HIV disease markers among GBV-C co-infected patients[18-22].

Furthermore, the effect of GBV-C co-infection among HIV-infected individuals who are taking highly active anti-retroviral therapy (HAART) is unclear. Recent analyses have sought to explore the relationships among HIV, GBV-C and HAART[13, 23-27]. All previous studies have examined GBV-C infection that preceded or was concurrent with HIV infection and the time sequence of the relationship between GBV-C viremia, HIV disease markers, and exposure to HAART has not been fully investigated. An analysis that can account for both acquisition of GBV-C infection and also time-updated HAART status and HIV disease markers would be valuable in clarifying the relationship between HIV and GBV-C. We examined the effect of both existing GBV-C infection and new GBV-C RNA acquisition via transfusion on all-cause mortality and on markers of HIV disease progression. Here we report on survival and changes in HIV viral load (VL) based on the known dates of GBV-C acquisition and changes in HAART status, HIV VL and CD4+ cell count in a cohort of transfusion-naïve HIV-infected subjects receiving transfusions.

4.2. Methods

The Viral Activation Transfusion Study (VATS) was a double-blind clinical trial initiated in July 1995 to evaluate the effects of leukoreduced versus non-leukoreduced allogeneic transfusion on patients infected with HIV and cytomegalovirus (CMV)[28, 29]. Details of subject selection and findings of the clinical trial have been previously described[28]. Briefly, participants were transfusion-naïve at baseline, with symptomatic anemia that required red cell transfusions. Participants were randomized to receive either a filter-leukoreduced or standard non-leukoreduced blood unit. Given the enrollment criteria, study participants had advanced HIV disease (78% had an AIDS-defining illness); their mean baseline plasma HIV viral load was 4.5 $\log_{10}$ copies/ml (SD= 1.1), median baseline CD4 count 15 cells/µl (IQR= 3-73), and median survival time 8.4 month (IQR=2-21.8) post initial transfusion. At last observation, the mean HIV viral load for those who

survived was 4.3 log_{10} copies/ml (SD= 1.3), and the median CD4 count was 22 cells/µl (IQR= 3-129.5).
Study samples were collected and stored at -70º C pre-transfusion (baseline), weekly post-transfusion for one month and quarterly thereafter; pre-transfusion and weekly post-transfusion samples were collected for all additional transfusion episodes. In June 1996, about one year following the start of the accrual period for VATS, HAART was introduced into HIV treatment and became available to some but not all study subjects. At baseline, 24% of recruited subjects were receiving HAART (three or more antiretroviral drugs, including at least one protease inhibitor or non-nucleoside reverse transcriptase inhibitor), 55% were treated with some other combination of antiretroviral therapy, and 21% were not receiving antiretroviral treatment[29].

Of the 531 enrolled subjects, 489 (92%) had paired pre-transfusion and final samples available for GBV-C evaluation. All available paired (baseline and final visit) plasma samples were tested for GBV-C E2-antibody, using the anti-GBenv microplate EIA assay and for RNA using the quantitative GBV-RNA RT-PCR assay (both assays provided by Roche Diagnostics, Penzberg, Germany). All interim blood samples from individuals with evidence of incident GBV-C viremia or acquisition of GBV-C antibody between the pre-transfusion and final samples were tested by both antibody and RNA assays. Interim samples were also tested for GBV-C RNA if the final sample showed evidence of RNA clearance during the study. The final sample was retested following interim testing to confirm RNA positivity if it was the only RNA-positive sample.
For the current analysis we used the VATS limited-access public use dataset provided by the National Heart Lung and Blood Institute (NHLBI), combined with the GBV-C results provided by Blood Systems Research Institute (BSRI, San Francisco, CA). Written informed consent was obtained from all VATS study subjects [28], and this supplemental study protocol was approved by the Committee for the Protection of Human Subjects at the University of California, Berkeley, USA.

4.3. Statistical Analysis

Study subjects were divided into groups based on GBV-C status at baseline: GBV-C RNA positive, E2-antibody positive, or both GBV-C markers negative[19]. Missing GBV-C RNA and E2-antibody status for interim samples was imputed by carrying the most recent measurement forward.

We analyzed all VATS subjects as a single cohort (cohort (1)), and as two sub-cohorts, according to their GBV-C status at baseline. Thus, cohort (2) consisted of VATS subjects who were E2-antibody negative at baseline (n=328) and cohort (3) consisted of subjects with no evidence of prior or active GBV-C infection at baseline (i.e. who were RNA and E2-antibody negative; n=294). In addition, we stratified cohort (1) by time-updated HAART status at baseline and study follow-up: cohort (1a) included subjects who never received HAART during VATS study follow-up, and subjects who started HAART at some point later and were censored when started HAART (n=371), and cohort (1b) included subjects who were receiving HAART at baseline as well as those who initiated HAART during follow-up (n=265).

Categorical variables were compared using the Chi-square test and continuous variables were compared using one way ANOVA when normally distributed or, alternatively, by the Kruskal-Wallis rank test. Time to death (i.e. all-cause mortality) was analyzed using Kaplan –Meier estimates and the log-rank test according to baseline GBV-C RNA and E2-antibody status. Cox proportional hazard regression models were used to calculate unadjusted and adjusted hazard ratios (HR) and 95% confidence intervals (CI) as predicted by time-updated GBV-C and HIV disease markers, and baseline characteristics. Subjects were followed from date of VATS study entry to either death, loss to follow-up, or censoring at clinical trial conclusion (up to 3.5 years after baseline). At each point in time (t) GBV-C RNA and E2-antibody status and HAART use were categorized as binary time-updated variables. HAART use was coded as "yes", if the subject received HAART between the last visit ($t-1$) and time t. Log_{10} HIV VL and CD4+ T lymphocyte count (square root transformed) measured at time $t-1$ were entered into the models as continuous time-updated variables. Baseline characteristics including, sex (male/female), race (white/non-white), and HIV risk behavior (heterosexual sex, men having sex with men, and injecting drug use) were tested as binary time-fixed variables. Failure was defined as all-cause mortality between t and $t+1$ for cohorts (1), (1a), (1b), (2), and (3); all-cause mortality or virological progress (> 1 $\log_{10}$ increase in HIV VL between t and $t+1$) for cohort (1a)[9], and all-cause mortality or virological failure (detection of two consecutive plasma HIV VL >500 copies/ml at t and $t+1$ if at least one plasma HIV VL ≤ 500 copies/ml was detected after initiation of HAART) for cohort (1b)[23].

Proportional hazards assumptions were tested using log-log survival curves, the global test for proportionality based on Schoenfeld residuals, and testing interaction of the binary time-updated GBV-C variable with log of time (p=0.08). All comparisons were two sided with a 5% significance level using STATA 10.1 (StataCorp, College Station, Texas, United States).

4.4. Results

4.4.1. Mortality among all VATS subjects relative to GBV-C status - cohorts (1) and (2)

Of the 489 subjects tested for GBV-C RNA and E2-antibody comprising cohort (1), 294 (60%) were RNA and E2-antibody negative, 34 (7%) were RNA positive, and 161 (33%) were E2-antibody positive at baseline. These three groups were mutually exclusive. For all subjects the median follow-up time from baseline to final visit was 8.4 months (IQR=2-21.8 months). At last day of follow-up, 261 (53%) subjects were GBV-C RNA and E2-antibody negative, 67 (14%) were RNA positive and 161 (33%) were E2-antibody positive (Figure 1). In no case were subjects simultaneously RNA and E2-antibody positive, at any time point. Table 1 provides an overview of subject characteristics at baseline and last day of follow-up according to their baseline GBV-C RNA and E2-antibody status. Subjects with GBV-C viremia at baseline had slightly longer follow-up time and significantly lower HIV VL and higher CD4 cell counts at both visits than those without GBV-C viremia at baseline (p<0.001). At baseline, more

subjects with detectable GBV-C RNA were receiving HAART as compared with GBV-C RNA negative subjects (p=0.04).

Kaplan-Meier estimates of survival were significantly higher for subjects with GBV-C viremia at baseline as compared to those who were GBV-C RNA negative at baseline, regardless of baseline E2-antibody status (Figure 2, p=0.02 for cohort (1); p=0.03 for cohort (2), data not shown).

Current GBV-C viremia status, higher current CD4 cell count, lower current HIV viral load, current HAART use, and being a man who reported having sex with men predicted lower mortality in univariate Cox regression analyses of cohort (1) (unadjusted HR for GBV-C RNA= 0.32, 95% CI= 0.18, 0.56) (Table 2). The association between mortality and time-updated GBV-V RNA was no longer significant after adjusting for other covariates (adjusted HR=0.54, 95% CI=0.29, 1.02). Similarly, univariate and multivariate Cox regression analysis of cohort (2) showed a significant unadjusted hazard ratio for time-updated GBV-C RNA (unadjusted HR= 0.34, 95% CI=0.19, 0.59) and a non-significant trend towards lower mortality among subjects with current GBV-C viremia after adjusting for time-updated HIV VL, CD4 cell counts, HAART status, E2-antibody and baseline HIV risk behavior (adjusted HR= 0.61 (0.33, 1.15).

4.4.2. Mortality among GBV-C negative subjects who acquired GBV-C RNA during follow-up - cohort (3)

VATS subjects were receiving blood transfusions during study follow-up, and thus were at risk of parenteral GBV-C transmission via transfusion (see chapter 3, which documented an 8% per transfused unit increased rate of GBV-C infection in VATS patients). We restricted our sample to those who were GBV-C RNA and E2-antibody negative at baseline (cohort 3) to compare hazard of all-cause mortality between subjects who presumably acquired GBV-C via transfusion and those who remained GBV-C RNA and E2-antibody negative during study follow-up. Two-hundred ninety-four subjects were included in this sub-analysis, of those, 39 (13%) became GBV-C RNA positive between baseline and last follow-up visit, 25 (9%) acquired E2-antibody, and 230 (78%) remained GBV-C negative. In the unadjusted analysis, GBV-C acquisition was associated with a substantial reduction in mortality, with hazard ratio of 0.23 (95% CI=0.09, 0.57) (Table 3). In a Cox proportional hazard regression analysis adjusting for time-updated HIV VL, CD4 cell counts, HAART status, E2 –antibody and baseline HIV risk behavior, the relative hazard of mortality in the GBV-C RNA positive group was 0.31 (95% CI= 0.11, 0.86), as compared with the GBV-C RNA negative group (Table 3).

4.4.3. Stratified analysis for virological failure

We performed stratified analysis according to HAART status at baseline and during study follow-up to examine mortality or virological progress/failure in cohorts (1a) and (1b) (data not shown). The Cox proportional hazard regression analysis of subjects before initiation of HAART (follow-up started at baseline and censored by initiation of HAART if subjects started HAART later), cohort (1a), showed a non-significant trend towards lower mortality or virological progress among GBV-C RNA positive subjects after adjusting for other covariates (unadjusted HR=0.50 (0.28, 0.90); adjusted HR= 0.63, 95%

CI= 0.34, 1.19). Similarly, the hazard of mortality or virological failure among those on HAART who were receiving HAART at baseline or when started HAART during study follow-up, cohort (1b), was not significantly different between GBV RNA positive versus negative patients in the multivariate regression model (unadjusted HR= 0.65, 95% CI= 0.39, 1.08); adjusted HR= 0.91, 95% CI= 0.53, 1.55).

4.5. Discussion

This study provides evidence that acquisition of GBV-C infection is associated with lower mortality among patients with advanced HIV disease. This association was independent of HIV risk behavior, HAART status, and HIV VL and CD4 cell count measured prior to current GBV-C RNA status among patients who were GBV RNA and E2 antibody negative at baseline. GBV-C acquisition during study follow-up is likely as a consequence of transfusion transmission from asymptomatic viremic donors (the 8% incident infection rate per unit transfused in the VATS patients is very similar to the rates of GBV-C viremia reported among blood donors in the US and Europe) (reviewed in[30]). Our analysis also shows that GBV-C viremia was associated with a non-significant trend towards lower mortality and less virological failure among HIV-infected VATS subjects, regardless of their HAART exposure status.

To our knowledge this is the first report showing a survival benefit of incident GBV-C infection among patients who were infected with HIV prior to GBV-C acquisition. All previous studies on the effect of GBV-C co-infection have described prevalent GBV-C infection which likely preceded HIV infection (reviewed in[31]). It remains an open question whether improved survival observed among patients with GBV-C viremia is due to GBV-C viremia serving as a marker for high CD4 cell counts (either because the increased CD4 count provides more target cells for GBV-C replication or because it correlates with lower HIV viral loads and thus increased risk of acquisition and persistence of GBV-C viremia) or if GBV-C viremia itself improves survival[32-35].

Our analysis is strengthened by our ability to evaluate time-updated GBV-C RNA status (including GBV-C loss) as a predictor of all-cause mortality, given HIV VL, CD4 cell count, and HAART status prior to GBV-C measurement, thus, establishing a clear temporal sequence between HIV disease markers, GBV-C viremia and mortality.

There are a few reports on the effect of GBV-C on survival in the presence of HAART (reviewed in[34]). We examined the effect of time-updated HAART using two approaches. First, we considered current HAART exposure as a potential confounder, assuming no effect modification between HAART and GBV-C in cohort (1). We then stratified our cohort by its HAART status, cohorts (1a) and (1b), to examine changes in HIV viral load and mortality in each group. A major assumption in all survival analyses was that censoring was independent of the outcome in the absence of censoring, given the covariates included in the model. In cohorts (1), (2) and (3), censoring occurred primarily due to end of study, and thus is expected to be non-informative. In cohorts (1a) and (1b), however, subjects were also right censored if they started or stopped HAART,

respectively. Inclusion of time-updated HIV VL and CD4 cell count, both major determinants of ART treatment decisions, improves the plausibility of this assumption. We did not find a significant effect of GBV-C RNA on mortality in subcohorts (1a) or (1b) when we adjusted for HIV VL and CD4 counts. However, a trend towards lower mortality among GBV-C RNA-positive subjects was consistent in all models and sub-cohorts. We should also note potential misclassification of "HAART" status due to binary categorization of the "HAART" variable. The "no HAART" group included subjects who never received any antiretroviral therapy, as well as those who were receiving some combination of drugs that did not qualify as HAART. Given the above limitations, either by adjusting for HAART or stratifying by HAART status, we found results consistent with previous reports of better or neutral response to HAART in GBV-C viremic subjects (reviewed in[34]).

A common finding in previous studies was that being GBV-C viremic during late stages of HIV infection is associated with better HIV disease outcomes[34]. Willimas et al. found GBV-C viremia detected 5-6 years after HIV seroconversion was associated with significantly longer survival, but no significant effect if GBV-C was measured 12-18 months after HIV seroroconversion. They concluded that stage of HIV infection may be an explanation for inconclusive findings of the previous studies[14].[21] Unfortunately, we were not able not estimate the date of HIV seroconversion for VATS subjects. Given the clinical status of the VATS cohort (78% had an AIDS defining illnesses at baseline), we know that all subjects had advanced HIV disease with presumably several years since HIV seroconversion[29].

Our study has a number of limitations. First, our estimate of survival benefit related to GBV-C infection may be an underestimate, as E2-antibody positive subjects were classified with GBV-C RNA negative ones. Among 294 subjects who were GBV-C RNA and E2-antibody negative at baseline (cohort (3)), 25 subjects became E2-antibody positive in the final sample, despite remaining GBV-C RNA negative. This could be due to either passive antibody transfer via transfusion or formation of E2-antibody by the subject in response to transient undetected GBV-C viremia. Misclassification of transient GBV-C viremic subjects as GBV-C negative would be expected to bias our estimated hazard towards the null.

Second, the VATS cohort had a relatively short follow-up time, up to 3.5 years. Most previous studies have examined the effect of GBV-C co-infection for a period of 4-8 years (reviewed in[34]). We cannot rule out the possibility that we might have seen an effect if VATS cohort had been followed for a longer period of time. It is noteworthy that in all models and sub-cohorts we examined, the direction of the relationship between GBV-C viremia and survival was towards better survival among GBV-V viremic group.

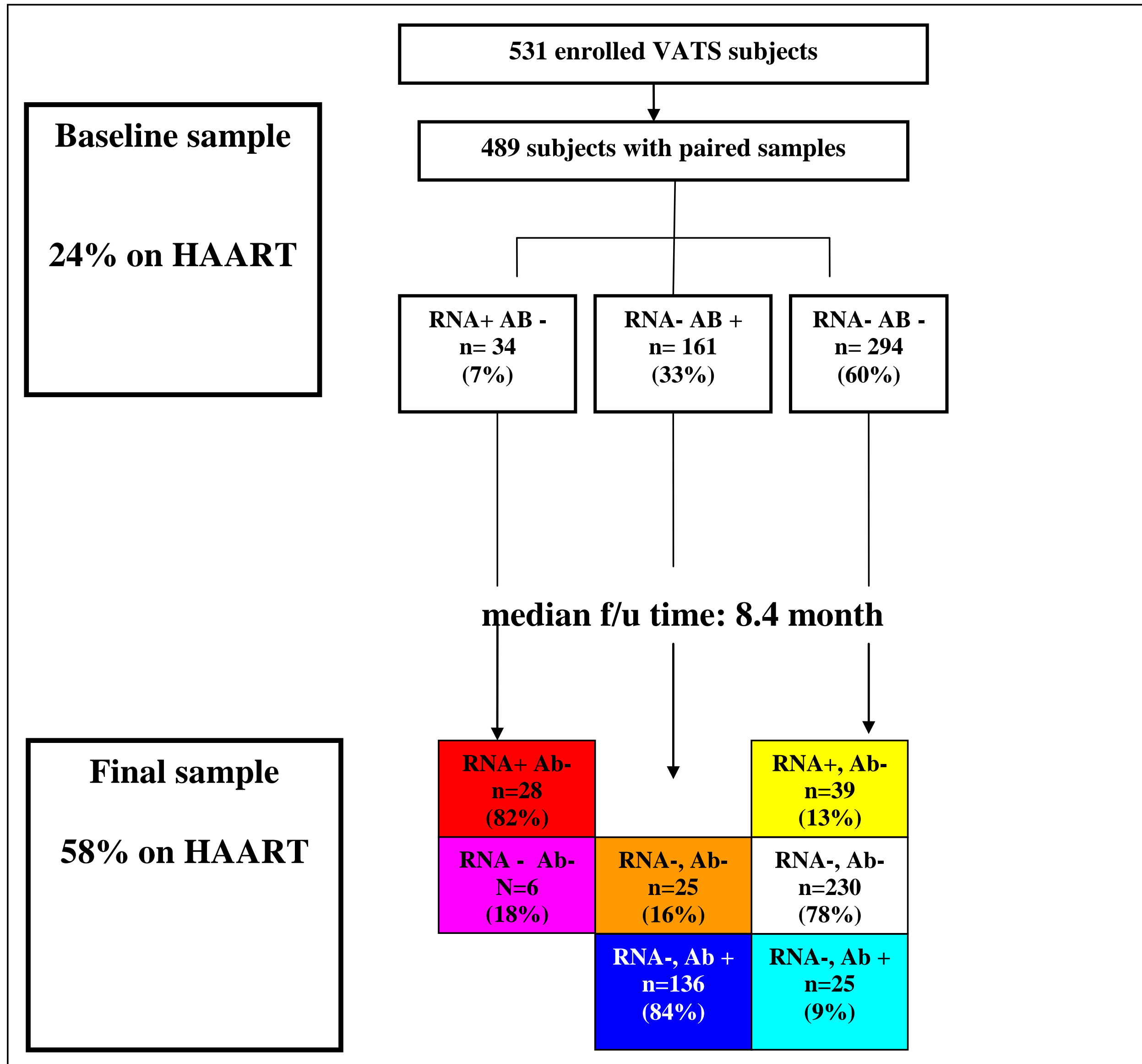
Third, neither the unadjusted associations between time updated GBV-RNA and mortality, nor these associations adjusted for time updated CD4, HIV VL, and HAART use are expected to provide unbiased estimates of the effect of GBV-C viremia on survival. The unadjusted analyses are subject to residual confounding; higher CD4 counts and lower HIV VL during the course of follow up both may have predisposed subjects to

acquisition of GBV-C RNA and increased survival. Analyses that adjust for time updated CD4 count and HIV viral load address this concern to some extent; however, the resulting estimates cannot be interpreted as either the immediate effect of current GBV-C status or as the cumulative effect of GBV-C viremia, both because of potential confounding by past GBV-C status not controlled by adjustment for current CD4 count and HIV VL and because time updated HIV VL and CD4 count are potentially affected by past GBV viremia. The association between GBV-C acquisition and mortality among initially GBV-C negative subjects (cohort (3)) may exclude potential confounding by past GBV-C status, but still is not perfect due to possible direct effect of past CD4 count (nadir CD4 count) and HIV VL on mortality. Alternative analysis methods such as marginal structural models and longitudinal G-computation (maximum likelihood estimation) are required to estimate the cumulative effect of GBV-C on HIV disease outcomes while appropriately adjusting for time-varying confounders affected by prior GBV-C. Given the relatively small sample size and low prevalence of exposure (7%) we did not expect to observe a dramatic change in the findings if using these alternative methods.

Fourth, one has to be cautious in generalizing the findings of the current study, as VATS cohort enrolment started before availability of HAART. Thus, not all study subjects had access to HAART, which is currently the standard of care for HIV infection in many countries.

Nonetheless, we found that GBV-C viremia is associated with a trend towards lower mortality in HIV-infected patients, after adjusting for time-updated HIV VL, CD4 cell count, HAART status and E2 antibody, consistent with other reports. In addition, we found a significant reduction in mortality associated with acquisition of GBV-C viremia during follow up among HIV-infected subjects receiving transfusions, even after adjusting for time-updated HIV disease markers. There is substantial *in vitro* evidence demonstrating inhibition of HIV replication by GBV-C. Establishing clinical evidence for a causal relationship between GBV-C infection and HIV disease outcomes will require larger studies with more complex designs and methodology such as larger observational studies of incident GBV-C infections in HIV-infected transfusion recipients. Such studies can be designed to more directly characterize the acute and long term impact of GBV-C acquisition on virological, immunological and clinical consequences of HIV infection.

Figure 1: Patterns of GBV-C infection status at baseline and final sample – VATS (1995-1999)



Characteristic	GBV-C RNA positive n=34	GBV-C E2-antibody positive n=161	GBV-C negative n=294
Follow-up time in months			
Mean (SD)	17.0 (12.6)	11.2 (11.5)	12.9 (12.6)
Median (IQR)	17.5 (3.9-28.9)	6.6 (1.8-21.0)	9.2 (2.0-23.4)
HIV viral load ($\log_{10}$ per ml)			
Baseline, mean (SD)*	3.6 (1.2)	4.6 (1.0)	4.6 (1.1)
Last day of follow-up, mean (SD)*	3.4 (1.1)	4.4 (1.2)	4.4 (1.3)
CD4 cells per µl			
Baseline, median (IQR)†	71.5 (20-230)	11.0 (3-61)	15.0 (3-61)
Last day of follow-up, median (IQR) †	129.0 (50-230)	11.0 (2-93)	19.5 (3-118)
Antiretroviral exposure (HAART)			
Baseline, n (%)‡	14 (41.2)	40 (24.8)	64 (21.8)
By the last day of follow-up, n (%)	24 (70.6)	88 (54.7)	170 (57.8)
Age in years, mean (SD)*	37.3 (7.0)	40.3 (7.3)	37.6 (7.4)
Gender			
Male, n (%)	28 (82.4)	126 (78.3)	234 (79.6)
Race, n (%)			
White, non-Hispanic	17 (50.0)	94 (58.8)	146 (49.7)
Black, non-Hispanic	13 (38.2)	50 (31.3)	97 (33.0)
Other	4 (11.8)	16 (10.0)	51 (17.4)
HIV risk behavior, n (%)$			
Heterosexual sex	9 (26.5)	46 (28.8)	99 (33.7)
Men having sex with men	22 (64.7)	97 (60.6)	171 (58.2)
Injecting drug use ‡	9 (26.5)	53 (33.1)	63 (21.4)
Transfusion status			
Leukoreduced, n (%)	19 (55.9)	85 (52.8)	135 (45.9)

Table 1: Subject characteristics by baseline GBV-C status of 489 subjects with HIV infection, VATS, cohort (1), 1995-1999

‡p-value for Chi-2 test <0.05
* p-value for F-test <0.001
†p-value for Kruskal-Wallis rank test <0.001
$ HIV risk behavior groups are not mutually exclusive.

Figure 2: Kaplan-Meier estimates of survival according to GBV-C RNA and E2 antibody status at baseline, VATS, cohort (1), n=489

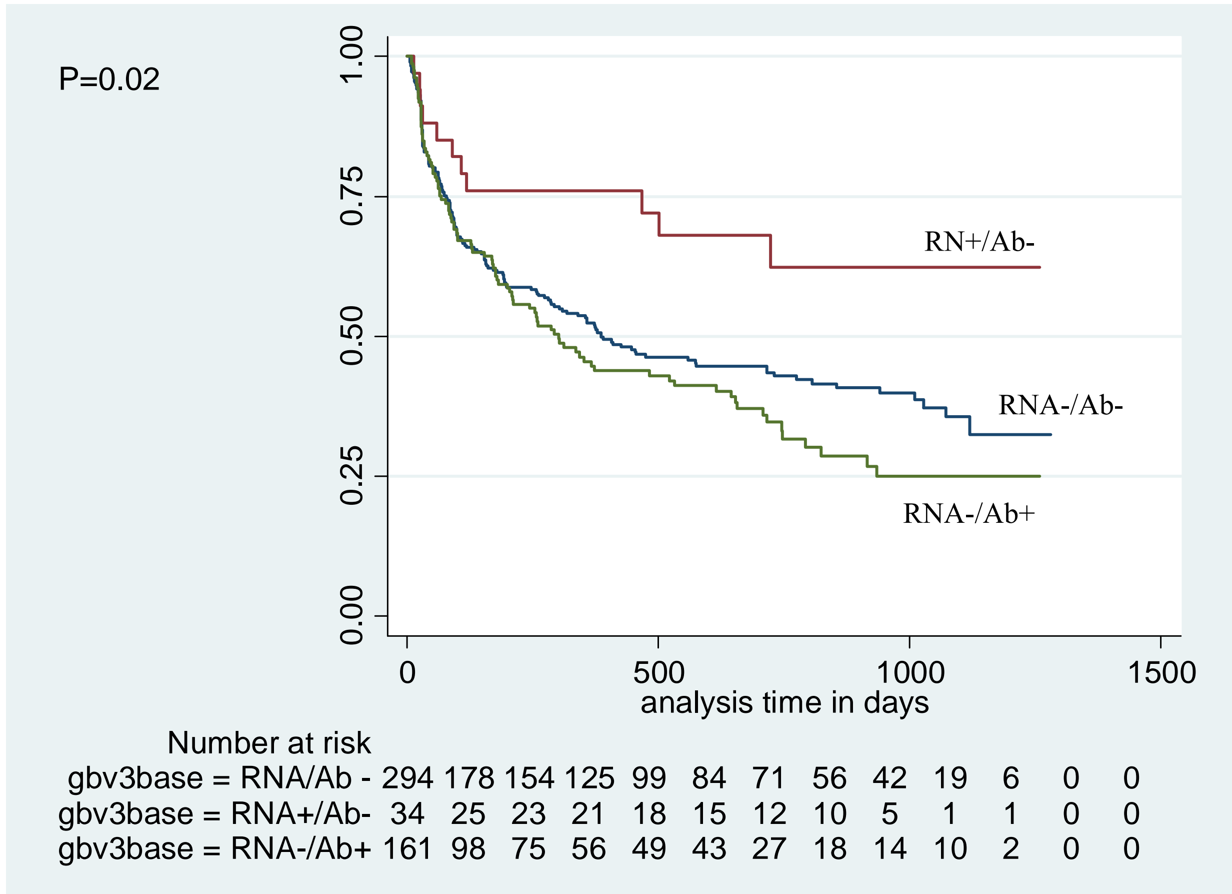


Table 2: Unadjusted and adjusted hazard ratio (HR) for mortality using Cox proportional hazard regression models, VATS, cohort (1), n=489

Characteristic	No of deaths	Unadjusted HR	95% CI	Adjusted HR*	95% CI
Time-updated					
GBV-C RNA negative GBV-C RNA positive	252 15	1.00 0.32	0.18, 0.56	1.00 0.54	0.29, 1.02
GBV-C E2-antibody positive †		1.09	0.85, 1.41	0.95	0.73, 1.23
Current use of HAART		0.35	0.26, 0.46	0.45	0.33, 0.61
HIV VL (log10/ml)		1.71	1.53, 1.91	1.09	0.94, 1.27
CD4 cells /µl (sqrt)		0.79	0.75, 0.83	0.85	0.81, 0.89
Baseline					
Heterosexual sex		1.18	0.91, 1.52	--	--
Injecting drug use		1.04	0.78, 1.38	--	--
Men having sex with men		0.77	0.60, 0.98	--	--
Male		0.75	0.55, 1.02	--	--
Non-white race		0.97	0.82, 1.14	--	--

* HR is adjusted for time-updated E2-antibody status, HAART status, HIV VL, and CD4 cell count at time *t-1* and HIV risk behavior (MSM),
† E2-antibody values are imputed for subjects with antibody +/+ or antibody -/- results at baseline/final sample.

Table 3: Unadjusted and adjusted hazard ratio (HR) for mortality using Cox proportional hazard regression models, VATS, cohort 3, n=294			
Characteristic	**No of deaths**	**Unadjusted HR (95% CI)**	**Adjusted HR* (95% CI)**
Time-varying			
GBV-C RNA negative GBV-C RNA positive	153 6	1.00 0.23 (0.09, 0.57)	 0.31 (0.11, 0.86)
Current use of HAART		--	0.47 (0.31, 0.69)
HIV VL (log10/ml)		--	1.20 (0.98, 1.48)
CD4 cells /µl (square root)		--	0.87 (0.82, 0.92)
GBV-C E2-antibody positive†		--	1.18 (0.57, 2.43)

* HR is adjusted for time-updated E2-antibody status, HAART status, HIV VL, and CD4 cell count at *t-1* and HIV risk behavior (MSM),
† E2-antibody values are imputed for subjects with antibody +/+ or antibody -/- results at baseline/final sample.

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5. Conclusion

GB virus type C (GBV-C) is transmitted to healthy and immunosuppressed individuals via transfusion. GBV-C inhibits HIV replication *in vitro,* and prior clinical studies have suggested an association between GBV-C infection and better survival among HIV-infected individuals. Our analysis of 489 HIV-infected transfusion naïve patients showed an increased risk of GBV-C acquisition with each additional units of blood transfused. Lower HIV (VL) and being on HAART were associated with GBV-C acquisition, controlling for the cumulative number of units of blood transfused, consistent with previous reports showing an inverse relationship between GBV-C viremia and HIV VL. We were able to clarify the temporal relationship between HIV VL, HAART and GBV-C acquisition using VATS dataset. Our findings showed that a higher HIV VL at baseline was associated with a reduced risk of subsequent GBV-C acquisition, controlling for HAART status and the cumulative number of units received. This finding may be an explanation for previous observations of higher HIV VL among GBV-C negative patients.

We also found a trend towards lower mortality and lower HIV VL in GBV-C viremic subjects after controlling for time-updated HIV VL, CD4 cell counts, and HAART status, confirming previous reports. In addition, for the first time, we examined acute GBV-C infection in relationship to mortality in advanced HIV-infected patients. Our analysis of GBV-C RNA and antibody negative sub-cohort showed acute GBV-C acquisition is associated with a significant reduction in all-cause mortality, after controlling for time-updated covariates.

Previous investigators have been trying to identify the mechanism by which GBV-C and HIV interact in hopes of finding novel effective treatments for HIV infection. Neither the unadjusted association between GBV RNA and survival, nor the association adjusted for time updated covariates can be interpreted directly as an estimate of the causal effect of GBV infection on mortality because of residual confounding by past GBV-C status and HIV markers. The small sample size and relatively short follow-up time of the VATS subjects limited our ability to reach significant findings. Larger longitudinal studies of HIV-infected patients receiving transfusions are needed to examine pre- and post-transfusion GBV-C and HIV markers, and subsequent virological and immunological changes in HIV disease and survival. Such studies will make possible application of analytic approaches such as marginal structural models that are able to appropriately adjust for time-varying covariates and thus provide improved estimates of the causal effect of GBV-C infection on survival.

6. Appendix

Acronyms

BSRI	Blood systems research institute
CI	Confidence interval
CMV	Cytomegalovirus
EIA	Enzyme linked immunoassay
GBV-C	GB virus type C
HAART	Highly active antiretroviral therapy
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HGV	Hepatitis G virus
HIV	Human immunodeficiency virus
HR	Hazard ratio
IQR	Inter-quartile range
IV	Intravenous
LR	Leukoreduced
MSM	Men having sex with men
MTCT	Mother-to-child transmission
NHLBI	National Heart, Lung and Blood Institute
OR	Odds ratio
RBC	Red blood cells
RT-PCR	Reverse transcriptase polymerase chain reaction
SD	Standard deviation
Sqrt	Square root
VATS	Viral activations transfusion study
VL	Viral load
WBC	White blood cells