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<td><strong>Author(s)</strong></td>
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Prevalence of GB virus type C viraemia in MSM with or without HIV-1 infection in Beijing, China

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SUMMARY

GB virus C (GBV-C) is frequently identified in patients co-infected with human immunodeficiency virus type 1 (HIV-1) due to the similar transmission routes. However, it remains unclear how these two viruses interact with each other and how one virus affects the replication of the other in the human body. In this study, we performed a case-control study to determine whether GBV-C viraemia could prevent the acquisition of HIV-1 infection, and a cohort study to determine the prevalence, genotypic characteristics and incidence of GBV-C infection in men who have sex with men (MSM) populations in Beijing, China. The prevalence of GBV-C infection in HIV-1-negative subjects was similar to that in HIV-1-positive subjects. Before HIV-1 acquisition, the prevalence of GBV-C was 17.7%, which increased to 27.2% at the acute stage and to 34% at the chronic stage. Genotype 3 was the major genotype of GBV-C in both groups. A significantly positive correlation was observed between the CD4+ T-cell counts and GBV-C viral loads at the acute stage of HIV infection. At the chronic stage (12 months later), this correlation was no longer significant, although it was still positive. Overall, this study demonstrated that pre-existing GBV-C viraemia could not prevent the acquisition of HIV-1 infection and transmission of HIV-1 significantly increased the prevalence of GBV-C viraemia.

Key words: Cohort study, GB virus C; human immunodeficiency virus type 1, men who have sex with men (MSM).

INTRODUCTION

GBV-C is a flavivirus that is closely related to hepatitis C virus (HCV) [1]. Transmission of GBV-C via parenteral, sexual, and vertical routes has been documented, and infection is common in many populations [2–7]. There are five major genotypes that are prevalent worldwide [8, 9]. Several studies have reported a strong association between the existence of GBV-C viraemia and the survival of HIV-infected patients [7, 10, 11]. However, other studies do not support this association [12–14]. In fact, two studies showed that patients co-infected with GBV-C and HIV-1 had an increased mortality rate [15, 16].
Mechanisms underlying the positive effects on the progression of HIV infection include decreased expression of CC chemokine receptor 5 (CCR5) on CD4+ T cells and direct inhibition of HIV replication by GBV-C [17, 18]. Recent findings suggest that HIV-1 patients with active GBV-C infection had a reduced percentage of T cells positive for CD38+CD4+, CD38+CD8+, CCR5+CD4+, and CCR5+CD8+, which might provide a key mechanism involved in the protection conferred by GBV-C against HIV-1 disease progression [19].

MSM are the second most vulnerable population for HIV-1 infections after intravenous drug users in China in recent years. In Beijing, 3.0–4.6% of MSM were found to be HIV-1 positive [20]. A cross-sectional study has shown a high prevalence of sexually transmitted infections and HCV to be present in HIV-1-positive MSM [21], but the infection rate of GBV-C in MSM has not been established. Here, we performed a cohort study to evaluate the prevalence of GBV-C viraemia in MSM for those with a clear date of HIV-1 infection and those without HIV-1 infection. We also analysed the association of GBV-C viraemia, syphilis, HCV and hepatitis B virus (HBV) with HIV-1 infection in this MSM cohort. Furthermore, we determined the potential effect of GBV-C viraemia on the progression of HIV-1 disease.

MATERIALS AND METHODS

Study sample

The subjects included in this study were selected from the participants in the AIDS High Risk Cohort Programme supported by the Beijing Science & Technology Committee and the study was approved by the Ethics Committee of the Beijing Youan Hospital. A total of 4236 MSM were enrolled in the study between 2007 and 2010. Written informed consent was obtained from all participants. At 2-month intervals, HIV-related clinical status was assessed, an interviewer-administered questionnaire was completed, and blood samples were obtained for detection of HIV-1 antibodies and HIV-1 RNA levels by immunoassay and RT–PCR (Roche, Sweden), respectively. Of 4236 MSM, 99 subjects who had a clear date of HIV-1 infection and 175 subjects without HIV-1 infection at the same visit as a control were selected from the cohort for the case-control study. The prevalence of GBV-C viraemia and antibodies against HCV, HBV surface antigen (HBsAg) and syphilis were compared between individuals with HIV-1 infection and controls without HIV-1 infection. In the cohort study, 99 HIV-1-positive subjects were detected for GBV-C viraemia at the acute stage of HIV infection and the chronic stage of HIV-1 infection. Acute stage is defined as negative HIV p24 antibody testing by enzyme-linked immunosorbent assay in the presence of detectable HIV-1 RNA or positive HIV p24 antibody testing and an evolving (≤3 bands positive) HIV Western blot [22]. Chronic stage is defined as having been infected for at least 6 months and usually for several years [23]. In our study, we collected the samples at 12 months post-HIV infection. CD4+ T-cell counts and HIV-1 viral loads were also determined at each time point.

RT-PCR for detection of GBV-C RNA

GBV-C RNA was extracted from the samples by the Qiagen viral RNA Mini kit (Qiagen, Germany). A Superscript First-Strand Synthesis kit (Invitrogen, USA) was used to create GBV-C complementary DNAs (cDNAs). Both first- and second-round PCRs were performed using primers that hybridize to 5' non-translated regions of GBV-C (GenBank accession no. NC_001710.1, 31–376 nt and 121–333 nt, respectively). Primers for the first-round RT–PCR were GBV-F1 (5'kACT GGG TGC AAG CCC CAG AAA CC) and GBV-R1 (5'kCTG GTC CTT GTC AAC TCG CCG). Primers for the second-round PCR were GBV-F2 (5'kGTG ATG ACA GGG TTG GTA GGT CGT) and GBV-R1 (5'kCTG GTC CTT GTC AAC TCG CCG). Primers for the second-round PCR were GBV-F2 (5'kGTG ATG ACA GGG TTG GTA GGT CGT) and GBV-R1 (5'kCTG GTC CTT GTC AAC TCG CCG). PCR products were detected on 1.5% agarose gels containing 0.5 mg/ml of ethidium bromide. The expected band sizes were 366 and 233 bp for the first- and second-round PCRs, respectively.

Quantification of GBV-C viral load

Absolute quantification requires a standard curve of known amounts of the amplicon per reaction. The substrates for standard curve were generated by subcloning the amplicon in a plasmid (pGEM-T easy, Promega). A standard curve of the GBV-C NCR plasmid was quantified using the universal QPCR method in a 25-μl reaction containing 1 × Taqman Universal PCR Master Mix (Applied Biosystems, USA), 400 nM of primer gbv-p1 (5'kAGC GCA CGG TCC ACA GGT GTT-3'), 400 nM of primer gbv-p2 (5'kGAC ATT GAA GGG CGA CGT GGA-3'), and
200 nM of fluorescent probe gbv-probe (5′-FAM-CCC TAC CGG TGG GAA TAA GGG CCC GAC TAMRA-3′) (Invitrogen). Serial tenfold dilutions of GBV-C NCR plasmid were used to cover a range of 10^4–10^7 molecules per reaction. Triplicate reactions of each dilution were pipetted into a 96-well plate and amplified in an ABI Prism 7900HT real-time PCR system (Applied Biosystems) at 50 °C for 30 min and then 94 °C for 2 min for the inactivation of the AMV reverse transcription enzyme, followed by 40 cycles of 94 °C for 10 s and 60 °C for 1 min. The data was analysed with Sequence Detection Systems (SDS) software (Applied Biosystems).

**Determination of nucleotide sequences and GBV-C genotypes**

The 627-bp PCR outer fragments of 5′-UTR were obtained by the primer pairs of GBV-OF1 (5′-GTG CAA GCC CCA GAA ACC GAC-3′, 36 nt, NC_001710.1) and GBV-OR1 (5′-GGG GCG CAA CAG TTT GTG AGG-3′, 641 nt, NC_001710.1). Subsequently, the 366-bp PCR products were obtained by primer pairs GBV-F1 and GBV-R1. The nucleotide sequences were determined using an ABI PRISM3730 XL Genetic Analyzer (ABI Biosystems, USA). The sequences were aligned with the sequences from reference isolates of GBV-C genotype 1 (U36380), genotype 2 (U44402 and AF081782), genotype 3 (D90601) and genotype 5 (AY949711) by Mega software (version 5.0). The phylogenetic trees were constructed by neighbour-joining as implemented in the software and the evolutionary distances were computed using the maximum composite likelihood method.

**Statistical analyses**

The odds ratios (OR) and 95% confidence intervals (CI) for the association between the occurrence of GBV-C viraemia and HIV acquisition was calculated. The occurrence of GBV-C viraemia between subjects prior to HIV-1 infection and those after acquisition of HIV-1 infection were compared by Pearson’s χ² test. The GBV-C viral loads at two time points were compared by paired Student’s t test. The median of HIV viral loads and CD4^+ T-cell counts between the GBV-C viraemia group and the GBV-C RNA-negative group in HIV-1-positive subjects were compared by Mann–Whitney U test. The relationship between HIV-1 viral loads and GBV-C viral loads, and CD4^+ T-cell counts were evaluated by linear regression analysis.

**RESULTS**

**HIV-1 acquisition was independent of GBV-C viraemia**

To determine the impact of GBV-C viraemia on HIV-1 transmission, we followed up 99 MSM subjects before and after they acquired HIV-1. Based on a cross-sectional analysis, we found that the prevalence of GBV-C viraemia in these 99 study subjects was 17.7% before HIV-1 infection, which was not significantly different from that (12.6%) of the 175 HIV-1-negative subjects (OR 1.36, P = 0.29) (Table 1), indicating that a pre-existing GBV-C infection probably did not play a significant role in preventing the acquisition of HIV-1. Similarly, the prevalence of HBV and HCV in the 99 HIV-1-positive subjects was not significantly different from that in the 175 HIV-1-negative subjects (Table 1). In contrast, the prevalence of syphilis in the 99 HIV-1-positive subjects (20.20%) was significantly higher than that in the HIV-1-negative subjects (3.42%, OR 5.89). These results indicated that HIV-1 acquisition was independent of the presence of HBV, HCV and GBV-C viraemia, but was highly associated with the presence of syphilis in the MSM population in Beijing.

**Occurrence of GBV-C viraemia was increased after HIV-1 acquisition**

During follow-up studies, the occurrence of GBV-C viraemia in the 99 HIV-1-positive subjects increased from 17.7% prior to HIV-1 acquisition (visit 1) to 27.2% at the time of HIV-1 acquisition (visit 2) (P = 0.09). After 12 months (visit 3), GBV-C RNA
was still positive in the 27 subjects that were positive at visit 2. Furthermore, an additional seven subjects had acquired GBV-C viraemia at visit 3, leading to a significant increase of GBV-C viraemia occurrence compared to visit 1 (34.3%, \( P < 0.01 \)). However, for the HIV-negative subjects, newly acquired GBV-C viraemia was not observed after 12 months. The occurrence of GBV-C viraemia was significantly higher in the HIV-1-positive subjects than in the HIV-1-negative subjects at visits 2 and 3, respectively (Fig. 1). For HIV-1-positive subjects at visit 2, the distribution of GBV-C RNA (27.2%) was similar with respect to age, HBV, HCV, syphilis co-infection and HIV-1 subtypes. The baseline clinical characteristics of 99 MSM with HIV-1 infections are given in Table 2.

### Table 2. Baseline clinical characteristics of 99 HIV-positive MSM with or without GBV-C infection

<table>
<thead>
<tr>
<th>GBV-C viraemia status</th>
<th>GBV-C positive (n = 27)</th>
<th>GBV-C negative (n = 72)</th>
<th>( P )</th>
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<tbody>
<tr>
<td>Median age (years)</td>
<td>25 (24–31)</td>
<td>30 (24–39)</td>
<td>0.064*</td>
</tr>
<tr>
<td>Median days of HIV infection</td>
<td>30 (17–30)</td>
<td>30 (28–46)</td>
<td>0.11*</td>
</tr>
<tr>
<td>HIV subtype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRF01-AE</td>
<td>15/27 (55.6%)</td>
<td>36/72 (50%)</td>
<td>0.62†</td>
</tr>
<tr>
<td>B</td>
<td>9/27 (33.3%)</td>
<td>21/72 (29.2%)</td>
<td>0.69†</td>
</tr>
<tr>
<td>B/C</td>
<td>3/27 (11.1%)</td>
<td>7/72 (9.7%)</td>
<td>1†</td>
</tr>
<tr>
<td>C</td>
<td>0</td>
<td>2/72 (1.4%)</td>
<td>1†</td>
</tr>
<tr>
<td>Not detected</td>
<td>0</td>
<td>6/72 (8.3%)</td>
<td></td>
</tr>
<tr>
<td>HBsAg positive</td>
<td>1/27 (3.7%)</td>
<td>1/72 (1.39%)</td>
<td>0.47†</td>
</tr>
<tr>
<td>Anti-HCV positive</td>
<td>0</td>
<td>1/72 (1.39%)</td>
<td>1†</td>
</tr>
<tr>
<td>Syphilis</td>
<td>7/27 (25.9%)</td>
<td>13/72 (18.1%)</td>
<td>0.385†</td>
</tr>
<tr>
<td>Median (IQR 25–75%)</td>
<td>4.29 (3.69–5.51)</td>
<td>4.83 (3.97–5.58)</td>
<td>0.31*</td>
</tr>
<tr>
<td>(log_{10} copies/ml)</td>
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<td></td>
</tr>
<tr>
<td>Median (IQR 25–75%)</td>
<td>423 (361–587)</td>
<td>506 (334–602)</td>
<td>0.46*</td>
</tr>
<tr>
<td>CD4 counts (cells/mm^3)</td>
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MSM, Men who have sex with men; IQR, interquartile range.
* Mann–Whitney test.
† Two-sided Pearson’s \( x^2 \) test.

![Fig. 1. The occurrence of GBV-C viraemia in subjects at different stages of HIV infection (n = 99) and HIV-negative subjects (n = 175). The x-axis represents the time during which the samples were collected. The y-axis represents the percentage of GBV-C viraemia. \( P \) values represent the statistical analysis between each comparison as shown in the figure. Compared by Pearson’s \( x^2 \) test, the significance level was set at 0.05. Visit 1: before HIV infection; visit 2: near HIV infection; visit 3: 12 months post-HIV infection.](image-url)
Following the determination of GBV-C prevalence in 99 HIV-1-positive and 175 HIV-1-negative subjects, the nucleotide sequences of PCR products were analysed to determine the GBV-C genotypes. Reference sequences of four genotypes (1, 2, 3, 5) were included in this analysis. Of the 34 samples from HIV-1-positive subjects, 30 (88.2%) were GBV-C genotype 3, while the remaining four (11.8%) were genotype 2 according to comparison with the reference strains (Fig. 2). Of the 22 samples from HIV-1-negative subjects, one (4.5%) sample was genotype 2, and 21 (95.5%) samples belonged to genotype 3. The genetic distances were small between the viruses from HIV-1-positive subjects and those from HIV-1-negative subjects (Figs 2, 3). These results indicated that the majority of GBV-C in MSM in Beijing belonged to genotype 3. During HIV-1 infection, both genotypes 2 and 3 were transmissible.
GBV-C viral loads at the acute and chronic stages of HIV-1 infection

The GBV-C viral load in each of 26 HIV-1-positive subjects [one case started antiretroviral therapy (ART) 3 months after HIV-1 acquisition in the follow-up study and was excluded from the analysis] at two time points (acute and chronic stages) of HIV infection were quantified with a real-time PCR. The GBV-C viral loads at the acute stage of HIV-1 infection ranged from $1.62 \times 10^3$ to $2.82 \times 10^6$ copies/ml with a median of $5.24 \log$ copies/ml. At 12 months post-HIV-1 acquisition (chronic stage), the viral loads ranged from $3.24 \times 10^3$ to $3.80 \times 10^6$ copies/ml with a median of $5.56 \log$ copies/ml. No significant difference was found between the GBV-C viral

Fig. 3. Phylogenetic tree of 22 GBV-C strains from 22 HIV-1-negative MSM subjects (indicated by NG prefix) based on the sequences of the 5'-UTR gene (300 bp). The phylogenetic tree was constructed by the neighbour-joining method and the evolutionary distances were computed using the maximum composite likelihood method of Mega software (version 5.0). Reference strains of genotypes 1 (U36380), 2 (U44402 and AF081782 from China), 3 (D90601) and 5 (AY949711) were obtained from GenBank. The scale bar represents 0.5% genetic distance (0.005 substitution per site).
Comparison of HIV-1 viral loads and CD4+ T-cell counts between subjects with and without GBV-C infection

To evaluate the potential effect of GBV-C co-infection on the replication of HIV-1, we analysed the HIV viral loads and CD4+ T-cell counts in the GBV-C-positive and -negative subjects at the acute and chronic stages of HIV-1 infection. In the GBV-C RNA-positive subjects, the median HIV viral load was 4.29 log copies/ml (IQR 3.69–5.51), and the median CD4+ T-cell count was 423 cells/mm$^3$ (IQR 361–587) at the acute stage. In the GBV-C RNA-negative subjects, the median viral load was 4.81 log copies/ml (IQR 4.00–5.57), and the median CD4+ T-cell count was 502 cells/mm$^3$ (IQR 327–622). Statistical analysis showed that both CD4+ T-cell counts and HIV-1 viral load were not significantly different between the two groups at the acute stage of HIV-1 infection ($P=0.34$, $P=0.5$, respectively) (Table 2).

In the follow-up study, one case in the GBV-C-positive group and two cases in the GBV-C-negative group started ART 3 months after acquiring HIV-1. Seven cases in the GBV-C-negative group acquired GBV-C viraemia 12 months post-HIV-1 acquisition. Thus, the case numbers in the GBV-C-positive and -negative groups became 26 and 63, respectively. The median of HIV-1 viral load for 26 GBV-C-positive subjects and 63 GBV-C-negative subjects was 4.65 log$_{10}$ copies/ml (IQR 3.86–5.03) and 4.41 log$_{10}$ copies/ml (IQR 3.99–4.97), respectively, at 12 months post-HIV-1 acquisition. Statistical analysis showed that HIV-1 viral load was not significantly different between these two groups ($P=0.47$). The median CD4+ T-cell counts were 431 cells/mm$^3$ (IQR 281–542) and 435 cells/mm$^3$ (IQR 313–613) in the GBV-C positive and negative groups, respectively. Statistical analysis showed that there was no significant difference between CD4+ T-cell counts in the GBV-C-positive and GBV-C-negative subjects ($P=0.31$).

We then conducted a correlation analysis between GBV-C viral load and HIV-1 viral load or CD4+ T-cell count at the acute or chronic stages of HIV-1 infection. At the acute stage, a significant positive correlation was observed between the GBV-C viral load and CD4+ T-cell count ($r=0.42$, $P=0.03$) (Fig. 5). HIV-1 viral load was inversely and not significantly correlated with the GBV-C viral load ($r=-0.17$, $P=0.39$). At the chronic stage (12 months post-HIV-1 acquisition), we performed the same analyses excluding the ART cases, and found a persistent positive correlation between GBV-C viral loads and CD4+ T-cell counts at this time point, but
the correlation was not significant ($r = 0.09, P = 0.67$) (Fig. 6). There was a persistent negative, but not significant, correlation between GBV-C viral load and HIV-1 viral load ($r = 0.2, P = 0.34$) (data not shown) at the chronic stage.

**DISCUSSION**

Previous studies have shown that GBV-C is prevalent in haemodialysis patients, commercial blood donors [24], intravenous drug users [25] and female commercial sex workers [26] in China. To our knowledge, the current study is the first to describe the prevalence of GBV-C viraemia in the MSM population with or without HIV-1 infection in China. The positive rate of GBV-C viraemia in subjects with HIV-1 infections was 27.2% (27/99) at the time of HIV-1 acquisition, which was much higher than that in the HIV-1-negative subjects ($P = 0.002$). These results are consistent with previous reports showing that the prevalence of GBV-C viraemia in HIV-1-infected individuals at 12 months post-HIV-1 infection, resulting in an incidence density of 9.7/100 person-years. None of the subjects acquired new GBV-C viraemia in the HIV-negative group during this period, indicating the high incidence density of GBV-C viraemia in the MSM populations with HIV infection. In our study, the occurrence of syphilis was high in HIV-1-positive subjects. This result was in accord with a previous cross-sectional study on MSM in Beijing [21]. In addition, we did not find a high prevalence of HCV and HBV in MSM with or without HIV-1 infections.

According to some reports of the past two decades, the major genotype of GBV-C is genotype 3. Few virus strains of genotypes 2 and 1 have been reported in different populations in China [31, 32]. Genotypic analysis in this study showed that MSM subjects were mainly infected with the genotype 3 strain of GBV-C (88.2%). Only four subjects were infected with genotype 2 strain and no subjects were infected with genotype 1 strain. Therefore, we concluded that the major GBV-C genotype prevalent in the MSM population was similar to that in other populations in China. Our results also agree with the viral genotype prevalent in other Asian countries [32, 34]. We attempted to identify if the genotypes had any effect on the viral loads of GBV-C. Our results showed that there was no significant relationship between the genotype and viral loads of GBV-C (data not shown). This differs from a previous report [34] showing that GBV-C viral loads were associated with genotype. This difference may be due to the small numbers of genotype 2 in our study or differences in the progress of HIV-1 within the study population.

Previous studies have shown that GBV-C replication in peripheral blood mononuclear cells decreases
the expression of HIV-1 co-receptors (chemokine receptors CCR5 and CXCR4) on the surface of CD4+ T cells and increases the production of various chemokines that serve as competitive inhibitors of the HIV-1 co-receptors [18, 35, 36]. It is known that there is vigorous viral replication at the acute stage during the natural history of HIV-1 infection. Thus, we speculate that there are some complicated interactions between the two viruses at the acute stage of HIV-1 infection. Our results showed that the GBV-C viral loads had an ascending trend from the acute (5.24 log copies/ml) to chronic (5.56 log copies/ml) stage (Fig. 4). Statistical analysis showed a sustained negative correlation between the GBV-C viral loads and HIV-1 viral loads in the acute and chronic stages of HIV-1 infection. Although, we were unable to conclude that the lower GBV-C viral loads at the acute stage of HIV-1 infection was directly caused by higher HIV-1 viral loads, our results provide some evidence that an inhibitory interaction between these two viruses might exist during the period of co-infection.

Previous studies have suggested that HIV RNA levels at later time points are better indicators of long-term disease progression compared to the levels when HIV-1 was acquired because the viral load reaches a stable mean or ‘set point’ around 1 year post-infection [37, 38]. In this study, both plasma HIV RNA and the CD4+ T-cell counts did not differ between GBV-C-positive and GBV-C-negative subjects at 1 year post-HIV infection. These results indicate that GBV-C viraemia could not increase CD4+ T-cell counts or reduce HIV-1 viral load during the 1-year period following HIV-1 infection. These results also suggest that there was no significant suppressive effect by GBV-C on HIV-1 replication in the year following HIV-1 infection. Williams et al. reported that GBV-C viraemia was significantly associated with the prolonged survival of HIV-positive men 5–6 years post-HIV-1 infection. However, the survival rate was not increased 12–18 months post-HIV infection [39]. Thus, a long-term follow-up study should be conducted on this cohort.

The molecular mechanisms by which GBV-C suppresses the replication of HIV-1 are poorly understood and the effect of GBV-C viraemia on the progress of HIV disease is currently controversial. Several studies have shown that GBV-C viraemia could assist the elevation of CD4+ T-cell counts because a positive correlation between GBV-C viral load and CD4+ T-cell counts was observed [40, 41]. Interestingly, in our study, a significant positive correlation between CD4+ T-cell counts and GBV-C viral loads ($r=0.42$, $P=0.03$) (Fig. 5) was observed at the acute stage of HIV-1 infection after 1 year, and the correlation was still positive but no longer significant ($r=0.09$, $P=0.67$) (Fig. 6). Because GBV-C RNA can replicate in CD4+ T cells, the decrease of CD4+ T cells during the course of HIV-1 infection implies a loss of target cells for GBV-C RNA. This might explain why the correlation between CD4+ T cells and GBV-C viral loads was weakened 1 year post-HIV infection as a consequence of a decrease in CD4+ T-cell counts. Our study can only reflect on the instance of male homosexuals who sexually transmit GBV-C, and does not include bloodborne transmission of HIV-1. In addition, the period of the current study is not long enough to observe the loss of GBV-C viraemia and the effect of sustained GBV-C viraemia on the progress of HIV/AIDS. In conclusion, this is the first cohort study, to the best of our knowledge, on GBV-C co-infection with HIV-1 in the MSM population of Beijing, China. Long-term observation is needed on this cohort to further reveal the correlation between GBV-C and HIV infection.

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DECLARATION OF INTEREST

None.

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