Plasma Epstein-Barr viral load measurement as a diagnostic marker of lymphoma in HIV-infected patients

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ABSTRACT

Background and objectives: To assess the use of the Epstein-Barr virus (EBV) viral load as a marker for lymphoma diagnosis in HIV-infected patients. We also aimed to identify the relationship between EBV viral load in plasma and the presence of EBV in lymphoma cells.

Patients and methods: Retrospective observational study of two HIV-infected populations: one of patients diagnosed with lymphoma and a control group. Thirty-nine patients with AIDS-related lymphoma (ARL) (32 non-Hodgkin's and 7 Hodgkin's lymphomas) and 134 HIV-positive individuals without neoplasia or opportunistic infections were studied. Blood samples were collected before lymphoma treatment in ARL patients. EBV viral load was measured in plasma by real-time quantitative PCR and the presence of EBV-EBER mRNA in lymphoma tumor was investigated by in situ hybridization.

Results: Patients with ARL had higher EBV viral loads than those without lymphoma: 24,180.5 (±773,387.6) copies/mL versus 2.6 (±21.6) copies/mL (p < 0.001). HIV-infected patients without lymphoma had negative or very low EBV load values. Among ARL patients, no correlation was found between EBV viral loads and CD4+ lymphocyte counts or between EBV and HIV RNA loads, or any other clinical or biological parameter. Cases with an EBV-EBER-positive lymphoma had higher EBV viral loads than those with EBER-negative tumors.

Conclusions: EBV viral load is a useful marker of lymphoma in HIV-infected patients, and may be a useful tool for early diagnosis and treatment.

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RESUMEN

Carga viral del virus de Epstein-Barr como marcador diagnóstico de linfoma en pacientes infectados por el VIH

Palabras clave:
VIH
Virus de Epstein-Barr
Carga viral
Linfoma no hodgkiniano
Linfoma de Hodgkin

Fundamento y objetivo: Evaluar el uso de la carga viral del virus de Epstein-Barr (VEB) como marcador para el diagnóstico de linfomas en pacientes infectados por el VIH. Identificar la relación entre la carga viral de VEB en plasma y la presencia del virus en las células del linfoma.

Pacientes y método: Estudio observacional retrospectivo de dos poblaciones de pacientes VIH: una de pacientes diagnosticados de linfoma y un grupo control. Se estudiaron 39 pacientes con linfoma asociado a infección por el VIH (32 linfomas no hodgkinianos y 7 de Hodgkin) y 134 individuos con infección por el VIH sin neoplasia ni infecciones oportunistas. Las muestras de plasma de los pacientes con linfoma fueron obtenidas en el momento del diagnóstico. La carga viral en plasma del VEB fue realizada mediante una PCR cuantitativa en tiempo real y la presencia de RNA VEB-EBER en los tumores fue investigada por hibridación in situ.

Resultados: Los pacientes con linfoma asociado a infección por el VIH tenían cargas virales de VEB más elevadas que los pacientes sin linfoma: 24.180,5 (±73.387,6) copias/mL frente a 26 (±21,6) copias/mL (p < 0,001). Los pacientes con infección por el VIH sin linfoma presentaron carga viral muy baja o negativa. En los pacientes con linfoma no se halló correlación entre la carga viral del VEB y el recuento de linfocitos...
Introduction

The mechanisms of lymphomagenesis of AIDS-related lymphomas (ARL) are still not completely known, but a number of factors have been identified: infection by transforming agents such as some herpesvirus, oncogenes or suppressor genes, and persistent B-cell stimulation by high levels of certain cytokines.\textsuperscript{1} The Epstein-Barr virus (EBV) is a human lymphotropic herpesvirus involved in the pathogenesis of lymphoma. It persists in latent form inside B lymphocytes as episomes in the cell nucleus and replicates during cellular division. In HIV-infected patients, the profoundly defective response of cytotoxic T lymphocytes may reactivation EBV infection, leading to uncontrolled lymphoproliferation and favouring the development of lymphoma.\textsuperscript{2,3} However, the precise role of EBV in ARL lymphomagenesis and its preference for specific anatomic sites and association to specific histopathology remain unclear.

The presence of EBV has been demonstrated in tumor cells from both non-Hodgkin’s lymphoma (NHL) and Hodgkin’s lymphoma (HL). The EBV genome can be detected in 60% of ARL cases, depending on the histological subtype of the lymphoma and its localization.\textsuperscript{5} HIV-related HL is almost always associated with EBV.\textsuperscript{5} Approximately 30–50% of AIDS-related systemic diffuse large B-cell lymphomas (DLBCL) contain EBV. In contrast, almost all cases of AIDS-related primary central nervous system lymphomas (PCL) contain EBV.\textsuperscript{6} Therefore, the pathogenesis of AIDS-related systemic NHL and PCL may be different. In Burkitt’s lymphoma, EBV is present in around 30% of cases, and lymphomagenesis includes, in addition to EBV infection, activation of the C-MYC gene and inactivation of P53.\textsuperscript{7}

Titration of EBV antibodies against early and nuclear EBV antigen is not a useful diagnostic tool to detect virus reactivation of EBV-related diseases in immunosuppressed individuals (e.g., transplant recipients, AIDS patients) because of the insufficient humoral response in these patients. Thus, simple unquantified molecular detection of EBV is poor, while quantification of plasma EBV DNA is a useful tool to detect EBV-associated diseases in immunosuppressed patients.\textsuperscript{8} The EBV virus apparently plays a role in ARL lymphomagenesis and EBV viral load might be elevated among HIV-infected patients concurrently developing lymphoma. Furthermore, high EBV viral loads may be associated with the presence of EBV in malignant cells. A few studies have been carried out to explore EBV viral loads in patients with ARL.\textsuperscript{9–11} Several investigators have shown that a rapid increase of EBV viral loads in peripheral blood or plasma is predictive of lymphoproliferative disorders in transplant recipients, nasopharyngeal carcinoma and other malignancies.\textsuperscript{9,12} Moreover, the therapeutic efficacy has been monitored from sequential measurements of EBV viral load in these disorders.\textsuperscript{13,14} From a technical standpoint, EBV viral load assays have been shown to be sensitive and specific. New commercially available kits based on real-time PCR technology are less time-consuming and reduce the risk of contamination by amplicons.\textsuperscript{15,16} These kits allow the sequential measurement of EBV viral loads on a high number of samples and patients.

The aim of this study was to assess the usefulness of EBV viral load as a marker for lymphoma diagnosis. EBV viral loads were compared in HIV-infected patients with and without lymphoma. We also identified the relationships between EBV viral loads and presence of virus in the tumor cells.

Methods

Patients

This retrospective study included 173 adult individuals coinfected with HIV and EBV (positive for VCA IgG antibodies).

Between 1998 and 2006, forty-nine consecutive cases of ARL (38 NHL and 11 HL) were diagnosed in a single institution. Ten ARL patients were excluded from the study because no plasma frozen samples were available. Thus, thirty-nine patients with ARL were included (32 NHL and 7 HL) in this study. The number of males and females was 28 (72%) and 11 (28%), respectively, and mean (SD) age was 40 (8 years). Twenty-five out of 32 NHL patients and 7 with HL were on highly active antiretroviral therapy (HAART) at the time of lymphoma diagnosis. The median duration of HAART was 45 months [range, 9–109]. Lymphoma diagnosis was performed by histological study of tissue biopsies. The following clinical and biological variables were measured: age, sex, risk activity, HAART, CD4 lymphocyte count, HIV viral load, histologic subtype of lymphoma, B symptoms and serum LDH. Following the guidelines of the Centers for Disease Control and Prevention (CDC), 4 ARL patients were classified as being in stage A2, 2 as in stage A3, 6 as in stage B3, 4 as in stage C2 and 12 as in stage C3.

A control group of 134 HIV-infected individuals without neoplasia or opportunistic infections was collected from the outpatient population of the HIV Unit of the Hospital Germans Trias i Pujol. Control cases were selected according to age, clinical and immunological status. There were 94 males (70.1%) and 40 females (29.9%), and the mean age was 43.6 years. From this population, 25 had a CD4 count ≤200/µL (24 on HAART); 54 had a CD4 count between 200 and 500/µL (25 on HAART) and 55 had a CD4 count ≥500/µL (25 on HAART).

In this study, we followed the rules of good clinical practice of the Germans Trias i Pujol University Hospital.

Biological samples

A specimen of blood with EDTA was collected after consent from all patients and controls included in the study. The specimens from ARL patients utilized for the current study were originally collected after the diagnosis of lymphoma and before starting the lymphoma treatment. The plasma samples of the control group were obtained at the time of routine clinical HIV follow-up visits, and were retrospectively assayed for EBV viral load. Plasma was separated by centrifugation and stored at −80 °C until processing. Tissue samples were routinely fixed, paraffin-embedded, and cut into sections (5–6 µm thick).

EBV detection in tissue samples

The EBV-encoded RNA (EBER) in the tumor was studied in 17 of 39 lymphoma cases. The EBER was detected by in situ hybridization with the INFORM EBER PROBE assay (Ventana Medical Systems, Tucson, USA) and the ISH VIEWBLUE detection system (Ventana Medical Systems) in a Benchmark LT automated instrument (Ventana Medical Systems).
EBV viral load

A total of 173 plasma samples of HIV-infected patients were analyzed for EBV viral load. DNA was extracted from 200 µL of plasma using the QIAamp® DNA mini kit (Qiagen, Hilden, Germany) in accordance with the manufacturer’s instructions, eluting the extracted DNA in 60 µL of buffer. Five µL were used for amplification. An internal control was added to the specimen to check extraction efficiency and the presence of PCR inhibitors.

EBV viral loads were determined by a commercial real-time PCR technique (RealArt EBV LC PCR kit, Artus, Hamburg, Germany). Amplification and detection were performed on the LightCycler instrument (Roche Diagnostics, Mannheim, Germany). The RealArt EBV LC PCR kit amplifies a 97-bp region of the EBV genome as well as the internal control. Amplified products were detected with specific fluorescence resonance energy transfer (FRET) hybridization probes. Four quantification standards and a negative control were included in each run. Copy numbers were detected with specific fluorescence resonance energy transfer (FRET) hybridization probes. Four quantification standards and a negative control were included in each run. Copy numbers per µL of plasma were calculated by the instrument from the generated standard curve. The detection limit of this assay is 5.78 copies/µL.

CD4+ lymphocyte counts and HIV-1 RNA load

CD4+ lymphocytes were counted with a FACSCalibur flow cytometer using Cell Quest software (Becton Dickinson, California, USA). Nucleic acids were extracted from 1 mL of plasma with a silica-based method as described by Boom et al. using the automated extraction instrument NucliSens® Easy Mag® (bioMérieux, Boxtel, The Netherlands). HIV-1 RNA viral load was quantified using a real-time NASBA method (NucliSens EasyQ®) in accordance with the manufacturer’s instructions.

Statistical analysis

A descriptive analysis of all variables was performed. EBV viral loads were expressed as mean number of copies/mL (standard deviation, SD). Bivariate tests (Student’s t-test, Mann–Whitney U-test and variance analysis when appropriate) were used to compare quantitative variables, and the chi-square test was employed to compare categorical variables. The Kruskal–Wallis H-test and the Mann–Whitney U-test (both non-parametric) were employed to calculate differences between mean EBV viral loads in HIV-infected patients with and without lymphoma. Survival probabilities were calculated according to the Kaplan–Meier method. The level of significance for all statistical tests was 0.05. Statistical analyses were performed using SPSS package software, version 15.

Results

The main clinical and biological characteristics of the ARL patients are listed in Table 1. EBV viral loads were undetectable in 11 out of 28 systemic NHL, 3 out of 4 PCL and 1 out of 7 HL cases. In the control group, 125 out of 134 had undetectable plasma EBV viral loads, and 9 showed low levels, with a median of 0.39 copies/mL [range 0.19–238.6]. EBV viral loads did not differ significantly between patients with PCL and those with systemic NHL. Table 2 shows EBV viral loads for HIV-infected patients with lymphoma and control groups of HIV-positive individuals without lymphoma. Patients with lymphoma had significantly higher EBV viral loads than patients without lymphoma (24180.5 [73387.6 versus 2.6 [21.6]). EBV viral loads were significantly different between these two groups regardless of the CD4 count control group. No correlations were found between plasma EBV viral load and CD4+ lymphocyte count at lymphoma diagnosis, between EBV viral load and HIV RNA load, nor between EBV viral load and any other clinical or biological variable. No differences were found between EBV viral loads of the 3 main lymphoma histological subtypes (DLBCL, Burkitt’s lymphoma and HL) (Table 3). Moreover, there were no differences between patients who were on HAART at the time of lymphoma diagnosis (n = 32) and those who were not (n = 7) (means of 24824.4 [78130] copies/mL and 28192.8 [60156] copies/mL, respectively).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Clinical characteristics of patients with AIDS-related lymphoma</th>
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<tbody>
<tr>
<td></td>
<td>AIDS-related lymphoma (N=39)</td>
</tr>
<tr>
<td></td>
<td>Number of patients %</td>
</tr>
<tr>
<td>Risk activity for HIV infection</td>
<td></td>
</tr>
<tr>
<td>IVDU</td>
<td>22 56</td>
</tr>
<tr>
<td>Homosexual</td>
<td>11 28</td>
</tr>
<tr>
<td>Heterosexual</td>
<td>6 16</td>
</tr>
<tr>
<td>Presence of a previous AIDS-defining illness</td>
<td></td>
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<tr>
<td>(n=36)</td>
<td>18 50</td>
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<tr>
<td>HAART prior to lymphoma diagnosis</td>
<td>32 82</td>
</tr>
<tr>
<td>B symptoms</td>
<td>25 64</td>
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<tr>
<td>Histologic subtype</td>
<td></td>
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<tr>
<td>Non-Hodgkin’s lymphoma</td>
<td>32 82</td>
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<tr>
<td>Systemic</td>
<td></td>
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<tr>
<td>Diffuse large B-cell</td>
<td>14 46</td>
</tr>
<tr>
<td>Burkitt’s lymphoma</td>
<td>8 25</td>
</tr>
<tr>
<td>Plasmablastic</td>
<td>2 6.5</td>
</tr>
<tr>
<td>Primary effusion lymphoma</td>
<td>1 3</td>
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<tr>
<td>MALTLymphoma</td>
<td>1 3</td>
</tr>
<tr>
<td>Non-specified</td>
<td>2 6.5</td>
</tr>
<tr>
<td>PCL</td>
<td>4 10</td>
</tr>
<tr>
<td>Hodgkin’s lymphoma</td>
<td>7 18</td>
</tr>
<tr>
<td>Mixedcellularity</td>
<td>3 43</td>
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<tr>
<td>Nodular sclerosis</td>
<td>2 29</td>
</tr>
<tr>
<td>Lymphocyte depletion</td>
<td>1 14</td>
</tr>
<tr>
<td>Non-specified</td>
<td>1 14</td>
</tr>
<tr>
<td>Serum LDH &gt; 270 U/L</td>
<td>15 39</td>
</tr>
<tr>
<td>Undetectable HIV RNA load</td>
<td>4 (N=31) 13</td>
</tr>
<tr>
<td>CD4 lymphocytes/µL (n=37)</td>
<td></td>
</tr>
<tr>
<td>&lt; 200</td>
<td>25 26</td>
</tr>
<tr>
<td>200–500</td>
<td>11 30</td>
</tr>
<tr>
<td>&gt; 500</td>
<td>1 2</td>
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</tbody>
</table>

NIVDU: intravenous drug users; HAART: highly active antiretroviral therapy; MALT: mucosa-associated lymphoid tissue; LDH: lactate dehydrogenase (normal values of up to 270 U/L); PCL: primary cerebral lymphoma; SD: standard deviation.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Epstein-Barr virus loads in patients with AIDS-related lymphoma and HIV-infected controls</th>
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<tr>
<td></td>
<td>N</td>
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<td></td>
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<tr>
<td>Lymphoma patients</td>
<td></td>
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<tr>
<td>NHL+HL</td>
<td>39</td>
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<tr>
<td>HIV-infected controls</td>
<td></td>
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<tr>
<td>CD4 &lt; 200/µL</td>
<td>25</td>
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<tr>
<td>CD4 200–500/µL</td>
<td>54</td>
</tr>
<tr>
<td>CD4 &gt; 500/µL</td>
<td>55</td>
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* Comparison between lymphoma patients and the different groups of HIV-infected controls.
The presence of EBER in tumors was investigated in 17 lymphoma cases. EBER was positive in 6 cases (2 DLBCL, 3 Burkitt’s lymphoma and 1 plasmablastic), and negative in 11 (8 DLBCL, 2 Burkitt’s lymphoma and 1 plasmablastic). EBER-positive cases had significantly higher plasma EBV viral loads than EBER-negative cases (p = 0.022) (Table 4). All cases with undetectable EBV viral load (7 out of 17) were EBER-negative (p = 0.017).

The median follow-up of ARL patients was 63 months. There were no significant differences in survival between patients with EBV loads higher and lower than the median (347.1 copies/mL). However, EBV viral loads ≥ 1000 copies/mL was a factor negatively affecting overall survival (OS). Patients with EBV < 1000 copies/mL, when compared with those with EBV viral loads ≥ 1000 copies/mL, had a significantly higher OS: 51% [range 31–71] and 17% [range 0–38] 3-year OS probability, respectively (Fig. 1).

Discussion

We retrospectively studied the presence of EBV viral loads in plasma samples from ARL patients, obtained at the time of lymphoma diagnosis, and before administration of any lymphoma treatment. Given that EBV apparently plays a role in lymphomagenesis and is present in tumor cells from a subset of lymphomas, we expected to find higher plasma EBV viral loads in patients with ARL compared to controls. To rule out a possible effect of HIV infection increasing plasma EBV viral loads, we also analyzed a control group of HIV-infected patients selected from an outpatient clinic and with a different degree of immunosuppression as measured by the CD4+ lymphocyte count. The absence of any other neoplasia or opportunistic infection, such as tuberculosis, leishmaniasis or Mycobacterium avium-complex, was checked by revision of clinical records from both patients and controls.

Few studies have been published on EBV viral load testing ARL patients. Among ARL patients, who had either NHL or HL we found high plasma EBV viral loads. HIV-infected patients with lymphoma had higher plasma EBV viral loads at the time of lymphoma diagnosis than HIV-positive individuals without lymphoma, regardless of the CD4+ lymphocyte count, as also found by Fan et al. However, in another study by Bonnet et al., no significant differences were found in whole blood EBV viral loads between ARL-related NHL and HIV-infected patients without AIDS. In the latter study, an inverse correlation was found between CD4+ lymphocyte count and whole blood EBV viral load in patients with AIDS-related NHL.

Circulating EBV DNA can be measured on several types of clinical specimens, such as whole blood, isolated peripheral blood mononuclear cells (PBMCs), serum or plasma. In HIV-infected patients, the EBV viral load in PBMCs increases rapidly after HIV infection; thus, this type of sample is not recommended for diagnosing lymphoma. Whole blood has been used because it can be obtained readily and contains all blood compartments that may harbour EBV. However, serum and plasma samples allow automated extraction and better standardization and reproducibility of methods than samples of PBMCs or of whole blood. In lymphomas, epimorphic or naked (fragmented) EBV DNA derived from apoptotic tumor cells, passes into the bloodstream. Several studies have shown that plasma EBV viral loads correlate with the occurrence of HL and that viral loads are rapidly eliminated from circulation after treatment of tumors in immunocompetent patients. Additionally, some authors have suggested that plasma EBV viral load may be a useful marker for diagnosis of EBV-associated NHL in AIDS patients.

In immunosuppressed patients, EBV DNA in plasma derives from viruses replicating in the EBV-infected circulating B cells. The viral load is thus of viral particles and not of free EBV DNA. For the above reasons, in this study we utilized plasma as the more desirable clinical specimen to measure EBV viral load as a tool for early diagnosis of lymphoma in HIV-infected patients.

HIV infection is associated with numerous immunologic abnormalities, including qualitative and quantitative defects in the T-cell population. Consequently, HIV-infected individuals have selective impairment of immune surveillance against EBV-infected B cells. These cells are present in increased numbers in the peripheral blood and lymphoid tissues and may be responsible for small clonal B-cell expansions announcing malignant transformation. However, we could not confirm the presence of high peripheral blood EBV viral loads in patients with counts of 100–400 CD4+ lymphocytes/μL as reported by Telenti et al. In addition, altered immune functions of CD4+ and, particularly, of CD8+ T-cells, have been reported in HIV-infected individuals.

Moreover, prolonged immunosuppression and B-cell stimulation are other important factors associated with ARL development. These additional factors have not been investigated in this study. Immunodeficiency is not the only factor to consider, since ARL also occurs in patients with mild degree of immunosuppression. Other factors, such as HAART and HIV RNA loads, could also have an important impact on the risk of ARL.
The increase in the CD4+ lymphocyte counts induced by HAART is widely thought to be directly responsible for the fall in the incidence of PCL and Kaposi sarcoma. In contrast, some studies have shown that, although it has diminished, the incidence of NHL and HL has not changed dramatically since HAART introduction.26–28 These reports, together with the fact that PCL is a lymphoma restricted to the central nervous system that appears in severely immunosuppressed patients, suggest a unique pathogenesis for PCL. In our study, no relationship was found between either HIV RNA load or HAART administration and EBV viral load. This suggests that antiretrovirals that are active against HIV do not affect EBV. In contrast, Bonnet et al.29 showed that the use of HAART for at least 6 months is associated with a decreased risk of NHL, although patients with prolonged uncontrolled HIV RNA load had an increased risk. Bonnet et al.28 also found that hepatitis B and C coinfections and previous herpesvirus infection (herpes simplex virus, varicella zoster virus, cytomegalovirus or Kaposi sarcoma) were not associated with a risk for NHL.

We did not find differences in EBV viral load between PCL and systemic NHL, nor among the most frequent histological subtypes (HL, DLBCL and Burkitt’s lymphoma). Perhaps due to the small number of cases in our study, results failed to find differences in EBV viral loads related to lymphoma subtype. This comparison would have provided additional information to help explain existing differences in lymphomagenesis. Further studies are needed to determine the links between the presence or absence of circulating EBV and lymphomagenesis in HIV-infected patients.

We found, as the study of Fan et al.,11 that EBER-positive cases had higher EBV viral loads than EBER-negative cases. Furthermore, all cases with undetectable EBV viral loads were EBER-negative. In fact, EBV is present within tumor cells in approximately half of all AIDS-related NHLs, and in almost all AIDS-related HLs and PCLs. Our data suggest that circulating EBV could facilitate infection of lymphoid cells by EBV. This observation is consistent with the findings by Gandhi et al.20 and Lei et al.30 These authors found high plasma EBV viral loads in patients who had EBV in the lymphoma cells; meanwhile EBV viral loads were not detectable in EBV-negative HL.

A viral load ≥1000 copies/mL was a factor which negatively affected the OS. No associations were found between EBV viral loads and any clinical or biological parameters, including IPI and lymphoma stage. A few published studies of HIV populations with lymphomas who have assessed EBV viral loads, but none have correlated viral load with OS. Thus, comparison of our results with literature values was difficult. Some authors have found that patients with nasopharyngeal carcinoma or NK/T cell lymphomas who had EBV viral loads higher than 7.3 × 10^7 or 1500 copies/mL, respectively, were associated with a lower OS.19,21

In conclusion, we have identified high plasma EBV viral loads in HIV-infected patients with ARL. The results of this study show that a subgroup of ARL patients has EBV DNA circulating in blood. Independently of the lymphoma histology or localization, the presence of high amounts of EBV may play a role in the lymphomagenesis of a subset of lymphomas. These observations suggest that EBV viral loads measured in plasma could be a useful tool for the clinical diagnosis of NHL and HL in HIV-infected patients. Further studies are needed to establish cut-off values of plasma EBV viral loads, which could help achieve an early diagnosis of ARL and, consequently, lead to prompt lymphoma treatment.

Conflict of interest

The authors declare no conflicts of interest.

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