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**Prospective evaluation of blood Epstein Barr virus DNA load and antibody profile in
HIV-related non-Hodgkin lymphomas**

Running head: EBV biomarkers in AIDS-lymphomas

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1 **Introduction**

2

3 Although the incidence of non-Hodgkin lymphoma (NHL) has decreased since the advent of
4 combined antiretroviral therapy (cART), NHL is still the first to second most frequent AIDS-
5 defining cancer in developed countries and is the first or second cause of cancer-related death
6 in HIV-infected individuals [1–3]. Epstein-Barr virus (EBV) is an oncogenic virus associated
7 with 20 to 80% of AIDS-related lymphomas. This virus is found in tumor cells of HIV-
8 infected patients with lymphomas more frequently than in the same lymphoma subtypes in
9 HIV-negative patients. Only 10% of diffuse large B cell lymphomas (DLBCLs), the most
10 common type of NHL, are associated with EBV in the general population, whereas EBV-
11 associated DLBCL represents 40% of DLBCL in HIV-infected patients [4,5].

12 Recent studies have underlined the synergistic role of HIV and EBV in lymphomagenesis.
13 Beyond HIV-induced immunosuppression increasing the pool of EBV-infected cells, HIV
14 may indirectly contribute through the induction of chronic B-cell activation or more directly
15 via the interaction of HIV variant protein P17 with the EBV oncoprotein, latent membrane
16 protein-1 (LMP-1) [6]. EBV-driven B-cell immortalization is achieved through the concerted
17 action of various latency proteins, such as LMP-1, which are able to dysregulate cellular
18 pathways that control growth or survival [7]. Recent evidence also suggests that EBV proteins
19 produced during viral replication or lytic infection may have pathogenic relevance in
20 lymphomas in the context of underlying impairment of immune responses [8,9].

21 The EBV DNA load may have a prognostic significance in patients with hematological
22 malignancies, such as DLBCL, regardless of the tumor EBV status defined by conventional
23 methods [10–12]. Few reports have examined EBV DNA in peripheral blood as a predictive
24 or prognostic marker for HIV-related lymphoma [12–17]. Reports investigating plasma EBV

25 DNA in HIV-infected patients with Hodgkin Lymphoma (HL) have provided conflicting
26 results due to differences between study populations according to cART and HIV-related
27 immune deficiency [13–15,18]. Two studies concluded that a high pretreatment plasma EBV
28 DNA load in HIV-infected patients with NHL is a poor prognostic marker, but they focused
29 only on patients not treated with rituximab [12,14]. In the current context of improving
30 prognoses for HIV-infected patients with lymphoma [19], we aimed to evaluate EBV markers
31 in a recent prospective cohort of HIV-patients with NHL who benefited from optimal
32 management of HIV infection and lymphoma including wide administration of rituximab, and
33 investigated the prognostic value of these markers.

34

35 **Materials and Methods**

36

37 **Patients**

38 This is an ancillary study of the prospective French Cohort of HIV-related lymphomas
39 (French National Agency for Research on AIDS and Viral Hepatitis ANRS-CO16
40 LYMPHOVIR cohort) [20,21]. This cohort enrolled consecutive HIV-infected patients with
41 newly diagnosed non-Hodgkin lymphoma (NHL $n=111$) and classical HL ($n=69$) in 22
42 centers between 2008 and 2015. The histological and clinical characteristics and outcome of
43 patients with HIV-NHL have been described in a previous report [21]. The research protocol
44 was approved by an ethics committee (Comité de Protection des Personnes, Ile-de-France
45 VII) and written informed consent was obtained from each patient. This study was registered
46 in ClinicalTrials.gov (NCT01164436).

47 **EBV in-situ detection**

48 Patients with HIV-NHL were analyzed for the presence of EBV in the tumor cells. EBV RNA
49 was detected in paraffin sections using *in situ* hybridization with fluorescein isothiocyanate
50 (FITC)-labelled EBV-encoded small RNA (EBER) 1+2 specific oligonucleotides (DAKO),
51 according to the manufacturer's instructions. The expression of the EBV latent membrane
52 protein (LMP1) was tested with the monoclonal antibody CS1-4 (DAKO) using the
53 streptavidin-biotin peroxidase labelling method with the LSAB Kit (DAKO).

54 **Quantification of the peripheral blood EBV DNA load**

55 DNA was isolated from 200 μ L whole blood (WB) using the MagNAPure LC instrument
56 (DNA Isolation kit, Roche Diagnostics) or plasma using the NucliSENS easyMAG instrument
57 (Biomérieux). The extracted DNA was aliquoted and frozen at -80°C before use. EBV DNA
58 loads were measured using the EBV R-gene quantification kit (Biomérieux) on a LightCycler

59 480 instrument (Roche Diagnostics) and expressed as the number of EBV DNA copies per
60 milliliter of sample, as previously described [22]. Any detection of EBV DNA in plasma or
61 WB, regardless of the level, was considered positive.

62 **EBV serology**

63 EBV markers in serum specimens stored at -80°C were determined by ELISA and indirect
64 immunofluorescence (IF) assays, as previously described [23]. The following markers were
65 determined using ELISA kits and an automated BEP III System (Siemens): Anti-EBV IgG or
66 IgA (Enzygnost, Siemens), anti-EBNA IgG (Theradiag), and anti-EA(D) IgG antibodies
67 (Platelia, Biorad). Analyses were performed according to the manufacturers'
68 recommendations. IF assays were carried out using in-house slides for the detection of anti-
69 VCA IgG, anti-VCA IgA, anti-EA (D+R) IgG, and anti-EA IgA antibodies, as previously
70 described [37]. High anti-EBV IgG antibody levels or elevated titers of anti-VCA IgG and
71 anti-EA IgG antibodies were defined by values ≥ 520 AU/mL, $\geq 1:640$, and $\geq 1:80$,
72 respectively, in accordance with routine laboratory experience [24]. Positive controls for anti-
73 VCA IgA and anti-EA IgA antibodies consisted of sera from nasopharyngeal carcinoma
74 (NPC) patients. A reactivation profile was defined by high anti-EBV IgG titers by ELISA and
75 high anti-VCA IgG antibody titers by IF. If the results between the ELISA and the IF assay
76 were discordant, the reactivation profile was defined by high anti-EBV IgG antibody levels or
77 high anti-VCA IgG antibody titers combined with high anti-EA IgG antibody titers
78 determined by IF and ELISA.

79 **Statistical analysis**

80 Patients were selected based on the following criteria: diagnosis of NHL with at least one
81 follow-up within the cohort, no previous lymphoma history, no missing values for EBV DNA
82 load in WB or plasma at diagnosis, and no missing values for CD4 counts and HIV-1 RNA at

83 diagnosis. Furthermore, the following criteria were applied for the sub-analysis of the
84 comparison of EBV variables at diagnosis and the six-month follow-up: no disease
85 progression at six months and no missing values for EBV DNA load in WB or plasma at six
86 months. Continuous variables are expressed as medians and interquartile ranges (IQR) and
87 compared using the Wilcoxon signed-rank test or Mann-Whitney test, when appropriate.
88 Categorical variables are expressed as frequencies and percentages and were compared using
89 the McNemar or Fischer test, when appropriate.

90 Follow-up was defined from HL diagnosis to the last follow-up. Progression-free survival
91 (PFS) was measured from lymphoma diagnosis to progression, relapse, or death. The
92 probability of PFS was estimated using the Kaplan–Meier estimate and differences were
93 compared using the log-rank test. Univariate logistic regression was performed to identify
94 factors associated with a high EBV viral load in WB and plasma. Covariables considered in
95 this analysis were CD4 count, Ann-Arbor stage, HIV-1 RNA load, prior AIDS defining event,
96 histological subtype, in situ EBV detection, and EBV serological profile. Statistical tests were
97 two-tailed and $p \leq 0.05$ was considered statistically significant. All statistical analyses were
98 performed using SAS software version 9.3 (SAS Institute Inc., Cary, NC, USA).

99

100 **Results**

101

102 **Clinical characteristics of patients at baseline**

103 Of the 111 HIV-infected patients enrolled in the ANRS-CO16 LYMPHOVIR cohort with a
104 diagnosis of NHL between 2008 and 2015, the pretreatment EBV DNA load was assessed
105 both in WB and plasma for 76 (59 men and 17 women, median age: 49.5 years) (Table 1).
106 The median HIV-1 RNA viral load was 572.5 copies/mL (IQR, 40–99536.5) and median CD4
107 T-cell count at diagnosis 269/ μ L (IQR, 116–428). The histological distribution of NHL was

108 as follows: DLBCL (n = 40), Burkitt lymphoma (n = 12), plasmablastic lymphoma (n = 10),
109 marginal zone lymphoma (n = 4), primary effusion lymphoma (n = 3), post-transplant
110 lymphoproliferative disease (PTLD)-like (n = 2), and others (n = 5). Eighteen of 43 (42%)
111 tested cases for in-situ EBV were positive. The histological distribution of NHL according to
112 EBV in-situ detection is shown in supplementary Table 1.

113 **EBV DNA load in whole blood and plasma**

114 Among the 76 patients diagnosed with NHL, the median pretreatment WB and plasma EBV
115 DNA values were 3,970 (IQR, 269–14400) and 0 (0–377.5) viral copies/mL, respectively.
116 pretreatment WB EBV DNA positivity was associated with a positive plasma HIV-1 RNA
117 load (Relative Risk (RR), 4.42 [1.33; 14.72], p = 0.02) and an NHL histological subtype other
118 than DLBCL (p = 0.03). A multivariable analysis confirmed the association between WB
119 EBV DNA detection and HIV loads > 50 copies/mL at diagnosis (RR 3.46 [1.00; 12.01],
120 p=0.05) when taking into account in the analysis AIDS status, EBER in situ expression and
121 NHL subtype (data not shown). Pretreatment plasma EBV DNA positivity was associated
122 with EBV in-situ detection (RR 10.62 [2.38; 47.49], p = 0.002), but not WB EBV DNA
123 (Table 1). The median pretreatment WB and plasma EBV DNA values were 11,400 (IQR,
124 3290-47550) and 309 (IQR, 43-1987) for patients with EBV-positive tumors and 2,580 (IQR,
125 117-10300) and 0 (IQR, 0-260) for patients with EBV-negative tumors, respectively (p = 0.05
126 and p = 0.006). Focusing our analysis on the subgroup of patients with DLBCL (n = 40), WB
127 EBV DNA, but not plasma EBV DNA, correlated with HIV-1 RNA load (p = 0.05). However,
128 the detection of EBV DNA in WB or plasma before treatment was not associated with EBER
129 detection (p = 0.58 and p = 0.95, respectively) in this subgroup. Estimated two-year PFS did
130 not differ according to pretreatment WB EBV DNA: 82% (95% CI [72%;91%]) in patients
131 with detectable pretreatment EBV DNA in WB versus 67%, (95% CI [43%;91%]) among
132 those with undetectable pretreatment EBV DNA in WB or according to plasma EBV DNA:

133 76% (95% CI [62%;91%] among patients with detectable pretreatment EBV DNA in plasma
134 versus 81% (95% CI [69%;93%]) among those with undetectable pretreatment EBV DNA in
135 plasma (Fig. 1). Similarly, there was no association with this outcome among patients with
136 DLBCL (supplementary Fig. 1).

137 We evaluated how WB or plasma EBV DNA loads evolved in patients responding to
138 chemotherapy, by comparing the EBV DNA loads between the diagnosis and the six-month
139 follow-up for 53 patients in remission at six months (Table 2). The proportion of patients with
140 WB and plasma EBV DNA (+) significantly decreased from 81 to 23% ($p < 0.0001$), and
141 from 43 to 8% ($p < 0.0001$), respectively. The detection of EBV DNA in WB or plasma at six
142 months was not associated with PFS (data not shown).

143 **EBV serology**

144 All patients were positive for anti-EBV IgG and anti-VCA IgG antibodies at NHL diagnosis
145 and 57% had a reactivation serological profile. There was no significant association between a
146 reactivation serological profile and WB or plasma EBV DNA detection ($p = 0.39$ and 0.11 ,
147 respectively) (Table 1). The serological profile did not differ between patients with EBV-
148 negative tumors and those with EBV-positive tumors and was not associated with outcome
149 (data not shown). At six months, we noted a significant decrease in anti-EA IgG antibody titer
150 ($p = 0.01$) and a trend towards a decrease in anti-EBV IgG antibody levels ($p = 0.07$) (Table
151 2).

153 **Discussion**

154
155 Here, we investigated EBV biomarkers in a recent prospective cohort of HIV-patients
156 diagnosed with NHL. Diverse forms of EBV DNA can be detected in peripheral blood [25].

157 Latent cell-associated episomal EBV DNA is, released from benign, latently infected cells or
158 from EBV tumor cells. It can be measured in PBMCs or whole blood. In contrast, cell free
159 virions DNA are excreted in the setting of lytic, replicative infection DNA. It can be
160 measured in plasma or whole blood. These two forms of EBV DNA have been shown to
161 coexist in the peripheral blood of HIV patients with or without lymphomas [26–28]. Given
162 the potential clinical utility of studying cell-free and cell-associated specimens in HIV
163 patients with NHL [14,16], we explored EBV DNA both in plasma and WB specimens, these
164 compartments reflecting respectively, cell free EBV DNA only and both cell free and cell
165 associated EBV DNA. We report a frequency of plasma EBV DNA detection of 45% at
166 diagnosis, a value similar to those reported in recent studies carried out in HIV-patients with
167 NHL [12,14] and higher than values reported for HIV-negative patients with lymphoma (12-
168 16%) [14,29], HIV-patients without lymphoma (16%), and HIV-negative immunocompetent
169 subjects (3%) [30]. An analysis of these results according to EBV tumor status showed
170 higher plasma EBV DNA detection in EBV(+) NHL patients than EBV(-) NHL patients.
171 These results are consistent with those of previous studies on HIV-uninfected and HIV-
172 infected patients with EBV(+) lymphomas [14,30–33]. Plasma EBV DNA is considered to be
173 a more specific tumor marker for EBV(+) lymphomas than EBV DNA in WB or PBMC [30].
174 We show a frequency of WB EBV DNA detection (80%) similar to that reported in a study
175 examining peripheral blood mononuclear cell (PBMC) EBV DNA detection in HIV-NHL
176 patients [16]. The lack of association between detection of EBV DNA in the WB and EBV in
177 situ detection suggests EBV replication in non-tumor cells. We also observed that EBV DNA
178 detection in WB before treatment was associated with detectable HIV-1 RNA load. The link
179 between WB EBV DNA and HIV-1 RNA load has been previously described in HIV-infected
180 patients without lymphoma [34].

181 EBV DNA in peripheral blood has been investigated as a tumor marker in EBV-associated
182 lymphomas. Recent reports on HIV-associated HL suggest that a high pretreatment level of
183 plasma EBV DNA is a marker of poor prognosis, but we and others did not find such an
184 association [13–15,18]. The lack of prognostic significance of pretreatment WB or plasma
185 EBV DNA loads in our study contrasts with the results from two studies suggesting that
186 positive plasma EBV DNA load was a potential prognostic marker in HIV-NHL patients
187 [12,14]. The patients included in these studies were not treated with rituximab and one of the
188 studies included patients with pejorative markers of HIV infection and frequent
189 immunodeficiency (60% of the patients had a CD4 count $< 200/\mu\text{L}$ at diagnosis and only 30%
190 had undetectable HIV-1 RNA) [14]. Most of the patients included in our study received
191 rituximab, which is associated with improved outcomes for patients with HIV-associated
192 lymphoma [35]. Second, the patients showed differences in HIV-infection and immune-
193 deficiency markers, with more patients under cART or with undetectable HIV-1 RNA load
194 and CD4⁺ cells $> 200/\mu\text{L}$ in our study. The OS estimates found in these studies were $< 65\%$,
195 which is inferior to those recently reported for HIV-NHL patients [21,36]. Several studies
196 analyzed the outcomes of HIV-NHL patients according to EBV tumor status and showed that
197 the presence of EBV in the tumor had no prognostic value for HIV-patients with NHL [37,38].
198 These results are consistent with ours, which show that plasma EBV DNA load correlates
199 with EBV in situ status but has no prognostic significance in HIV-NHL. We also investigated
200 whether EBV DNA load in peripheral blood could be a marker for monitoring patients after
201 chemotherapy, as suggested by Muncunill et al. [14], and observed a dramatic decrease of
202 WB and plasma EBV DNA load at six months after the initiation of chemotherapy in patients
203 without relapse. However, we were unable to show that WB or plasma EBV DNA detection
204 at six months after chemotherapy is associated with a worse outcome due to the small number
205 of patients experiencing relapse in our study population.

206 A few studies have explored the antibody response to EBV in HIV-infected patients with
207 lymphoma. We found a high frequency (57%) of patients with a high level of IgG antibodies
208 directed against EBV lytic antigens but this reactivation serological profile was not associated
209 with outcome. High levels of anti-VCA/EA IgG antibodies have been observed in patients
210 with EBV(+) HL [39], HIV-infected patients without lymphoma [27,28], and HIV-infected
211 patients with lymphoma (HL and NHL) [15,40]. The relationship between such anti-EBV
212 antibodies and NHL risk in HIV-uninfected patients is still debated and may depend on the
213 NHL subtype and the EBV tumor status [41–43]. We observed a decline in antibody IgG
214 levels against lytic antigens in patients who were in remission six months after chemotherapy,
215 concomitantly with the decrease of EBV DNA load, supporting restauration of the anti-EBV
216 immune response and control of EBV replication after successful treatment.

217 The strength of our study was the inclusion of patients with HIV-NHL for whom the
218 management of HIV infection and lymphoma was optimal and associated with a good
219 survival. However, our study had several limitations, in particular the absence of controls.
220 Moreover the small number of unfavorable events may have limited the statistical power.

221 In conclusion, we found that plasma EBV DNA loads at diagnosis vary according to the EBV
222 tumor status of HIV-NHL patients and that WB EBV DNA loads correlate with HIV load.
223 Overall, our results do not support the hypothesis that blood EBV DNA and EBV serology are
224 prognostic markers for HIV-related lymphoma in the modern cART era. The significant
225 decrease of the EBV load in WB and plasma during the follow-up may suggest that these
226 markers could be useful for monitoring patients after chemotherapy deserving further
227 evaluations.

228

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232

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238 R LANCAR performed the statistical analysis.

239 M ALGARTE-GENIN, H HENDEL-CHAVEZ and Y TAOUFIK performed the data and
240 biological collection.

241 F BONNET, MC MEYOHAS, M PARTISANI, B MARCHOUD and N MOUNIER enrolled
242 the patients and collected clinical and biological data.

243 S PREVOT coordinated the pathological analysis.

244 A FILIPPOVA performed EBV analyses

245

246

247 **Conflicts of interest**

248 The authors declare no competing financial interests.

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Figure Legends

Figure 1. Progression-free survival of HIV-NHL patients according to pretreatment whole blood (A) or plasma (B) EBV DNA load. **A.** Two-year PFS of patients with detectable pretreatment EBV DNA in WB: 82%, 95% CI [72%;91%]. Two-year PFS of patients with undetectable pretreatment EBV DNA in WB: 67%, 95% CI [43%;91%]. Median follow-up, 48 months. **B.** Two-year PFS of patients with detectable pretreatment EBV DNA in plasma: 76%, 95% CI [62%;91%]. Two-year PFS of patients with undetectable pretreatment EBV DNA in plasma: 81%, 95% CI [69%;93%]. Median follow-up, 48 months.

Table 1: Univariate analysis of detectable pretreatment EBV DNA in plasma or whole blood in HIV-NHL patients

	N	Detectable			<i>p</i> -value	Detectable			<i>p</i> -value
		WB EBV DNA (n)	RR	95% CI		plasma EBV DNA (n)	RR	95% CI	
Age					0.74				0.51
Age			1				1		
Age+10			0.91	[0.53; 1.57]			0.87	[0.56; 1.34]	
Sex					0.66				0.74
Male	59	48	1			27	1		
Female	17	13	0.74	[0.20; 2.73]		7	0.83	[0.28; 2.48]	
Ann-Arbor stage					0.30				0.57
I-II	18	16	1			7	1		
III-IV	58	45	0.43	[0.09; 2.13]		27	1.37	[0.47; 4.03]	
Nadir CD4 count, /μL					0.56				0.29
≥ 100	41	34	1			16	1		
< 100	31	24	0.71	[0.22; 2.28]		16	1.67	[0.65; 4.28]	
CD4 count at diagnosis, /μL					0.14				0.82
≥ 200	48	36	1			21	1		
< 200	28	25	2.78	[0.71; 10.87]		13	1.11	[0.44; 2.84]	
HIV load at diagnosis, copies/mL					0.02^b				0.16
≤ 50	29	19	1			10	1		
> 50	47	42	4.42	[1.33; 14.72]		24	1.98	[0.76; 5.16]	
Prior AIDS events					0.15				0.23
No	48	41	1			24	1		
Yes	28	20	0.43	[0.14; 1.34]		10	0.56	[0.21; 1.45]	
DLBCL subtype					0.03^c				0.68
No ^a	36	33	1			17	1		
Yes	40	28	0.21	[0.05; 0.83]		17	0.83	[0.33; 2.04]	
<i>In situ</i> EBV detection					0.14				0.002
Negative	25	19	1			8	1		
Positive	18	17	5.37	[0.59; 49.22]		15	10.62	[2.38; 47.49]	
EBV serological profile at diagnosis					0.39				0.11

Normal	24	18	1		9	1	
Reactivation	32	27	1.80	[0.48; 6.79]	19	2.44	[0.82; 7.22]

Footnotes: EBV: Epstein-Barr Virus; WB: Whole Blood; CI: confidence interval; RR: risk ratio; NA: not applicable. ^a Others histological subtypes included Burkitt lymphoma (n=12), plasmablastic lymphoma (n=10), marginal zone lymphoma (n=4), primary effusion lymphoma (n=3), posttransplant lymphoproliferative disease-like (n=2), others (n=5).

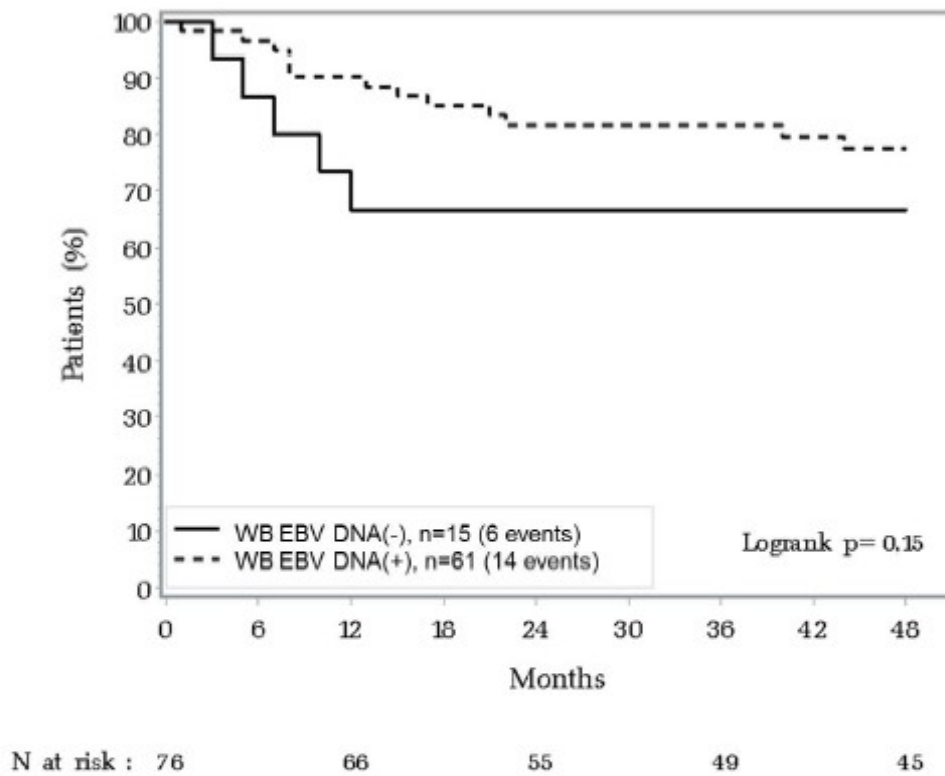
Table 2: Comparison of EBV DNA load and antibodies at diagnosis and 6 months after initiation of chemotherapy in patients in remission at 6 months

	Pretreatment (n=53)		Six-month follow-up (n=53)		<i>p</i> -value
	(%)	Median (IQR)	(%)	Median (IQR)	
WB EBV DNA					
Positive	43	81	12	23	<0.0001
> 4000 copies/mL	26	49	3	6	<0.0001
Values (copies /mL)		3970 (269–14400)		0 (0–0)	<0.0001
Plasma EBV DNA					
Positive	23	43	4	8	<0.0001
> 600 copies/mL	9	17	0	0	0.004
Values (copies/mL)		0 (0–342)		0 (0–0)	<0.0001
Anti-EBV antibodies					
Anti-EBV IgG, AU/mL		600 (330–1000)		570 (280–960)	0.07
Anti-VCA IgG, titer		640 (240–1280)		480 (240–940)	0.10
Anti-EA IgG , titer		80 (40–320)		80 (40–160)	0.01

Footnotes: EBV: Epstein-Barr Virus. WB: Whole blood. EA: Early Antigen. VCA: Viral Capsid Antigen. IQR: interquartile range. *P*-values were determined by the McNemar test (categorical variables) or Wilcoxon signed-rank test (continuous variables).

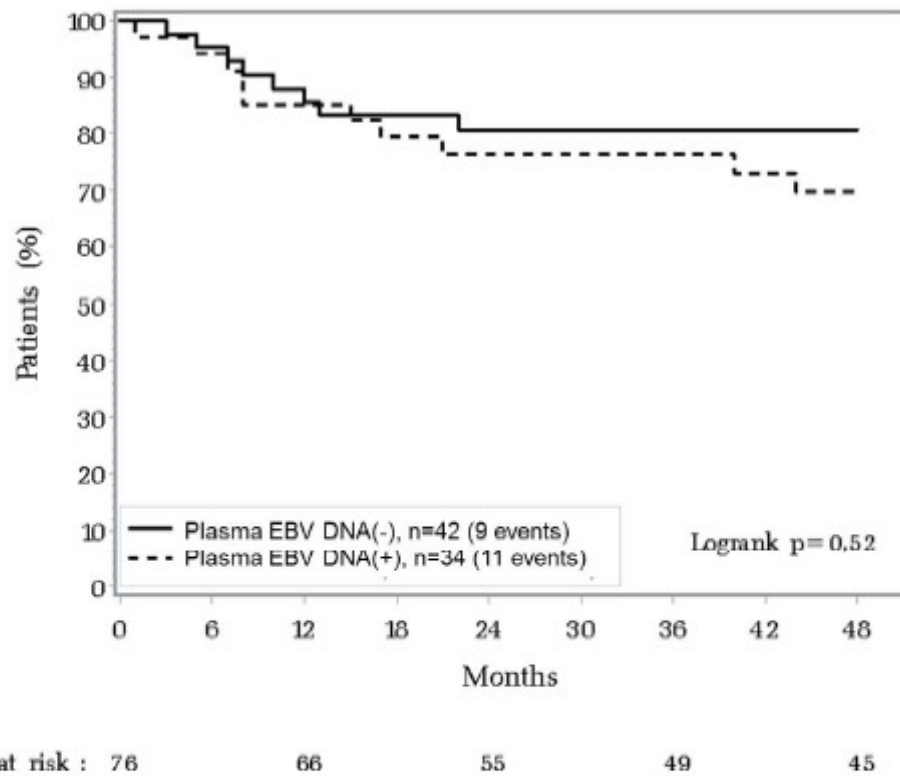
Figure 1: Progression-free survival of HIV-NHL patients according to pretreatment whole blood (A) or plasma (B) EBV DNA load.

A



Two-year progression-free survival of patients with detectable pretreatment EBV DNA in whole blood: 82%; 95% CI [72%;91%]. Two-year progression-free survival of patients with undetectable pretreatment EBV DNA in whole blood: 67%; 95% CI [43%;91%]. Median follow-up, 48 months.

B



Two-year progression-free survival of patients with detectable pretreatment EBV DNA in plasma: 76%; 95% CI [62%;91%]. Two-year progression-free survival of patients with undetectable pretreatment EBV DNA in plasma: 81%; 95% CI [69%;93%]. Median follow-up, 48 months.