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Keywords:	KSHV-DNA, HIV, immunodeficiency, homosexual relationship

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Table 1. Demographic, virological, and immunological characteristics of untreated HIV-seropositive patients according to the presence of KSHV DNA in PBMCs.

	All patients (n=481)	KSHV neg (n=407)	KSHV pos (n=74)	<i>p</i> value
Mean age in years ($\pm$ SD)	40.3 ($\pm$ 11.2)	41.7 ($\pm$ 11.7)	40.0 ($\pm$ 11.1)	0.26
Risk factor for HIV infection:				
*unprotected homosexual relationship, (%)	282 (64.4)	226 (61.4)	56 (80.0)	0.003
Patients with primary HIV infection, n. (%)	102 (21.2)	92 (22.6)	10 (13.7)	0.09
**Patients with B subtype (%)	401 (88.5)	339 (88.1)	62 (91.2)	0.45
Mean CD4+ cell count / μ l ($\pm$ SD)	449 ($\pm$ 288)	461 ($\pm$ 291)	389 ($\pm$ 264)	0.06
Patient CD4+ cell count, n (%)				0.08
<200 cells	87 (18.1)	70 (17.2)	18 (24.3)	0.025
200-350 cells	112 (23.3)	89 (21.9)	22 (29.7)	
>350 cells	282 (58.6)	248 (60.9)	34 (46.0)	
Percentage of CD4+ cells ($\pm$ SD)	22.1 ($\pm$ 10.6)	22.3 ($\pm$ 10.5)	21.2 ($\pm$ 10.6)	0.44
HIV RNA cp/ml ($\pm$ SD)	268061 ($\pm$ 872183)	280623 ($\pm$ 940158)	200150 ($\pm$ 304285)	0.50
HIV DNA cp x 10 ⁶ PBMCs ($\pm$ SD)	2893 ($\pm$ 4429)	2799 ($\pm$ 4483)	3340 ($\pm$ 4162)	0.38

* % of 438 evaluable patients

** % of 453 evaluable data

Table 2
Univariate analysis of 60 untreated HIV-seropositive patients with KSHV DNA
in PBMCs and for whom plasma samples were available/analysed

	All patients (n=60)	KSHV pl pos (n=33)	KSHV pl neg (n=27)	<i>P</i> value
Mean age in years (±SD)	42.1 (±11.5)	44.1 (±12.8)	39.2 (±8.8)	0.10
Risk factor for HIV infection: unprotected homosexual relationship (%)	47 (78)	29 (87.9)	18(66.6)	0.37
Patients with primary HIV infection, n. (%)	7 (11.6)	5 (15.1)	2 (7.4)	0.46
Patients with B subtype (%)	55 (91.6)	29 (87.8)	26 (96.3)	0.27
CD4+ cell count/µl (±SD)	404 (±267)	325 (±258)	513 (±244)	0.006
Patient CD4+ cell count, n. (%)				0.002
<200 cells	13 (21.7)	13 (39.3)	0 (0)	
200-350 cells	19 (31.7)	9 (27.2)	10 (37.0)	
>350 cells	28 (46.6)	11 (33.3)	17 (63.0)	0.01
Percentage of CD4+ cells (±SD)	22.1 (±10.4)	19.2 (±11.4)	25.8 (±7.7)	0.019
HIV RNA cp/ml (±SD)	197691 (±311073)	224658 (±313325)	162095 (±310812)	0.45
HIV DNA cp x 10 ⁶ PBMCs (±SD)	3446 (±4362)	3252 (±3786)	3786 (±5407)	0.67

Table 3 Multivariate analysis of untreated HIV-seropositive patients with KSHV DNA in PBMCs according to the presence of KSHV DNA in plasma

	p	OR	95% CI
Patients with age > median age	0.70	0.06	-0.24-0.35
Risk factor for HIV infection			
unprotected homosexual relationship	0.55	0.09	-0.22-0.40
CD4+ cell count/ml	0.03	0.001	<0.001-0.001
Patients with >median HIV RNA cp	0.28	-0.17	-0.48-0.14

**KSHV DNA viraemia correlates with low CD4+ cell count in Italian males
at the time of diagnosis of HIV infection**

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Short title: KSHV viraemia in patients infected with HIV

Abstract

To evaluate the relevance, virological and immunological markers of Kaposi sarcoma herpesvirus 8 (KSHV) viraemia in Italian male patients at the time of diagnosis of infection with HIV-1, 481 men infected with HIV were recruited consecutively. The presence of KSHV DNA was evaluated in peripheral blood mononuclear cells (PBMCs) and in plasma and correlated with demographic and viro-immunological parameters.

Seventy-four patients had KSHV DNA detected in PBMCs. By univariate analysis, the presence of KSHV DNA was associated significantly with unprotected homosexual relationships ($p=0.003$) and it was significantly higher in patients with $CD4^+$ cell <350 ($p=0.025$). By multivariate analysis, homosexual relationships were associated independently with KSHV DNA in PBMCs (OR: 3.25; 95% CI: 1.1-9.7; $p=0.035$). Among the 74 patients with KSHV DNA detected in PBMCs, plasma samples from 60 were analysed and 33 were positive for KSHV DNA. The $CD4^+$ cell counts and percentages were significantly lower in patients with KSHV DNA in both PBMCs and plasma as compared to patients with only KSHV DNA in PBMCs ($p=0.006$ and $p=0.019$, respectively). Among the patients with KSHV DNA detected in PBMCs, all 13 patients with $CD4^+$ cells count <200 had detectable levels of KSHV in their plasma. By multivariate analysis adjusted for the epidemiologic and virological parameters, low $CD4^+$ cell count was the only independent variable associated with the presence of KSHV DNA in plasma (OR, 0.001; 95% CI, <0.001 -0.001; $p=0.03$).

In HIV-positive antiretroviral therapy-naïve males, KSHV active replication as detected by KSHV DNA in plasma was associated significantly with low $CD4^+$ cell count.

Key words: KSHV DNA, HIV, immunodeficiency, homosexual relationship.

Introduction

Kaposi's Sarcoma-associated herpesvirus (KSHV) is linked causally to primary effusion lymphoma, multicentric Castleman disease, and all epidemiological forms of Kaposi's sarcoma (KS) (Boshoff and Weiss, 1998). The prevalence of KSHV varies between countries and populations. Variable routes of transmission, both sexual and non-sexual, have been described (Schulz & Moore, 1999; Hengge et al., 2002). In areas with low seroprevalence, KSHV appears to be acquired predominantly through sexual contact, and sex between men may be an important route of transmission (Schulz & Moore, 1999). Familial clustering of KSHV is observed frequently in endemic regions, where nasal secretions and saliva may be the sources of KSHV transmission (Plancoulaine et al., 2002; Andreoni et al., 1999; Rezza et al., 2000).

Variable seroprevalence of KSHV has been reported among subjects infected with HIV (Campbell et al., 1999; Laney et al., 2004)). In Italy, its overall prevalence in HIV-seropositive people in 1997-98 was demonstrated to be 31.4%, an increase from the 1987-88 prevalence of 14.6%, suggesting a spread of infection among HIV-seropositive subjects, and possibly from them to the HIV-negative community, by sexual and non-sexual routes (Parisi SG, Sarmati L et al., 2002).

HIV and KSHV are able to influence each other, and there is in vitro evidence that suggest the ability of one virus to facilitate the replication of the other. HIV activates lytic cycle replication of KSHV, and KSHV lytic antigens interact with HIV *Tat* to increase the cell's susceptibility to HIV infection (Zeng et al., 2007; Varthakavi et al., 2002). In vivo, HIV-1 infection has been associated with the presence of KSHV in any mucosal surface, and the presence of KSHV DNA in PBMCs is predictive of the development of KS in patients infected with HIV (Moore et al., 1996; Taylor et al., 2004, Engels et al., 2003). KSHV-DNA was more detected frequently in people infected with HIV and in the oropharynx and

1 peripheral blood of Ugandans with endemic and epidemic KS (Johnston et al., 2009). KSHV
2 DNA is detectable in KS tissues (Chang et al., 1994), in PBMCs from patients with KS and
3 multicentric Castleman disease, and in PBMCs and effusion fluid samples from patients with
4 primary effusion lymphoma (Marcelin et al., 2007). Among KS patients, KSHV DNA has
5 been detected in both plasma and PBMCs, and often no consistent difference between
6 biological compartments is detected (Lin et al 2009). KSHV-infected cells were detected at a
7 very low frequency in the inguinal lymph nodes of HIV-1-seropositive subjects without
8 KSHV-associated diseases (Campbell et al., 2005), suggesting that the latent infection of
9 lymph node cells provides a mechanism for the persistence of KSHV in KSHV/HIV-1-co-
10 infected people.

11 Although effective HIV suppression has been correlated with the regression of KS after
12 antiretroviral therapy (Martinez et al., 2006), the persistence of KS despite seemingly
13 effective antiretroviral therapy (e.g., an undetectable HIV load) is not rare (Krown et al.,
14 2008). However, the interactions between immune responses, KSHV replication, and their
15 relative contribution to KS have not been well characterised to date.

16 The aim of the study was to evaluate the prevalence of KSHV DNA viraemia in male patients
17 from northeastern Italy with chronic and acute HIV-1 infections at the time of HIV diagnosis.
18 Moreover, correlations between KSHV viraemia and the virological and immunological
19 parameters of HIV infection were evaluated.

Materials and Methods

Study population

Four hundred and eighty one men infected with HIV-1 were enrolled at six Infectious Diseases Units located in Veneto, Italy. The patients were recruited consecutively from July 1, 2004 to December 31, 2008 after obtaining their written informed consent to participate in the study. The ethics committee of the hospitals gave approval for the study.

The eligibility criteria included being of Italian origin, >18 years of age, and having antiretroviral-drug-naïve status.

Blood samples were submitted to the Laboratory of Virology at the University of Padua, stored within 6 hours from collection, and analysed subsequently.

A primary or recent HIV infection was defined by the presence of either of the following: (i) a negative or indeterminate HIV antibody-ELISA associated with HIV RNA-positive plasma; or (ii) an initially negative test for HIV antibodies followed by positive serology within 18 months.

The HIV RNA plasma viral load assessment (using Roche Cobas AmpliPrep-Cobas TaqMan HIV-1, version 1 assay, F. Hoffmann-La Roche, Diagnostics Division, Basel, Switzerland) and HIV genotypic analysis to detect subtype were performed as described previously (Parisi et al., 2007).

CD4+ cell counts and percentages were determined at enrolment. Blood collected in EDTA was separated into plasma and cells by Ficoll-Paque Plus density gradient centrifugation.

Aliquots of plasma and dry pellets of 2×10^6 PBMCs were stored at -80°C until use.

KSHV DNA analysis

The extraction and purification of DNA from cells and plasma samples was performed using the QIAmp Blood kit (Qiagen, Inc., Chatsworth, CA). KSHV DNA from cell and plasma samples was detected using a real-time polymerase chain reaction (RT-PCR; with TaqMan

1 probe, Applied Biosystems, Foster City, CA) of open reading frame 26 (ORF26), as described
2 previously (White and Campbell, 2000). The nucleotide sequence targeted by the primers and
3 probes is conserved highly among the three major subgroups of KSHV (Poole et al., 1999).
4 During all DNA extractions and purifications, precautions were taken to reduce the risk of
5 false-positive results.

6 Cellular HIV DNA quantitation

7 The Real-Time TaqMan protocol published by Viard and colleagues (Viard et al., 2004) was
8 used to quantify the HIV DNA copy number in PBMCs. The cell line 8E5, containing one
9 copy of integrated HIV DNA in each cell, was used to build a standard curve. The sensitivity
10 of the test was 13.3 copies/ 10^6 PBMCs.

11 Statistical analysis

12 The variables initially taken into account were age, risk factors for HIV infection (men who
13 had sex with a man vs. other risk groups), primary HIV infection, HIV subtype (B subtype vs.
14 other subtypes), CD4+ cell count, HIV RNA, and HIV DNA load. Among these, age was a
15 quantitative variable, while primary HIV infection, HIV subtype, and the presence of drug
16 resistance mutations were binary (yes/no) variables and were coded as 1/0. CD4+ cell count,
17 HIV RNA, and HIV DNA were both quantitative and discrete variables.

18 Univariate analyses were performed using all of the covariates, taking them both (when
19 appropriate) as continuous variables, as well as categorising by clinically relevant values. Chi
20 square and Kruskal-Wallis tests were used to assess differences between patient groups with
21 reference to categorical and continuous variables, respectively. The significance level was set
22 at 0.05 and all *P* values were two-tailed. To obtain an adjusted analysis while accounting for
23 all possible risk factors, a multiple logistic regression model was used. Age, HIV-RNA level,
24 and all of the significant variables in the univariate analysis were included in the multivariable
25 model.

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1 All of the analyses were performed on an intention-to-treat basis using SPSS for Windows
2 13.0 (SPSS Inc, Chicago, Illinois).
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Results

Of the 481 patients enrolled, in 43 cases (8.9%) it was not possible to define their risk for HIV infection. Of the remaining 438 patients, 282 were homosexual, 90 were heterosexual and 66 were intravenous drug abusers. Additionally, 102 patients had a primary HIV infection and 379 patients had a chronic infection.

Patients with chronic HIV infections were older (41 ± 11.4 vs. 37.6 ± 10.2 years, $p=0.008$) and had CD4+ cell counts (413.8 ± 273.4 vs. 603.9 ± 297.5 cells/ μ l, $p<0.001$) and percentages (21.2 ± 10.2 vs. $26.6 \pm 10.5\%$, $p<0.001$) significantly lower than the patients with primary infections.

Table 1 presents the population of patients infected with HIV studied according to the presence of KSHV DNA in PBMCs and different epidemiological and viro-immunological parameters.

Of the 481 enrolled patients, 74 (15.4%) had KSHV DNA detected in their PBMCs. By univariate analysis, unprotected homosexual relationships and a CD4+ cell count <350 cells/ μ l were associated significantly with the presence of KSHV DNA in PBMCs ($p=0.003$ and $p=0.025$, respectively).

Patients with primary HIV infections had a lower (but not significantly lower) rate of KSHV DNA positivity in PBMCs in comparison to patients with chronic infections. No significant differences in HIV RNA or HIV DNA levels were found between KSHV DNA-positive and KSHV DNA-negative patients.

By multivariate analysis adjusted for different epidemiological and virological parameters, having a homosexual relationship (OR: 3.25; 95% CI: 1.1-9.7; $p=0.035$) was the only parameter that was associated independently with the presence of KSHV DNA in PBMCs.

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3 1 Table 2 presents the univariate analysis of the population of HIV/KSHV co-infected male
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5 2 patients studied in relation to the simultaneous presence of KSHV DNA in PBMCs and
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7 3 plasma.
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10 4 Among the 74 subjects with KSHV DNA detected in PBMCs, the presence of KSHV DNA in
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12 5 plasma was analysed for 65 subjects from whom plasma specimens were available. Of the 65
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14 6 patients, 38 (58.4%) also had KSHV DNA-positive plasma. Overall, five patients had an
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16 7 ultimate diagnosis of KS and were excluded from the evaluation. Among the 60 non-KS
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18 8 patients, 33 (55%) had KSHV DNA detected in both PBMCs and plasma; 28 (85%) of these
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20 9 patients had a chronic HIV infection and 5 had a primary HIV infection. The CD4+ cell
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22 10 counts and percentages were significantly lower in patients with detectable levels of KSHV
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24 11 DNA in both PBMCs and plasma compared to patients with KSHV DNA detectable only in
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26 12 PBMCs ($p=0.006$ and $p=0.019$, respectively). Moreover, among patients with KSHV DNA
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28 13 detected in PBMCs, all 13 subjects with CD4+ cell counts <200 cells/ μ l also had KSHV DNA
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30 14 detectable in plasma.
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36 15 In the multivariate analysis (Table 3) adjusted for the different epidemiologic and virological
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38 16 parameters, CD4+ cell count was the only independent variable associated with the presence
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40 17 of KSHV DNA in the plasma of co-infected HIV/KSHV patients (OR, 0.001; 95% CI,
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42 18 <0.001 -0.001; $p=0.03$).
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46 19 A subgroup of 20 patients with KSHV DNA detected in PBMCs and plasma were re-analysed
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48 20 for the presence of KSHV DNA in PBMCs and plasma in a follow-up study.
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51 21 Nine subjects still had KSHV DNA detectable in PBMCs and plasma after a mean time of 19
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53 22 months. Six of these subjects were still highly active antiretroviral therapy (HAART)-naïve
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55 23 and three were on HAART, two of whom were HIV plasma viraemic at the time of the second
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57 24 analysis. Of the 14 patients who were successfully HAART-treated (<50 HIV RNA
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59 25 copies/ml), 11 were KSHV DNA-negative in PBMCs and plasma after a mean time of 17
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26 months.

Moreover, among the 14 patients treated with HAART, the 11 patients who were negative for KSHV DNA had a mean increase in their CD4+ cell counts of 249 cells/ μ l (range 57-603), whereas the 3 treated patients with detectable levels of KSHV DNA had CD4+ cell count increases of 57, 97, and 74 cells/ μ l.

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Discussion

The presence of KSHV DNA in a cohort of patients at the time of their initial diagnosis of HIV infection was studied. Out of the 481 evaluated subjects, 74 (15.3%) were found to have detectable levels of KSHV DNA in their PBMCs. Approximately half of the subjects found to be PBMC-positive had concomitant active KSHV replication in their plasma.

The presence of KSHV DNA in PBMCs is predictive of the development of KS in HIV-infected patients, and KSHV DNA has been detected in up to 70% of HIV-related KS cases (Whitby et al., 1995; Moore et al., 1996). Previous studies have reported variable results on the prevalence of KSHV DNA in HIV-1-infected people without KS. Laney and colleagues found KSHV DNA in the PBMCs of 28.9% of patients without KS, a rate almost double that of the cohort in this study (Laney et al., 2004). By contrast, Campbell et al (1999) found that 18% of KSHV/HIV-1 co-infected people without KS have detectable KSHV DNA in their PBMCs.

The lower prevalence in the cohort of this study may be due to the distribution of risk factors for HIV, with only 67% of our subjects being homosexual compared to >90% in the previous studies. The median CD4+ cell values were lower in the studies by Campbell and by Laney than they were in the cohort of this study.

In this study, patients with detectable KSHV DNA had lower CD4+ cells count compared to patients without detectable KSHV DNA. Using multivariate analysis, CD4+ cell count was found to be correlated negatively with active KSHV replication. By contrast, any correlation between KSHV DNA detection and either HIV RNA, HIV DNA viral load, or HIV subtype was not found. The lack of a correlation between the presence of KSHV DNA and HIV viraemia seems to confirm previous observations that assign a greater role to the immune response (in particular the NK-type response) and CD4+ cell recovery rather than to HIV replication in the control of KS in AIDS patients (Sirianni et al., 2002; Cattelan et al., 2001).

Higher levels of KSHV DNA (although not significantly so) were found in the group of

1 subjects with chronic HIV infections than in those with acute infections. This result agrees
2 with the significant correlation between KSHV DNA and low CD4+ cell count and seems to
3 confirm that it is the immunological deficiency (which is more pronounced in advanced HIV
4 infection) rather than the level of HIV replication that favours KSHV viraemia. The relevance
5 of the immune response to the control of KSHV replication is also demonstrated by the results
6 of a study in which KS risk was found to be associated positively with a reduced number of
7 lymphocytes, including CD4-positive cells (<457 cells/mL;) (Brown et al., 2006).

8 Almost 60% of patients with KSHV DNA-positive PBMCs also showed the presence of
9 HHV8 in plasma. Absolute CD4+ cell counts and percentages were significantly lower in
10 patients who were KSHV DNA-positive in both plasma and PBMCs compared to patients
11 positive only in PBMCs. Again, the active replication of KSHV was associated significantly
12 with a low number and low percentage of CD4+ cells, and this confirms the more relevant
13 role of immune response in controlling KSHV replication.

14 Previous studies have indicated a correlation between KSHV replication and KS pathogenesis
15 (Whitby et al., 1995; Cannon et al., 2003; Lorenzen et al., 2002; Smith et al., 1997; Broccolo
16 et al., 2002; Engels et al., 2003). In individuals infected with HIV the use of HAART has a
17 role in reducing the frequency of KSHV viraemia, mostly because of the immuno-restorative
18 properties of HIV treatment (Gill et al., 2002; Rizzieri et al., 1997; Lebbe et al., 1998).
19 Treatment with a regimen that includes a protease inhibitor was associated with the clearance
20 of KSHV DNA in KS lesions and PBMCs, and with the regression of KS lesions in AIDS
21 patients (Lebbe et al., 1998; Blum et al., 1997). Furthermore, a number of reports have linked
22 tumour regression after the initiation of HAART to the restoration of immune function
23 (Dupont et al., 2000; Marcelin et al., 2004), and it has been reported that KSHV DNA amount
24 in PBMCs rebounded in patients with KS after a short interruption of efficient antiretroviral
25 therapy (Parisi et al., 2002).

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1 Longitudinal observations on KSHV viraemia are limited. In a large cohort of homosexual
2 men who seroconverted to KSHV antigens, KSHV viraemia persisted for up to 6 months
3 (Goudsmit et al., 2000). This issue was addressed in a subgroup of patients from the cohort of
4 this study. KSHV viraemia persisted in the majority of patients who were untreated or treated
5 unsuccessfully with HAART. By contrast, all of the patients on successful HAART cleared
6 KSHV from their blood after a mean period of 17 months.

7 Before drawing any conclusions, some potential biases of the study must be addressed. The
8 cross-sectional design of the study does not allow conclusive explanation of the correlation
9 between KSHV viraemia and low CD4+ cell count. However, although the small number of
10 patients enrolled in this longitudinal study may limit its conclusiveness, the low CD4+ cell
11 counts in patients on HAART was associated significantly with active replication of KSHV.

12 Studies in a larger population are required to confirm this result. Finally, the lack of risk
13 factors for HIV infection for less than 10% of the patients may have caused misclassification,
14 potentially biasing the correlation between unprotected homosexual relationships and KSHV
15 viraemia.

16 In conclusion, KSHV DNA-positive results were observed in approximately 15% of an HIV-
17 positive cohort of Italian male subjects. The active replication of KSHV was associated
18 significantly with a low number of CD4+ cells, and it was more frequent in patients with
19 chronic than acute HIV infections. A longitudinal study of a limited number of patients
20 demonstrated a direct correlation between immune recovery during HAART and the
21 disappearance of KSHV DNA.

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2 **Potential conflicts of interest: No conflicts**

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For Peer Review

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