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Lymphoproliferative response after stimulation with soluble leishmania antigen (SLA) as a predictor of visceral leishmaniasis (VL) relapse in HIV+ patients

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ABSTRACT

The introduction of HAART resulted in the decrease of *Leishmania*/HIV co-infection cases; nevertheless, the number of relapses remains high and secondary prophylaxis is recommended. However, secondary prophylaxis is not necessary in all patients, and presents a high risk of toxicity and an elevated cost. Our aim was to study whether specific cellular response to *Leishmania infantum* (measured by cell proliferation response after stimulation with soluble *Leishmania* antigen (SLA)), could be a useful tool to attempt a secondary prophylaxis withdrawal. In June 2009 an outbreak of leishmaniasis by *Leishmania infantum* was declared in the southeast of Madrid, and since January 2013, we recruited 10 HIV+ patients that had been treated for visceral leishmaniasis. 6 patients had positive SLA-cell proliferation test. The mean CD4 cell counts of those patients with positive SLA were 140 cel/mm3 and 40 cel/mm3 in those with negative SLA test. 3 patients with positive SLA-cell proliferation test (CD4 count: 336, 307, 625) were not on prophylaxis, and the other 3 patients (CD4 count: 152, 189, 359) were on secondary prophylaxis that was withdrawn after the positive SLA-cell proliferation test with no posterior relapses (mean follow up 60 weeks). From the 4 patients, which had negative SLA-cell proliferation test and continued on prophylaxis, 3 had positive PCR for *Leishmania* at the end of the follow-up and 2 presented clinical relapses.

The performance of SLA-cell proliferation test can be a useful tool that can permit us to try withdrawal of the prophylaxis in *Leishmania*/HIV co-infected patients with low $CD4^+$ counts under clinical supervision, diminishing risk of toxicity and cost.

1. Background and objectives

Leishmaniasis is an infectious vector borne disease caused by the protozoan *Leishmania spp.,* which is transmitted by the bite of female sandflies of the *Phlebotomus* genus. Leishmaniasis is widely distributed throughout the world, and it is endemic in 98 countries, with an estimated incidence of 2 million new cases per year. Visceral leishmaniasis (VL) is the most severe clinical form because of its high morbidity and mortality. There are approximately 58,000 annual deaths and 235,7000 disability-adjusted life years lost caused by VL, although it is thought to be a high under-reported rate (Alvar et al., 2012).

In the Mediterranean region, VL is caused by *L. infantum* specie and it is considered to be hypoendemic (Monge-Maillo et al., 2014). In this region, the majority of the cases have been reported in Albania, Turkey, Italy, and Spain. *Leishmania*/HIV coinfection has been an emergent problem in the last two decades and has been reported in 35 endemic countries being Spain the one reporting most of the cases (Desjeux and Alvar, 2003). The number of new cases has decreased in Europe after the introduction of the HAART therapies in the end of the 90 s (López-Vélez et al., 2001; Fernández Cotarelo et al., 2003; Ter Horst et al., 2008).

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Table 1
Clinical characteristics of HIV patients at the moment of the visceral leishmaniasis diagnosis.

	Sex	Age	HIV RF[a]	Origin	CD4 count (cells/μl)	HAART[b]	Treatment (mg/kg)	Secondary Prophylaxis
Patient 1	Male	44	PDU	Spain	99	No	Liposomal amphotericin B(30)	Antimonials
Patient 2	Male	33	Hetero	Nigeria	4	No	Liposomal amphotericin B(32)	Liposomal amphotericin B
Patient 3	Male	34	Hetero	Peru	29	No	Liposomal amphotericin B(30)	Liposomal amphotericin B
Patient 4	Female	41	Hetero	Equatorial Guinea	305	TDF/FTC/EFV	Liposomal amphotericin B(40)	Liposomal amphotericin B
Patient 5	Male	46	Hetero	Spain	46	No	Liposomal amphotericin B(22)	Antimonials
Patient 6	Male	33	Hetero	Nigeria	322	No	Liposomal amphotericin B(40)	Liposomal amphotericin B
Patient 7	Female	18	Vertical	Equatorial Guinea	16	No	Liposomal amphotericin B(21)	Liposomal amphotericin B
Patient 8	Male	54	MSM	Spain	104	TDF/FTC+ LPVr	Liposomal amphotericin B(39)	Liposomal amphotericin B
Patient 9	Male	44	PDU	Spain	46	No	Liposomal amphotericin B(31)	Antimonials
Patient 10	Male	37	MSM	Poland	48	No	Liposomal amphotericin B(40)	Liposomal amphotericin B

[a] HIV RF: Risk factor for HIV infection.
[b] HAART: highly active antiretroviral therapy.

The immunosuppressed status of HIV patients is a well-known risk factor for developing visceral leishmaniasis, but also for a poor clinical response and high risk of relapse after treatment (van Griensven et al., 2014). Therefore, it is recommended to maintain secondary prophylaxis after the treatment of the first episode of VL (López-Vélez et al., 2001; Alvar et al., 2008; WHO, 2010; Panel, 2016). However, this secondary prophylaxis is not necessary in all patients; besides, it presents a high risk of toxicity and an elevated cost. Thus, it is preferable to maintain the secondary prophylaxis only in those patients who show predicting factors of relapse. Until now, several predictor factors associated with treatment failure have been described in co-infected patients: the incapability of the patient to increase the CD4 cell count, having a history of previous relapses, and a CD4 cell count below 100 cel/μl (Cota et al., 2011). These aspects are taken into account to maintain secondary prophylaxis. Nevertheless, there have been VL relapses described in patients with CD4 cell counts which are higher than 200 cel/μl (Ter Horst et al., 2008; Villanueva et al., 2000). In addition, HIV-seropositive patients with severe and prolonged reduction of CD4 cell counts did not develop VL (Kubar et al., 1998). This fact, suggests the presence of other factors than CD4 cell levels, not yet described, that might affect the therapy response, and represent a great difficulty in establishing a safe CD4 cell count in which the withdrawal of secondary prophylaxis is secure.

HAART therapy alone seems to be insufficient to prevent VL relapse (Ter Horst et al., 2008; van Griensven et al., 2014; Cota et al., 2011; Casado et al., 2001). Despite the fact that HAART therapy increases the CD4 cell count, the studies performed with VL patients receiving HAART, showed a similar rate of relapse than studies carried out in the pre HAART era (van Griensven et al., 2014; Cota et al., 2011; Villanueva et al., 2000). Therefore, there are no secure recommendations for the maintenance of secondary prophylaxis. Some experts recommend maintaining it indefinitely. Some others suggest to withdraw it in selected patients who have >200–350 cel/μl $CD4^+$ cell counts and have not presented any relapses in the last 6 months, with undetectable viral load for more than 3 months, and preferably with negative PCR for *Leishmania* in blood. There are even recommendations of reinitiating prophylaxis if the $CD4^+$ cell counts fall below 200 cel/μl (WHO, 2010; Panel, 2016; Casado et al., 2001; Berenguer et al., 2000).

In the other hand, since it is not easy to define the criteria to keep the secondary prophylaxis or not, another possible approach is to establish, with a higher degree of accuracy, the patients who do not need secondary prophylaxis. This would help to reduce toxicity and high costs of unnecessary treatments.

In immunocompetent patients with VL, the successful response to therapy depends on the activation of the IFN-gamma, which produces a Th1 subset of CD4 positive *Leishmania*-specific T cells, resulting in a complete cure without relapses (Kemp et al., 1993). The lymphoproliferative response after stimulation with soluble antigen of *Leishmania* (SLA), has been proposed as an immunological marker of a good *Leishmania*-specific Th1 response after the infection (Hailu et al., 2005), so it could be useful as a predictor of cure and would indicate that there is no risk of relapse after a first episode of visceral leishmaniasis.

In June 2009 an outbreak of leishmaniasis by *Leishmania infantum* was declared in the southeast of Madrid, with most of the cases reported in the city of Fuenlabrada. More than 400 cases have been reported, 160 VL, being the 10% of them in HIV+ patients (Arce et al., 2013), and 13 have been treated in the Hospital of Fuenlabrada. Under these circumstances, our aim was to evaluate the utility of the SLA-specific cell proliferation test as a useful tool to assess the withdrawal of secondary prophylaxis in HIV+ patients, without a posterior risk of relapse, and also to enable us to predict relapses when specific cellular response is lost. This procedure would improve the therapeutic management of these patients, the healing criteria definition, and would facilitate the possibility of withdrawing the secondary prophylaxis to *Leishmania*/HIV- coinfected patients under certain situations.

2. Material and methods

The ethical committee of Fuenlabrada University Hospital approved the study, and all patients gave their consent for the performance of the blood test. We considered recruitable, all HIV positive patients that were followed-up in our hospital during the five years of the study, that presented an episode of visceral leishmaniasis, and who gave their consent for the extraction of blood samples, considering non-recruitable, patients that were under 18 years old, and those that did not wanted to donate their consent.

Since the 2009 outbreak until 2015, 13 HIV infected patients have been diagnosed of visceral leishmaniasis. All the clinical data of adult patients with VL during the outbreak was prospectively collected, and after the hospital discharge, there was a patient follow-up until 1st of September 2015. From these 13 patients, two patients had already been treated of a previous visceral leishmaniasis episode. We were able to perform the follow up in 10 patients, this was due to the loss of the follow up of a patient that did not return to the hospital, and two other patients that died of *Leishmania* non-related diseases (cirrhosis and progressive multifocal leukoencephalopathy).

The clinical characteristics of the patients at the moment of the diagnosis of visceral leishmaniasis are presented in Table 1.

Since January 2013, we recruited all these 10 HIV+ patients that had been treated for VL, for SLA tests. Blood samples were collected to perform cell proliferation assay stimulated with soluble antigen of *Leishmania* test (SLA test), IFAT serology, and quantitative PCR for *Leishmania*. At least two samples per patient were collected during the study period. The physicians in charge of the follow up of these patients were informed of the results of the tests, which enabled them to decide the best therapeutic measure in each case.

Table 2
Patient characteristics at the beginning of the follow-up (first test).

	Weeks[a]	HAART[b]	Adherence	CD4 Counts (cells/μl)	SLA[c]	PCR Leishmania (parasites/μg)	IFAT[d]	Rk39	Secondary Prophylaxis before tests	Secondary Prophylaxis after tests	Relapse
Patient 1	61	TDF/FTC+DRVr	Correct	68	Negative	809	1/640	+	Yes	Continued	Relapse
Patient 2	99	TDF/FTC+DRVr	Irregular	7	Negative	327	1/320	+	No[e]	Restarted	Relapse
Patient 3	72	ABC/3TC+DRVr	Correct	154	Negative	3326	1/160	+	Yes	Continued	No
Patient 4	37	TDF/FTC/EFV	Correct	336	Positive	Negative	1/40	+	No	No	No
Patient 5	329	DRVr+RAL+ETV	Correct	307	Positive	Negative	1/160	Negative	No	No	No
Patient 6	48	ABC/3TC+EFV	Correct	625	Positive	Negative	1/80	Negative	No	No	No
Patient 7	106	TDF/FTC/RPV	Correct	189	Positive	Negative	1/40	Negative	Yes	Withdrawn	No
Patient 8	234	TDF/FTC+LPVr	Correct	359	Positive	Negative	1/80	Negative	Yes	Withdrawn	No
Patient 9	50	ABC/3TC+LPVr	Correct	136	Negative	Negative	1/80	Negative	Yes	Continued	No
Patient 10	83	TDF/FTC/EFV	Correct	152	Positive	Negative	1/40	Negative	Yes	Withdrawn	No

[a] Time after treatment in weeks since the end of the treatment and the performance of the tests.
[b] HAART: highly active antiretroviral therapy. TDF: Tenofovir; FTC: Emtricitabine; DRVr: Darunavir/ritonavir; ABC: Abacavir; 3TC: Lamivudine; EFV: Efavirenz; RAL: Raltegravir; ETV: Etravirine; RPV: Rilpivirine; LPVr: Lopinavir/ritonavir.
[c] Stimulation with *Leishmania* antigen.
[d] Immunofluorescent antibody titre.
[e] Secondary prophylaxis was indicated, but there was no adherence to the treatment.

All statistical analyses were conducted using the statistical software package SPSS 11.5 (Chicago, IL, USA). The quantitative variables appear as the medias and standard deviations. Normal quantitative variables were compared using an analysis of variance (ANOVA) test.

The following definitions were used:

- Exposed to *Leishmania* (infected): defined as patients with documented infection by at least one of the next test:
  - ◯ Visualization of *Leishmania* by microscope in bone marrow.
  - ◯ Isolation of the parasite from PBMCs in NNN medium culture.
  - ◯ DNA detection by PCR in blood or other tissue samples.
  - ◯ Positive by serology using rK39 technics (qualitative) or by IFI dilution superior to 1/40.
  - ◯ Positive cell proliferation after SLA stimulation (stimulation index higher than 1.74).
- Relapse: reappearance of clinical disease after response to initial treatment.

2.1. Laboratory methods

1. Preparation of soluble *L. infantum* antigen (SLA): *L. infantum* antigen extract was prepared from sonicated promastigotes (JPC strain, MCAN/ES/98/LLM-722), and protein quantification was performed using Bicinchoninic acid (BCA) (Thermo Scientific Pierce BCA Protein Assay Kit, USA).
2. Stimulation of peripheral blood mononuclear cells *in vitro*: Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples, and cultured in triplicate for 5 days in the presence of SLA (10 microgr/ml). Cell proliferation was measured by bromodeoxyuridine incorporation using the Cell Proliferation Biotrak ELISA kit (General Electric Healthcare Life Sciences, UK). The results are shown as stimulation index (SI) for the antigen. The cut-off for positive lymphoproliferation was calculated as the mean + 3 SD (standard deviation) of the SI for 51 *Leishmania*-exposed but negative HIV subjects and it corresponds to a value of SI = 1.74.
3. Immunofluorescent antibody titre (IFAT) analyses of plasma samples were performed using 2×105 *L. infantum* promastigotes in PBS per well (MCAN/ES/98/LLM-722). Plasma was assayed as two-fold serial dilutions from 1/20 to 1/640 in PBS to determine total IgG levels using fluorescein isothiocyanate-conjugated goat anti-human IgG (Fluoline G) (Bio-Mérieux, France) diluted 1/200, and threshold titer for positivity was set at 1/40.
4. rK39-ICT dipsticks were purchased from Leti Laboratories and the corresponding test was performed following the manufacturer's instructions.
5. DNA isolation and real time PCR: DNA isolation and real time PCR (qPCR) were performed as described by Cunha et al. (2013). Briefly, DNA was extracted from 100 μL of peripheral blood by conventional phenol-chloroform extraction and eluted in 100 μL of sterile distilled water. For qPCR, 1000 nM of R223 and 500 nM of R333 primers (Sigma-Aldrich, USA) for the small subunit rRNA (SSUrRNA) sequence were used. Total DNA was used as a template in touchdown qPCR reactions involving the LightCycler FastStart DNA Master SYBR Green I kit (Roche Applied Science, Switzerland).

NNN culture of PBMCs: The presence/absence of *Leishmania* was checked for every week up to 4 weeks.

Table 3
Characteristics of the patients at the end of the follow-up (last test).

	Follow-up time (days)	HAART[a]	Adherence	Secondary prophylaxis	Last CD4 counts.	SLA[b]	PCR *Leishmania* (parasites/μg)	IFAT[c]	Rk39
Patient 1	167	3TC + DRVr + MRC	Correct	Yes	121	Negative	29	1/160	+
Patient 2	161	TDF/FTC + DRVr	Irregular	Yes	68	Negative	2888	1/160	–
Patient 3	138	ABC/3TC + DRVr	Correct	Yes	111	Negative	4092	1/160	+
Patient 4	92	TDF/FTC/EFV	Correct	No	601	Positive	Negative	Negative	+
Patient 5	394	DRVr + RAL + ETV	Correct	No	322	Positive	Negative	1/80	Negative
Patient 6	160	ABC/3TC + EFV	Correct	No	867	Positive	Negative	1/40	Negative
Patient 7	161	TDF/FTC/RPV	Correct	No	345	Positive	Negative	1/80	Negative
Patient 8	300	TDF/FTC + LPVr	Correct	No	219	Positive	Negative	1/80	Negative
Patient 9	146	ABC/3TC + RAL	Correct	Yes	166	Negative	Negative	1/40	Negative
Patient 10	189	TDF/FTC/EFV	Correct	No	263	Positive	Negative	1/40	Negative

[a] HAART: highly active antiretroviral therapy. 3TC: Lamivudine; DRVr: Darunavir/ritonavir; MRC: Maraviroc; TDF: Tenofovir; FTC: Emtricitabine; ABC: Abacavir; EFV: Efavirenz; RAL: Raltegravir; ETV: Etravirine; RPV: Rilpivirine; LPVr: Lopinavir/ritonavir.
[b] Stimulation with *Leishmania* antigen.
[c] Immunofluorescent antibody titre.

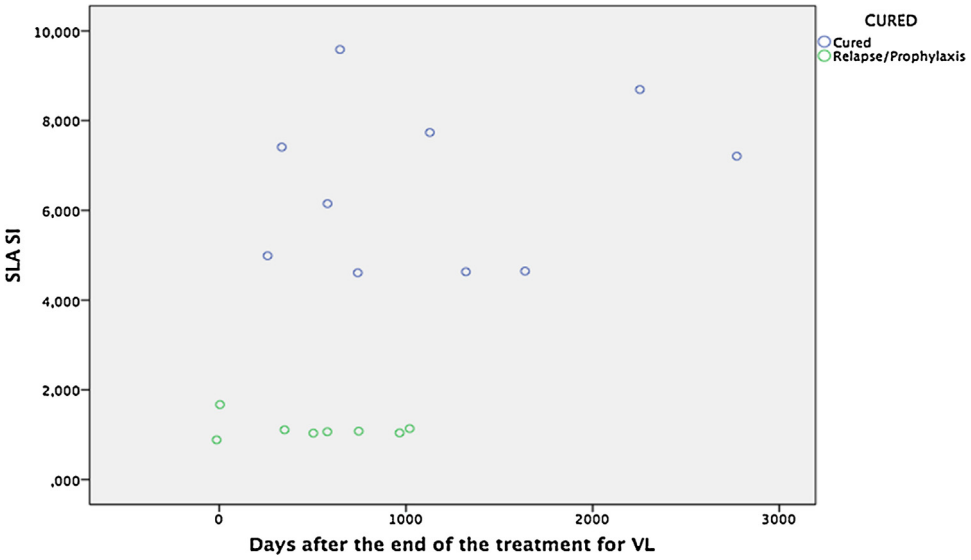

Fig. 1. Soluble antigen of *Leishmania* (SLA) Stimulation Index (SLA SI), after the end of VL treatment. Positive test: SLA SI ≥ 1.74.

3. Results

Clinical findings regarding VL in the 10 patients we followed-up, did not differ from those found in inmunocompetent patients. Most of the patients presented VL manifestations; including weight loss, hepato-splenomegaly, and pancytopenia, with the exception that fever appeared only in 6 patients (67%). All patients were treated with liposomal amphotericin B followed by secondary prophylaxis after the end of the treatment (Table 1). Table 2 shows the characteristics of the patients at the beginning of the follow up, when the first test was made. Six out of ten patients had positive SLA-cell proliferation test. The mean CD4 cell counts of those patients with positive SLA were 140.2 cel/mm3, and 40.5 cel/mm3 in those with SLA-negative cell proliferation test ($p = 0.22$). Three patients with positive SLA-cell proliferation test were not on prophylaxis (it had been withdrawn before the beginning of the study), and the other 3 patients (CD4 count: 152, 189, 359) were on secondary prophylaxis. Nevertheless, secondary prophylaxis was withdrawn in these patients after the positive SLA-cell proliferation test was obtained with no relapses (mean duration of the follow up: 60 weeks). Four patients with negative SLA-cell proliferation test continued on prophylaxis, 3 of them had positive PCR for *Leishmania* at the end of the follow up, and 2 presented clinical relapses and were re-treated. The clinical status of all the patients at the end of follow up is showed on Table 3. Fig. 1 shows the results of SLA Stimulation Index (SLA SI) in cell proliferation tests and Fig. 2 the comparative media results in cured patients versus relapsing patients and patients still in prophylaxis.

4. Discussion

The introduction of HAART for the treatment of HIV infection has resulted in the decrease of HIV/*Leishmania* co-infection cases, which is associated with the increase of CD4 cell count induced by this treatment. The combination of HAART and secondary prophylaxis for VL has achieved a lower incidence of VL cases in HIV/*Leishmania* co-infected patients (López-Vélez et al., 2001). However, the number of relapses remains high in some patients on HAART and prophylaxis, mainly in patients with low CD4 cell count (Alvar et al., 2008). Classically, the CD4 cell count <200 cel/μl has been the parameter related to the risk of developing primary VL and has also shown to be determinant for relapses (Cota et al., 2011). Therefore, it has traditionally been used to decide whether or not, a patient should be administered secondary prophylaxis (Alvar et al., 2008; WHO, 2010; Panel, 2016; Ter Horst et al., 2008; Kubar et al., 1998). Visceral leishmaniasis, unlike other opportunistic infections, can occur with CD4 cell counts that are higher than 200 cel/μl (Ter Horst et al., 2008; Villanueva et al., 2000). There is evidence that other factors, different from the CD4 cell counts, are involved in the susceptibility of HIV+ patients to present a pri-

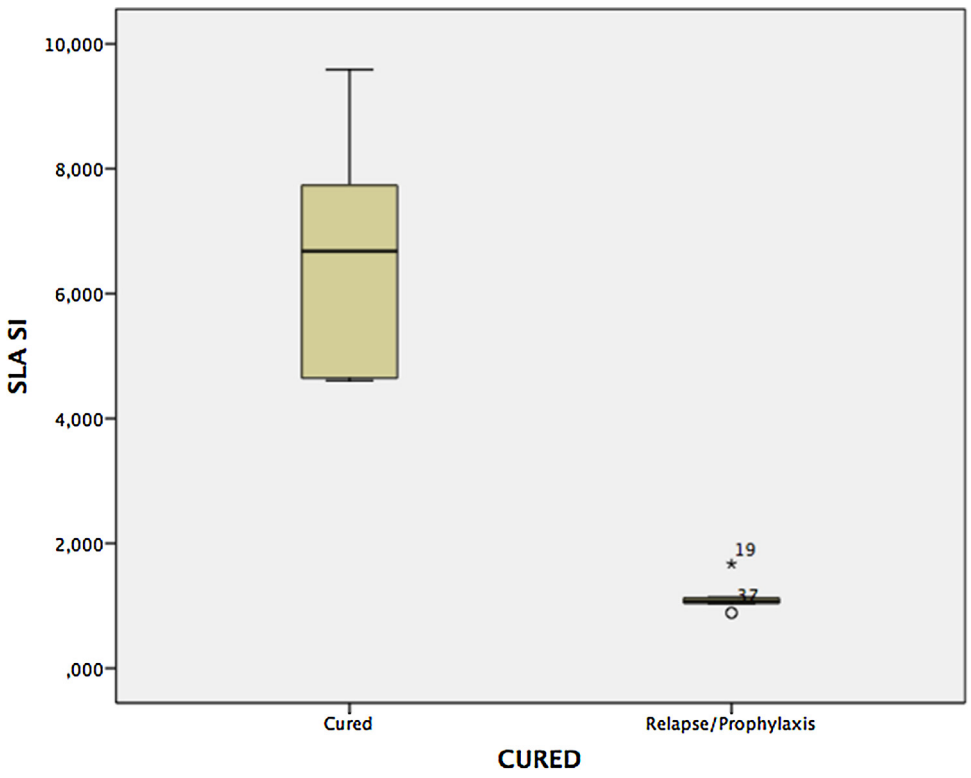

Fig. 2. Soluble antigen of *Leishmania* (SLA) Stimulation Index (SLA SI), in relation to cured patients, after the end of VL treatment.
Positive test: SLA SI ≥ 1.74.
ANOVA $p < 0.001$.

mary *Leishmania* infection and posterior relapses (Alvar et al., 2008; Kubar et al., 1998). Apart from the general immunosuppression status expressed by the CD4 cell count, our aim in this work, was to confirm whether the specific cellular response to *Leishmania infantum* (measured by cell proliferation response after stimulation with soluble antigen of *Leishmania* (SLA)), was a useful tool to assess the existence of *Leishmania*-specific T cell memory clones, capable to keep the parasitic infection under control, to avoid relapses and, in consequence, attempt a secondary prophylaxis withdrawal in these patients.

Follow up of the co-infected patients after treatment showed that 6 out of 10 presented a positive SLA-cell proliferation test. All of these patients were left without prophylaxis for at least 60 weeks and presented no relapses during the whole period. The four remaining patients who were followed up presented a negative SLA test. One of them, with low CD4 cell count (<100 cel/μl) took irregularly HAART and abandoned secondary prophylaxis, presenting a relapse. Another patient with low CD4 cell count (<100 cel/μl), under regular HAART and secondary prophylaxis, also presented a relapse. The two other patients with negative SLA test, continue with secondary prophylaxis, and have not presented any relapses up to the moment. Nevertheless, it is interesting to point out, that one of these patients presents positive PCR for *Leishmania,* which suggest*s* the presence of parasites in peripheral blood. These results support the fact that SLA-cell proliferation positive test is indicative of a cell-mediated immune response against the parasite and a good predictive marker of non-appearance of relapses in HIV patients co-infected with *Leishmania*.

All the patients in our cohort who presented a negative SLA-cell proliferation test had less than 200 CD4 cel/μl. However, we observed that 2 of the 6 patients with adequate cell response had less than 200 CD4 cel/μl counts when the prophylaxis was withdrawn, and 4 out of these 6 patients without prophylaxis at the present time have less than 350 CD4 cel/μl count. Although the current recommendations are to use the CD4 count levels in order to recommend the maintenance or withdrawal of secondary prophylaxis, there is no clear evidence for this statement (WHO, 2010; Panel, 2016). Previous studies reveal the importance of maintaining a good CD4 cell count level, as well as developing an specific T cell response against *Leishmania* after treatment, in order to diminish the number of relapses (Moreno et al., 2000; Bourgeois et al., 2008). It is well known that co-infection has a synergic effect, facilitating the increase of HIV viral load and progression to AIDS, and that it favours uncontrolled multiplication of the parasites (van Griensven et al., 2014), finally reducing the capability of HAART therapy to increase CD4 counts. Besides, it has been shown that there is an specific interaction between *Leishmania* and HIV at a cellular level that mainly affects macrophages and dendritic cells, and that may result in the impairment of cell mediated immune response (Alvar et al., 2008). In consequence, in coinfected patients, specific immunity to *Leishmania* is not only related to the CD4 count. Functional activity of these cells, as assessed in SLA cell proliferation test, should be taken into account, as a small number of memory T cells could be enough to build up a sufficient secondary immune response (Moreno et al., 2000).

During the outbreak in Madrid, a study in solid organ transplant recipients was performed, in order to test exposure and confirmation of cure in patients treated of VL. In this study, SLA tests were performed, and two patients did not present a positive lymphoproliferative response. One of them was serologically positive by IFAT and developed VL a few days after, and the other patient, was a patient that had been already treated for VL and was the only patient (out of six patients with previous VL episodes) who showed no lymphoproliferative response and relapsed four months after the test was performed. These results, although with their limi-

tations, reinforce our hypothesis in immunodepressed individuals (PLOS Neglected Tropical Diseases, 2016).

Another laboratory parameter, which shows encouraging results when applied to follow up of coinfected patients, is the quantitative determination of parasite load by PCR. Various studies using real-time PCR demonstrated that relapses are preceded by an elevation of parasite levels measured by this method, in peripheral blood (Bourgeois et al., 2008; Antinori et al., 2007; Bossolasco et al., 2003; Mary et al., 2006). It is not well known how often these tests should be performed, nor which levels are clearly associated to a relapse. In our cohort, we found 3 patients with positive PCR, including the two patients that relapsed. The third one was asymptomatic but actually presented a higher parasitic load than the rest of the other patients that had presented clinical relapses. All these three patients presented negative SLA-cell proliferation test. The results of both parameters, lymphoproliferative response after stimulation with SLA and PCR presented no variations along the period of the study, which ranged from 55 to 112 weeks. This fact reinforces our results, which are consistent, showing a relationship between positive PCR and negative SLA-cell proliferation test, which supports the idea that both techniques could be used as complementary tests to follow up coinfected patients after the withdrawal of secondary prophylaxis.

There are limitations to our study, and one of them was the small number of patients. Our results have limitations inherent to observational studies, which do not allow drawing any definitive conclusions because of the requirement of comparative clinical trials to be done. The unexpected outbreak did not allow us to design a randomized controlled trial, nor an observational prognostic study in which other factors associated with VL relapse were also studied. However, we think that our findings are consistent and promising enough to be investigated in future trials.

Another condition that has to be taken into account is that implementation of PBMC SLA stimulation assays may be difficult in certain situations, because is laborious and time-consuming. However, this technic has already been used in HIV patients with *Leishmania*, although this is the first time that it is used to study relapses and as a prognostic marker (Moreno et al., 2000). Due to the complexity of some these laboratory tests, there has been development of whole blood assay (Quantiferon) techniques to detect asymptomatic individuals in areas where *Leishmania infantum* is endemic (Ibarra-Meneses et al., 2016), but there are no results published in *Leishmania*/HIV coinfection. The aim of the development of this technic is to have an easy rapid test that can help us to detect patients with a higher risk of relapse or development of the disease in HIV exposed patients in areas with high endemicity. There have been results published with these techniques assessing latent tuberculosis infection in HIV patients, although there are still certain limitations concerning these results in patients with severe immunosuppression (Cattamanchi et al., 2011; Aichelburg et al., 2014).

In conclusion, we think that the performance of lymphoproliferative response test after stimulation with soluble antigen of *Leishmania* (SLA), can be a tool which may enable us to withdraw the prophylaxis in patients under frequent clinical supervision that have not achieved >200 cel/μl CD4 cell counts. This could diminish the administration of drugs with many side effects, that are expensive, and which have proven to be insufficient to prevent relapses. Further investigation is needed and we suggest the incorporation of SLA test in future trials.

Conflicts of interest

All authors report no conflicts of interest relevant to this article.

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