Title: Urinary lipoarabinomannan as predictor for the tuberculosis immune reconstitution inflammatory syndrome

Running Head: Urinary lipoarabinomannan in TB IRIS

Anali Conesa-Botella MD*1,2, Marguerite Massinga Loembé MSc PhD*3, Yukari C. Manabe MD4,6,7, William Worodria MMed4,5,7, Doreen Mazakpwe MBChB5,7, Kenneth Luzinda MBChB5,7, Harriet Mayanja-Kizza MBChB, MMed, MSc 4,5,7, Mitra Miri MSc8, Olive Mbabazi BSc4, Olivier Koole MD, MSc 1, Luc Kestens Msc, PhD3,9, Robert Colebunders MD, PhD1,2

1 Department of Clinical Sciences, Institute of Tropical Medicine, Antwerp, Belgium
2 Epidemiology and Social Medicine, University of Antwerp, Belgium
3 Department of Microbiology, Institute of Tropical Medicine, Antwerp, Belgium
4 Infectious Diseases Institute, Makerere University College of Health Sciences, Kampala, Uganda
5 Makerere University College of Health Sciences/ Mulago Hospital, Kampala, Uganda
6 Department of Medicine, Division of Infectious Diseases, Johns Hopkins University School of Medicine, Baltimore, Maryland
7 Infectious Diseases Network for Treatment and Research in Africa (INTERACT), Kampala, Uganda
8 Interdepartmental Neuroscience Program, Yale University, New Haven, CT, USA
9 Department of Biomedical Sciences, University of Antwerp, Belgium
Abstract

Background

Upon initiation of antiretroviral therapy (ART), 15.7% (95%CI: 9.7%–24.5%) of tuberculosis (TB)-HIV co-infected individuals experience paradoxical worsening of their clinical status with exuberant inflammation consistent with Immune Reconstitution Inflammatory Syndrome (IRIS). We investigated whether a positive urinary TB lipoarabinomannan (LAM) antigen ELISA test prior to ART initiation was associated with development of paradoxical TB-IRIS.

Methods

In a prospective observational cohort in Mulago Hospital, Kampala, Uganda, we measured pre-ART urinary LAM concentrations in HIV-infected patients on TB treatment. Patients who developed TB-IRIS (according to the International Network for the Study of HIV-associated IRIS (INSHI) case-definition) were compared to patients who remained TB-IRIS free for at least 3 months.

Results

Twenty-six individuals with TB-IRIS and 64 without TB-IRIS were included in the analysis. The median time to TB-IRIS was 14 days (IQR: 11-14). Univariate analysis showed that a positive pre-ART urinary LAM test (OR: 4.6 [95%CI: 1.5-13.8], p=0.006) and a CD4 count <50 cells/ml (OR: 21 [95%CI: 2.6–169.4], p=0.004) were associated with an increased risk of TB-IRIS. In multivariate analysis only a baseline CD4 T-cell count <50 cells/ml was predictive of IRIS (p<0.004). Sensitivity and specificity of a
positive pre-ART urinary LAM test to diagnose IRIS was 80.8% (95%CI: 60.6–93.4) and 52.4% (95%CI: 39.4–65.1), respectively.

Conclusion

If CD4 count testing facilities are available, pre-HAART urinary LAM testing has no added value to predict TB-IRIS. In case CD4 counts are not available, a positive LAM test could contribute to the early identification of patients at increased risk for TB-IRIS.

Word count: abstract: 250 words

Keywords: HIV, Tuberculosis, HAART, Immune reconstitution, Lipoarabinomannan
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¹ Department of Clinical Sciences, Institute of Tropical Medicine, Antwerp, Belgium
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³ Department of Microbiology, Institute of Tropical Medicine, Antwerp, Belgium
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⁵ Makerere University College of Health Sciences/ Mulago Hospital, Kampala, Uganda
⁶ Department of Medicine, Division of Infectious Diseases, Johns Hopkins University School of Medicine, Baltimore, Maryland
⁷ Infectious Diseases Network for Treatment and Research in Africa (INTERACT), Kampala, Uganda
⁸ Interdepartmental Neuroscience Program, Yale University, New Haven, CT, USA
⁹ Department of Biomedical Sciences, University of Antwerp, Belgium
* equal contribution

Corresponding author:
Anali Conesa-Botella
Institute of Tropical Medicine
2000 Antwerpen
Belgium
aconesa@itg.be

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Methods

In a prospective observational cohort in Mulago Hospital, Kampala, Uganda, we measured pre-ART urinary LAM concentrations in HIV-infected patients on TB treatment. Patients who developed TB-IRIS (according to the International Network for the Study of HIV-associated IRIS (INSHI) case-definition) were compared to patients who remained TB-IRIS free for at least 3 months.

Results

Twenty-six individuals with TB-IRIS and 64 without TB-IRIS were included in the analysis. The median time to TB-IRIS was 14 days (IQR: 11-14). Univariate analysis showed that a positive pre-ART urinary LAM test (OR: 4.6 [95% CI: 1.5-13.8], p=0.006) and a CD4 count <50 cells/ml (OR: 21 [95% CI: 2.6–169.4], p=0.004) were associated with an increased risk of TB-IRIS. In multivariate analysis only a baseline CD4 T-cell count <50 cells/ml was predictive of IRIS (p<0.004). Sensitivity and specificity of a
positive pre-ART urinary LAM test to diagnose IRIS was 80.8% (95%CI: 60.6–93.4) and 52.4% (95%CI: 39.4–65.1), respectively.

Conclusion

If CD4 count testing facilities are available, pre-HAART urinary LAM testing has no added value to predict TB-IRIS. In case CD4 counts are not available, a positive LAM test could contribute to the early identification of patients at increased risk for TB-IRIS.

Word count: abstract: 250 words

Keywords: HIV, Tuberculosis, HAART, Immune reconstitution, Lipoarabinomannan
Introduction

According to the most recent WHO estimates\(^1\), the burden of tuberculosis (TB) worldwide was 9.4 million incident cases and 14 million prevalent cases in 2009\(^2\). Co-infection with HIV accounted for about 11-13% of incident TB cases. Africa bears the largest burden of worldwide cases with 80% of co-infected individuals diagnosed on the continent. In resource-limited settings, smear microscopy is still commonly used for TB disease screening, despite its sub-optimal performance in HIV patients with advanced immuno-suppression and atypical disease presentation\(^3\). Accordingly, the Global Plan to Stop TB recently emphasized the need for sensitive and cost-effective tools for improved TB diagnostic at the point-of-care (POC).

Detection of circulating *Mycobacterium tuberculosis* (Mtb) lipoarabinomannan (LAM) antigen in TB suspects, a surrogate marker of infection, is among the portfolio of new POC tests for TB diagnostics. A urinary-based commercial LAM-ELISA assay, the Clearview TB® ELISA (Inverness Medical Innovations, USA) has been evaluated in various populations. Reported sensitivity was higher in HIV-positive individuals (21%-67%), but inversely correlated with CD4 T-cell counts\(^4\)-\(^9\), indicating that LAM detection assays could play a role as rapid rule-in test for Mtb in HIV-infected individuals with advanced immunosuppression.

Upon highly active antiretroviral treatment (HAART) initiation, 15.7% (9.7%-24.5%) of TB-HIV infected individuals experience a paradoxical deterioration of their clinical status\(^10\), a condition known as TB immune reconstitution inflammatory syndrome (IRIS) or immune reconstitution disease (IRD). TB IRIS can present as the sudden clinical inflammatory manifestation of occult sub-clinical TB disease (unmasking TB
IRIS) or as the worsening of a successfully treated TB infection (paradoxical TB IRIS). IRIS is postulated to result from a rapid and unbalanced restoration of pathogen-specific immune responses following HAART initiation\(^{11}\). A rabbit model proposed that IRIS was dependent on antigen load at the time of immune reconstitution\(^{12}\). In humans, low CD4 T-cell counts at the start of HAART, a shorter delay between TB therapy and HAART, and the presence of disseminated TB were reported risk factors for TB IRIS\(^{13}\). Based on data from a small study it has been hypothesized that a higher LAM antigen load pre-HAART may also be predictive of TB IRIS\(^5\).

We sought to evaluate the performance characteristics of pre-HAART urinary LAM as a predictor for TB IRIS in a prospective observational cohort of patients with HIV infection starting HAART while on TB treatment in Kampala, Uganda.

Methods

Study population

This sub-study was nested in a prospective cohort study of HIV-infected adults on treatment for active TB disease, HAART naïve and qualifying for HAART initiation. Patients were recruited at the National Tuberculosis and Leprosy Program (NTLP) clinic, Mulago Hospital in Kampala, Uganda between December 2007 and July 2010. Patients were followed up for 6 months to 2 years for the development of paradoxical TB IRIS. Eligibility criteria for the cohort study were: 1) adult (>18yrs) living within a 20 kilometre radius of the hospital, 2) confirmed TB-HIV infection according to World Health Organization (WHO) guidelines 3) starting TB treatment or having taken TB treatment for less than two months and 4) eligible for HAART according to Ugandan
national ART treatment guidelines (CD4 count ≤250 cells/µL). Individuals without a treatment supporter were excluded. The main findings of the cohort study have been published elsewhere. For inclusion in the LAM sub-study individuals needed: 1) to have been followed for at least 3 months, 2) not to have developed a non-TB related type of IRIS.

**Definitions**

TB infection was defined according to the WHO TB/HIV guidelines based on clinical examination, acid fast bacilli stain microscopy, solid medium mycobacterial culture and chest X-rays. Where indicated, histology and cytology of aspirates (lymph nodes, pleural fluid, abscesses) and ultrasound scan were additionally performed.

TB IRIS was defined according to the International Network for the Study of HIV associated IRIS (INSHI) case-definition. Study participants who developed signs and symptoms suggestive of another type of IRIS, or study patients with late onset IRIS (ie: fulfilling INSHI criteria but occurring more than 3 months after HAART initiation) were excluded from analysis.

Non TB IRIS cases were patients with a follow-up of at least 3 months who did not develop signs and symptoms suggestive of TB IRIS.

**Sample collection**

Urine collection started July 2008. Urine samples were collected pre-HAART, after 1 and 3 months of HAART and at IRIS. A midstream urine sample was collected in a sterile urine container, kept at 4°C while waiting for transportation on the same day.
Samples were aliquoted in polypropylene tubes and kept frozen at -80°C until analysis. Urine gravity was measured by refractometry (URC/Nalpha, Atago, Japan).

*LAM-ELISA assay*

LAM was measured using the Clearview TB® ELISA (Inverness Medical Innovations, U.S.A.), a direct antigen sandwich immunoassay, following manufacturer’s instructions. On the day of analysis frozen urine aliquots were thawed, boiled 30 min at 95°C and centrifuged. Supernatant was collected for analysis. Each plate included in duplicate: a standard curve with purified LAM antigen (received from S. Svenson and B. Hamasur, Karolinska Institute, Sweden\(^\text{17}\)), 100μl of the positive and negative control provided with the Clearview TB® ELISA kit and 100μl from each sample. The plate was read at 450nm and the ELISA test was considered valid if the mean of the positive controls minus the mean of the negative controls was between 0.3-0.5. Results were considered positive if the optical density was at least 0.1 OD above the signal of the negative control.

*Ethical considerations*

The study was approved by the Makerere University Faculty Ethics committee, the Mulago Hospital Research committee and the Uganda National Council of Science and Technology. Informed consent was obtained from all study participants.

*Statistical analysis*
All statistical analyses were performed using Stata statistics software (version 10.2; Stata Corp., Texas, USA). Data was summarized by count or median and interquartile range (IQR) for non-normally distributed variables. Normality was assessed using graphical methods and confirmed by D’Agostino and Pearson omnibus normality test.

Differences in LAM concentration medians were compared using the Wilcoxon rank-sum test and the Wilcoxon signed rank test for paired data. Test performance characteristics (sensitivity and specificity) of LAM were estimated in TB IRIS and non TB IRIS cases, using the "diagt" Stata component.

Logistic regression was used to study predictors of IRIS. A threshold of p<0.1 was used for variable inclusion in the multivariate model, which was then simplified by backward elimination. The following variables were analyzed: LAM concentration, age, sex, pre-HAART CD4 count, sputum smear, smear Lowenstein-Jensen cultures, type of clinical TB presentation, and the time from TB treatment start to HAART initiation. Linear regression was used to assess the association between LAM positivity and degree of culture positivity, sputum grading (negative, scanty or 1+, 2+, 3+), or the type of TB (Sputum Smear (SS) negative (-) pulmonary (P) TB, SS positive (+) PTB, Extrapulmonary (EP) TB ± PTB). All p values reported were two-sided at alpha of 0.05.

Results

Study participants

Three hundred and two patients were enrolled in the cohort study. Inclusion into the LAM sub-study started 9.5 months after the start of the cohort study. Therefore urine
was obtained for only 133 individuals. Forty-three patients were excluded: 5 patients developed late onset IRIS, 10 a non TB related form of IRIS, and 28 did not complete the 3 months of follow up. In total, 90 individuals were included in the analysis of which 26 were classified as TB IRIS and 64 as non TB IRIS (Figure 1).

The median (IQR) time of follow up for all patients in the sub study was of 332 days (168-353) and the median (IQR) time to IRIS was of 14 days (11-14). Non TB IRIS individuals were followed up for a median (IQR) of 333 days (170-413.5). Baseline characteristics of sub-study patients are presented in Table 1. All individuals had normal serum creatinine levels at study inclusion (reference range: 0.5-1.2 mg/dl). TB IRIS and non TB IRIS patients were comparable for all variables except pre-HAART CD4 T-cell count and delay in starting HAART after the onset of anti-tuberculous therapy. Patients who developed TB IRIS had a lower CD4 T-cell count [20.5cells/mm³ (11-40) vs 71 (26-158), p=0.0001] and a shorter time between TB treatment and the initiation of HAART [36 days (24-58) vs 52 days (33-65.5), p=0.037] than the non TB IRIS group.

Pre-HAART LAM positivity is predictive of IRIS

Performance characteristics of the LAM assay to predict IRIS were as follows: sensitivity: 21/26 (80.8%, [95%CI: 60.6%-93.4%]) and specificity: 31/64 (52.4%, [95%CI: 39.4%-65.1%]).

In patients with positive pre-HAART urinary LAM, the odds ratio for developing TB IRIS after the initiation of HAART was 4.6 (95%CI: 1.5-13.8, p=0.006). Moreover, the median pre-HAART LAM concentration was significantly higher in patients who developed TB IRIS compared to those not developing IRIS (1.25ng/ml [IQR: 0.2-7.1] vs.
0.1ng/ml [IQR: 0-0.5], p=0.001). There was no significant difference in urinary LAM concentration between IRIS and non IRIS patients at month 1 and month 3 of follow up.

Variation in LAM concentrations during HAART

Urinary LAM concentration decreased significantly during follow up in IRIS and non IRIS individuals (Figure 2A). Urine from 8 patients was collected at IRIS time-point. There was no increase in LAM concentration at the time of IRIS (Figure 2B). In individuals who developed TB IRIS, pre-HAART LAM concentration was not associated with the duration of TB treatment prior to HAART (p=0.567). LAM positivity was negatively correlated with CD4 T-cell counts (OR: 4.66 [95%CI: 2.43-8.93], p<0.0001)

LAM and TB

Sputum culture positivity at time of enrollment was associated with LAM positivity (n=45; OR: 6.75 [95%CI: 1.69-26.67], p=0.008). However, no significant association was observed between LAM positivity and sputum smear positivity grading (n= 69; p=0.29), type of TB (n=90; p=0.15), duration of TB treatment (n=88; p=0.506), nor Tuberculin Skin Test (TST) result (n=89; p=0.110). Patients with a positive baseline LAM result were TST negative and vice versa (Figure 3).

Risk factors for the development of TB IRIS (table 2)
In the univariate model, a positive pre-HAART urinary LAM test result (p=0.006) and a pre-HAART CD4 T-cell count below 50 cell/mm³ (p=0.004) were associated with increased risk of TB IRIS. LAM concentration was inversely associated with CD4 T-cell count (p=0.043). In multivariate analysis, a CD4 T-cell count below 50 cells/mm³ increased the risk of developing TB IRIS by 21 (95%CI: 2.6-169.4, p=0.004) compared to those with CD4 T-cell count >100/mm³.

Discussion

In this prospective observational cohort of HIV patients on TB treatment in Kampala, Uganda, we showed an association between LAM and TB IRIS. Baseline urine LAM concentration was significantly higher among patients who subsequently developed paradoxical TB IRIS. Moreover the LAM levels decreased during HAART treatment. As consistently reported elsewhere, CD4 counts were lower among the TB IRIS group.

Our results corroborate data from a small study in South Africa comparing 5 TB IRIS cases and 17 controls. In this study all 5 TB IRIS cases (100%) but only one control patient tested positive for LAM at TB diagnosis prior to ART initiation.

LAM is released by metabolically active bacteria. This might explain the observed association between LAM positivity and sputum culture positivity, and the apparent lack of association observed between LAM positivity and sputum smear grading.

In our study LAM concentration decreased during HAART and no transitional increase was observed at TB IRIS. This suggests that TB IRIS may be a feature of immune response against Mtb rather than due to an increase in Mtb antigen load. This
also might reflect that LAM is an imperfect measure of the antigen concentration at the site where TB IRIS occurs.

Anergy to TST has been reported in severely immuno-suppressed HIV infected individuals. In our cohort, all patients who tested TST negative had detectable LAM levels, while TST positive subjects did not have detectable LAM Ag in their urine. Severely immuno-suppressed individuals, anergic to TST, may present with higher LAM concentration as their disease is more likely to be more disseminated. Conversely, individuals with a strong response to TST are possibly more able to clear Mtb and LAM antigens faster during the anti-TB treatment and before initiation of HAART.

Our study was unable to evaluate the performance of urinary LAM for TB diagnosis as samples were collected a median of 44 days after beginning TB treatment. Ideally, LAM should be measured on a 24 hour urine collection or on first morning urine. In our study a urine sample was collected during the study visit. Urine specific gravity was similar in both groups indicating similarity in the TB IRIS and non TB IRIS patients in terms of urine concentration.

In conclusion, if CD4 T-cell count testing is available, a pre-HAART urinary LAM test has no added value to predict TB IRIS. When CD4 T-cell count is not available, a positive LAM test could indentify patients at increased risk of TB IRIS. The clinical utility of monitoring the LAM concentration during TB treatment needs to be investigated.
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References


<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total</th>
<th>All</th>
<th>TB IRIS</th>
<th>Non TB IRIS</th>
<th>p value</th>
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<tr>
<td>Gender male n (%)</td>
<td>92</td>
<td>49 (54.4)</td>
<td>14 (53.8)</td>
<td>35 (54.7)</td>
<td>0.942</td>
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<tr>
<td>Age (years) median (IQR)</td>
<td>92</td>
<td>31.5 (27;37)</td>
<td>30 (27;35)</td>
<td>32 (26.5;37.5)</td>
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<td>CD4 (cell/mm³) median (IQR)</td>
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<td>51 (19;118)</td>
<td>20.5 (11;40)</td>
<td>71 (26;158)</td>
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<td>0.57 (0.51;0.72)</td>
<td>0.64 (0.53;0.675)</td>
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<td>22 (44.9)</td>
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<td>22 (31.4)</td>
<td>5 (23.8)</td>
<td>17 (34.7)</td>
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<td>2+: n (%)</td>
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<td>9 (12.9)</td>
<td>3 (14.3)</td>
<td>6 (12.2)</td>
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<tr>
<td>3+: n (%)</td>
<td></td>
<td>8 (11.4)</td>
<td>4 (19.0)</td>
<td>4 (8.2)</td>
<td></td>
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<td>Culture positive: n (%)</td>
<td>46</td>
<td>33 (71.7)</td>
<td>12 (85.7)</td>
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<td>SS- PTB: n (%)</td>
<td>28</td>
<td>7 (28.9)</td>
<td>21 (32.9)</td>
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<td>SS+PTB: n (%)</td>
<td>35</td>
<td>12 (46.1)</td>
<td>23 (35.9)</td>
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<td>EPTB ± PTB: n (%)</td>
<td>27</td>
<td>7 (26.9)</td>
<td>20 (31.2)</td>
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<td>Urine specific gravity: median (IQR)</td>
<td>90</td>
<td>1.015 (1.010;1.017)</td>
<td>1.015 (1.012;1.017)</td>
<td>1.015 (1.010;1.018)</td>
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<tr>
<td>Delay between TB treatment and HAART initiation (days): median (IQR)</td>
<td>90</td>
<td>48 (31.63)</td>
<td>36 (24.58)</td>
<td>52 (33.65.5)</td>
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Table 2: Predictors of IRIS by logistic regression (univariate analysis).

<table>
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<th>OR</th>
<th>p value</th>
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<td>Pre-HAART LAM</td>
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<tr>
<td>Positive</td>
<td>51</td>
<td>21</td>
<td>4.6 (1.5-13.8)</td>
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<td>Negative</td>
<td>38</td>
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</tr>
<tr>
<td>Male</td>
<td>49</td>
<td>14</td>
<td>1.0 (0.4-2.6)</td>
<td>0.942</td>
</tr>
<tr>
<td>Female</td>
<td>41</td>
<td>12</td>
<td>1</td>
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<td>Age (years)</td>
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<tr>
<td>&lt; 31.5</td>
<td>45</td>
<td>15</td>
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<td>≥ 31.5</td>
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<td>CD4 (cells/mm³)</td>
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<td>&lt;0.001</td>
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<tr>
<td>&lt;50</td>
<td>44</td>
<td>21</td>
<td>21.0 (2.6-169.4)</td>
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<td>50-100</td>
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<td>≥100</td>
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<td>Negative</td>
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<td>Scanty or 1+</td>
<td>22</td>
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<td>2+</td>
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<td>3</td>
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<td>3+</td>
<td>8</td>
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<td>2.4 (0.5-12.0)</td>
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<td>13</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Type of TB</td>
<td></td>
<td></td>
<td></td>
<td>0.667</td>
</tr>
<tr>
<td>SS-PTB</td>
<td>28</td>
<td>7</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>SS+PTB</td>
<td>35</td>
<td>12</td>
<td>1.6 (0.5-4.7)</td>
<td>0.426</td>
</tr>
<tr>
<td>EPTB ± PTB</td>
<td>27</td>
<td>7</td>
<td>1.0 (0.3-3.5)</td>
<td>0.937</td>
</tr>
<tr>
<td>Delay of TB treatment (days)</td>
<td></td>
<td></td>
<td></td>
<td>0.382</td>
</tr>
<tr>
<td>&lt;14</td>
<td>3</td>
<td>1</td>
<td>2 (0.7-5.5)</td>
<td>0.180</td>
</tr>
<tr>
<td>14-56</td>
<td>51</td>
<td>17</td>
<td>2 (0.2-25.3)</td>
<td>0.593</td>
</tr>
<tr>
<td>≥56</td>
<td>35</td>
<td>7</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

- In the multivariate model (including pre-HAART LAM and CD4 T-cell count) only “CD4 T-cell count <50/mm³” was significant: p=0.004; OR: 21.0 (2.6-169.4)
Figure 1

TB-IRIS study
302 enrolled

LAM tested
133

Included for analysis
92

No urine stored (169)

Late onset IRIS (5)
Other type of IRIS (11)
Less than 3 months FU (25)

TB IRIS
26

Non TB IRIS
66
Figure 2A

Figure 2B
Figure 3
Figure 1: Flow chart

Figure 2A: Variation in LAM concentration during HAART follow up in TB IRIS (□) and non TB IRIS (■) individuals (IQR, median, and outliers (●)).

Figure 2B: Dynamic of LAM concentration on HAART in patients developing TB IRIS at different time points including TB IRIS (●).

Figure 3: Relation between TST reaction (mm) and LAM concentration (ng/ml).