

# Blood parasite load as an early marker to predict treatment response in visceral leishmaniasis in Eastern Africa

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**Summary:** Blood *Leishmania* parasite load, determined by qPCR, is a promising early biomarker to predict relapse in visceral leishmaniasis patients and might particularly be useful in the context of dose finding studies of new chemical entities.

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## ABSTRACT

**Background** In order to expedite the development of new oral treatment regimens for visceral leishmaniasis (VL), there is a need for early markers to evaluate treatment response and predict long-term outcomes.

**Methods** Data from three clinical trials were combined in this study, where Eastern African VL patients received various antileishmanial therapies. *Leishmania* kinetoplast DNA was quantified in whole blood with real-time quantitative PCR (qPCR) before, during and up to six months after treatment. The predictive performance of pharmacodynamic parameters for clinical relapse was evaluated using receiver-operating characteristic curves. Clinical trial simulations were performed to determine the power associated with the use of blood parasite load as a surrogate endpoint to predict clinical outcome at six months.

**Results** The absolute parasite density on day 56 after start of treatment was found to be a highly sensitive predictor of relapse within six months of follow-up at a cut-off of 20 parasites/mL (AUC 0.92, specificity 0.91, sensitivity 0.89). Blood parasite loads correlated well with tissue parasite loads ( $\rho = 0.80$ ) and with microscopy gradings of bone marrow and spleen aspirate smears. Clinical trial simulations indicated a >80% power to detect a difference in cure rate between treatment regimens if this difference was high (>50%) and when minimally 30 patients were included per regimen.

**Conclusion** Blood *Leishmania* parasite load determined by qPCR is a promising early biomarker to predict relapse in VL patients. Once optimized, it might be useful in dose finding studies of new chemical entities.

**Keywords:** visceral leishmaniasis, parasitemia, biomarker, pharmacodynamics, qPCR

## 1. INTRODUCTION

There is an urgent need to develop field-adapted oral efficacious treatments for the neglected tropical parasitic disease visceral leishmaniasis (VL), particularly in Eastern Africa. New candidates with different mechanisms of action have been identified and are progressing to clinical development [1]. To facilitate drug development, accurate tools are needed to evaluate treatment efficacy early after the treatment, which is a specific research priority for neglected tropical diseases according to the World Health Organization (WHO) [2,3]. This will allow early selection of promising drug regimens and will reduce the number of subjects exposed to regimens with poor efficacy.

Treatment evaluation is complicated, since initially cured patients can relapse due to recrudescence of parasites, which is a long-term event that is particularly difficult to predict [4]. Therefore, definitive cure in Eastern African VL clinical trials is generally assessed at six months after completion of treatment, defined as a negative parasitological test of cure at the end of treatment (absence of *Leishmania* amastigotes in spleen or bone marrow aspirate smears by microscopy), lack of VL clinical symptoms and no requirement for rescue treatment during 6 months follow-up. To speed up treatment evaluation, sensitive and specific biomarkers are needed to monitor treatment response and predict relapses. These biomarkers would be particularly useful in clinical trials with new chemical entities, where they could serve as a surrogate endpoint at an early time point after treatment.

Splenic aspiration is an invasive procedure, associated with risk of severe hemorrhage [5,6], and cannot be performed in patients with unpalpable or reduced spleen size at the end of treatment. Quantification of blood parasite load by real-time quantitative PCR (qPCR) can be an alternative: previous results suggest that positive blood parasite load after treatment is associated with a higher risk of VL relapse [7–19]. In HIV co-infected patients, blood parasite load >10 parasites/mL preceded clinical relapse [7]. However, risk of VL relapse in HIV co-infection is affected by other factors such as CD4 count [11]. In Eastern Africa, the region with the highest VL incidence globally, very limited *Leishmania* qPCR data has been published in the context of VL [20,21]; only a small study in 11 patients focused on the relation with clinical outcome [19].

To evaluate the pharmacodynamic potential of blood parasite load as a predictor for clinical relapse, we longitudinally quantified the blood parasite load using qPCR in patients from three multicenter Eastern African clinical trials. The first objective was to identify the most optimal predictor for VL treatment outcome at six months in terms of absolute or relative blood parasite load and time of sampling. Secondly, blood parasite loads were compared with tissue aspirate parasite loads to assess whether the parasite biomass in whole blood is reflecting that in the primary infected organs. Thirdly, the sensitivity of blood and tissue qPCR parasite loads were compared with microscopic readings of tissue samples. Lastly, the predictive power was quantified for different clinical trial scenarios with variable efficacy rates where this pharmacodynamic marker could hypothetically be used as early surrogate endpoint.

## 2. METHODS

### 2.1 Study sites and patients

Data originated from three Phase II open-label randomized clinical trials, to assess the safety and efficacy of different treatment regimens in the treatment of VL in Eastern Africa: LEAP0208 (NCT01067443 [21]); LEAP0714 (NCT02431143 [22]); and FEXI VL 001 (NCT01980199). Ethical approval was obtained from national and local Ethics Committees in Kenya, Sudan, and Uganda. Further patient and treatment details can be found in the Supplementary Material.

## 2.2 Clinical assessment of efficacy and sample collection

An initial cure was defined by improvement of clinical signs and symptoms of VL and a negative parasitological test of cure by microscopy at day 28. Patients who died or required rescue treatment before completion of study treatment were considered initial treatment failures. A definitive cure at day 210 (6 months) was defined as a patient who had initial cure and remained free of VL signs and symptoms, *i.e.*, no occurrence of relapse during the follow-up period and no requirement for rescue treatment.

Microscopic parasitological assessments on aspirate smears from lymph node, bone marrow, or spleen (LEAP0208 and FEXI VL 001), or spleen or bone marrow (LEAP0714) were performed at baseline and on day 28 in all studies; it was repeated at day 56, day 210 or in an unscheduled visit if clinically indicated, due to reappearance of VL signs and symptoms, suspecting of relapse. In LEAP0714 and FEXI VL 001, part of the tissue aspirate samples intended for microscopy were also collected to perform qPCR. Whole blood EDTA samples with a volume of 200  $\mu$ L were collected for pharmacodynamic assessment prior to treatment and nominally on day 3, 7, 14, 28, 56, 210 (LEAP0208), on day 3, 7, 14, 21, 28, 56 (LEAP0714), and on day 1, 3, 5, 8, 11, 14, 28, 56, 210 (FEXI VL 001).

## 2.3 Microscopy and molecular methods

Parasitological assessments in the studies were adapted according to the practice of tissue aspiration (spleen, bone marrow and lymph node) for VL diagnosis in the different countries. In LEAP0208, parasitological assessment by microscopy was done on lymph node aspirates (Dooka, Kassab), spleen aspirates (Kimalel) or bone marrow aspirates (all sites). In LEAP0714, spleen aspirates were collected or, under specific circumstances (see [22]), bone marrow aspirates. In FEXI VL 001, lymph node or bone marrow samples were collected. Aspirates were smeared on two slides per sample, stained with Giemsa and graded on a semi-quantitative logarithmic scale from 0 (no parasites in 1000 microscopic fields) to 6+ (>100 parasites per microscopic field). Measurements of the *Leishmania* parasite load in whole blood samples and tissue samples were performed using a qPCR method targeting *Leishmania* kinetoplastid DNA (kDNA). A detailed description of the DNA extraction, used primers, and qPCR protocol can be found in the Supplementary Material.

## 2.4 Data and statistical analysis

Data cleaning, statistical analysis, and clinical trial simulations were performed with R (version 3.5.1). qPCR data were excluded from the analysis for patients who were considered initial treatment failures, for samples collected after rescue treatment was given, or for samples considered unreliable. Absolute blood parasite concentrations and relative changes over time were evaluated for their ability to discriminate between cured and relapsed patients. Absolute and log-transformed data were checked for normality and equal variances using the Shapiro-Wilk test. Logistic regression was performed by an unpaired one-sided Wilcoxon signed rank test to compare blood parasite loads at baseline, day 28, and day 56 after start of treatment. Subsequently, receiver-operating characteristic (ROC) curves were generated with the R package “pROC” and “plotROC”. The area under the curve (AUC) was compared to find the most predictive parameter for clinical relapse in terms of follow-up day (day 14, day 28, or day 56 after start of treatment), and absolute parasite load or relative to baseline. Furthermore, the interplay between sensitivity and specificity of blood parasite load as a biomarker was evaluated and the optimal cut-off value was determined.

To evaluate the correlation between blood parasite load and tissue parasite load obtained by qPCR, Spearman's rank correlation rho was determined. The relationship between the two sources was determined by linear regression on log-transformed data, excluding data below the limit of quantitation (BLQ). The correlation between available matched qPCR blood and tissue loads and

microscopy gradings of aspirate smears was evaluated visually. To evaluate the sensitivity of the qPCR and microscopy method, the percentage of samples having detectable parasites was compared.

### 2.5 Surrogate endpoint evaluation

Finally, clinical trial simulations were performed to evaluate the predictive performance and power associated with the use of qPCR blood parasite load on either day 28 or day 56 as a surrogate endpoint to predict final clinical outcome at six months. For this we used non-inferiority clinical trial scenarios where a control treatment arm (90% cure rate at six months), representing current standard of care [1], was compared to an alternative treatment arm with lower, varying, cure rates (20%, 40%, 60%, and 80%). Patient populations ( $n = 10, 20, 30, 40, 50$ ) were sampled with replacement from the pool of cured ( $n = 143/147$ ) and relapsed ( $n = 30/32$ ) patients on day 28/56 in our original dataset. While the actual cure rate was pre-defined, the predicted cure rate of both populations was derived based on blood parasite load at day 28 or 56, based on the optimal cut-off. To simulate the performance of the qPCR procedure realistically, previously excluded and missing samples were included in these simulations. Fisher's exact test was used to test if these populations had significantly different predicted cure rates, based on blood parasite load. Per scenario, 1000 clinical trials were simulated. The power was defined as the number of times a significant difference was found between treatment arms and was considered adequate when  $>80\%$ .

### 3. RESULTS

In total, blood parasite loads were available from 177 patients, ( $n = 134$  for LEAP0208,  $n = 29$  for LEAP0714,  $n = 14$  for FEXI-VL-001), treated with 5 different treatment regimens. Overall, 15.8% of blood and 16.3% of tissue qPCR data had to be excluded (Table 1). Main reasons for exclusion of data were an unreliable or incomplete DNA extraction of the sample (based on glyceraldehyde 3-phosphate dehydrogenase (GAPDH)), bad sample quality, or insufficient sample material. None of the microscopic readings were excluded.

A difference in blood parasite load dynamics between cured and relapsed patients could be observed in all treatment groups (Figure 1). In total, cured patients had a significantly lower parasite load on day 28 ( $p = 3.91^{-06}$ ) and on day 56 ( $p = 2.58^{-14}$ ) (Table 2). Remarkably, cured patients also had a significantly lower baseline parasite load ( $p = 0.030$ ). This correlation has been demonstrated earlier for tissue baseline parasite loads detected by microscopy in HIV co-infected patients [23]. Baseline parasite loads were not significantly different between treatment groups.

The ROC AUC for absolute blood parasite load classifying clinical relapse (Figure 2) was highest on day 56 (0.92), compared to day 14 (0.71) and day 28 (0.74). The optimal cut-off value on day 56 was 20 p/mL, corresponding to a sensitivity of 89% and a specificity of 91%. ROC curves of relative parasite load at day 14, 28, or 56 in relation to baseline were also evaluated, resulting in comparable AUCs (0.93 on day 56), and thus the absolute parasite load was preferred since only a single sample is needed. Based on a threshold of 20 p/mL on day 56, 67.6% of patients in this study were correctly categorized as relapsed for day 210 outcome, taking into account missing samples to evaluate the overall performance of the sampling procedure, extraction, and qPCR method. Without missing samples, 85.2% of patients were correctly categorized as relapsed, representing the performance of the qPCR method. Relapsed patients not predicted at day 56 relapsed at day 68, 86, 108, and 112, whereas correctly predicted relapsed patients relapsed at day 102 (median) (IQR 64.5-136.5).

There was a significant correlation between matching log-transformed blood and tissue qPCR results ( $\rho = 0.80$ ), indicating an approximately 2 log higher parasite load in spleen, bone marrow, or lymph node compared to whole blood (Figure 3). In total, 302 blood qPCR samples and 71 tissue qPCR samples were compared to matching microscopy gradings of tissue aspirate smears (Figure 4 and 5). When stratified by tissue source, there was a positive trend between the two scores, especially

in samples from bone marrow and spleen. At start of treatment, parasites were detectable by microscopy in all tissue samples (microscopy grading >0), whereas 6% of matching blood qPCR samples were negative (Table 3). When no parasites were detected by microscopy on day 28, parasites were still detected by qPCR in 36% of blood samples (Table 3). Parasites were detectable by qPCR in all of the available tissue samples (data not shown).

In the clinical trial simulations, absolute blood parasite load on either day 28 or day 56 was evaluated as a surrogate endpoint to predict clinical cure for various treatment regimens, with a threshold of  $\leq 20$  p/mL based on the ROC curves. The power of different simulation scenarios is shown in Figure 6. Clinical trial simulations demonstrated that the power to detect a difference in cure rate was higher when blood parasite load on day 56 was used, instead of day 28, in accordance with the ROC curves. When blood parasite load on day 56 was used, clinical trials only achieved a >80% power when the difference in cure rate was high (>50%) between the hypothetical investigational regimen and a standard of care regimen with an efficacy of 90%, and when sufficient patients were included. For example, to identify an insufficient treatment regimen with 40% cure rate, at least 30 patients per treatment regimen need to be included. For alternative treatment regimens with higher cure rates, no adequate power was achieved with a sample size  $\leq 50$  subjects per treatment regimen.

#### 4. DISCUSSION

In this study, various parasitemia parameters were evaluated for their sensitivity and specificity in classifying and predicting final treatment outcome in a large Eastern African VL patient population. Absolute parasite load on day 56 was a highly sensitive predictor of relapse at a cut-off of 20 p/mL. When compared to other approaches, the surrogate marker can be assessed early (day 56 instead of 6 months) compared to IgG1 antigen detection [24] and more specific compared to antigen detection in urine [25]. The low cut-off value found in this study indicates that blood parasite loads as low as 20 p/mL are associated with a higher risk of disease relapse, even when patients do not yet present reoccurrence of clinical symptoms. Previously this has only been demonstrated in HIV co-infected patients, where values ranging from 0.03 – 42 p/mL indicated relapse [7,17,18,26].

A potential drawback of this biomarker is that blood represents only a proximal site for the total parasite biomass in the human host, of which the mainstay is resident in infected organs (e.g. liver, spleen and bone marrow). This is in line with our findings, as qPCR was approximately 2-log higher in tissue compared to whole blood. Another potential source of bias might be lingering kDNA of dead parasites in the circulation. However, a rapid clearance of circulating *Leishmania* kDNA immediately after treatment initiation has been shown previously, following clinical recovery [13]. Additionally, qPCR blood parasite load showed a good correlation with qPCR parasite load in tissue ( $\rho = 0.80$ ), indicating that whole blood is a good proxy compartment to monitor the parasite biomass in the infected tissues.

qPCR has been shown to be a sensitive method to measure blood parasite load previously [16,18,19,27,28], as well as in this study. Both blood and tissue qPCR parasite loads showed a correlation with microscopy gradings from aspirate smears; the clearest trend was observed between spleen qPCR and microscopy gradings. The observed correlation is in line with previous data from India [29]. qPCR analysis seems to be a more sensitive method, as parasites were detectable by qPCR in all tissue samples and in 76.7% of blood samples, compared to 60.5% of tissue samples by microscopy. The high sensitivity of qPCR on whole blood, as well as the convenience for the patient, suggest that qPCR is a suitable method for regular patient monitoring. Noteworthy, detectable qPCR blood or tissue parasite loads at end of treatment or during follow-up were observed in patients considered clinically cured. This could indicate that patients can still harbor *Leishmania* parasites at low levels, but nevertheless remain asymptomatic. In the context of a clinical trial this means negative blood qPCR loads cannot replace the gold standard of microscopic examination as a test of cure to

define initial cure and the clinical value of a positive qPCR in a patient without clinical signs and symptoms of disease remains to be defined. It could indicate the need for closer follow-up but not directly rescue treatment, as for an immunocompetent patient the immune system is expected to control the infection, conferring long-lasting protection [30,31].

To evaluate the usefulness of early blood parasite load as a surrogate endpoint for long-term clinical outcome in clinical trials evaluating novel drug regimens, clinical trial simulations were performed. The use of blood parasite load on day 56 might be suitable to identify insufficient treatment regimens or dose levels with a very poor cure rate of 40% or less and stop early for futility. However, the power will improve when the number of excluded samples can be reduced, *e.g.* by improving the performance of DNA extraction.

With the introduction of new chemical entities as clinical candidates for VL treatment, there is a need for better and more accurate tools to evaluate their efficacy at early time-points, as to allow for adaptive study design to select promising drug regimens and reduce the number of subjects exposed to regimens with poor activity. This is the first study that evaluated the predictive value of qPCR for long-term clinical outcome and its use as a surrogate endpoint in clinical trials for VL, by using a large dataset from different studies in Eastern African VL patients, including treatment regimens with different cure rates. The absolute parasite load on day 56 was a highly sensitive predictor of relapse at a cut-off of 20 p/mL, and its potential application has been shown by clinical trial simulations. However, this cut-off value is based on the studied data only, and the exact threshold and time-point may need to be optimized for future compounds, depending on their pharmacokinetic properties, treatment duration, and ultimately their effect on parasite dynamics. With the increase in molecular biology capacity in areas endemic for VL, we expect that it would be feasible to put this tool into practice in clinical trial settings. In the near future, validation of molecular biology tools in blood could be envisaged to replace the current invasive tissue aspiration procedures for parasitological diagnosis and treatment monitoring.

## **NOTES**

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### **CONFLICTS OF INTEREST**

None of the authors had any conflicts of interest.



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## TABLES

Table 1: Overview of the data used for logistic regression (Day 0, 28, 56) and ROC analysis (Day 14, 28, 56), specifying collected and excluded qPCR blood samples, qPCR tissue samples and microscopy scores derived from splenic or bone marrow aspirates.

Day	Study	Blood qPCR		Tissue qPCR		Microscopy score
		Collected samples	Excluded samples (%)	Collected samples	Excluded samples (%)	Available readings
0	LEAP0208	131	14 (11)	N/A	N/A	131
	LEAP0714	30	13 (43)	30	0 (0)	30
	FEXI VL 001	14	5 (36)	10	0 (0)	14
	Total	175	32 (18)	40	0 (0)	174
14	LEAP0208	139	18 (13)	N/A	N/A	N/A
	LEAP0714	30	12 (40)	N/A	N/A	N/A
	FEXI VL 001	14	5 (36)	N/A	N/A	N/A
	Total	183	35 (19)	N/A	N/A	N/A
28	LEAP0208	130	13 (10)	N/A	N/A	126
	LEAP0714	29	5 (17)	29	7 (24)	28
	FEXI VL 001	14	5 (36)	13	5 (38)	14
	Total	173	23 (13)	42	12 (29)	168
56	LEAP0208	136	12 (9)	N/A	N/A	8
	LEAP0714	29	2 (7)	1	0 (0)	1
	FEXI VL 001	13	4 (31)	4	1 (25)	N/A
	Total	178	18 (10)	5	1 (20)	9

Table 2: Blood parasite loads quantified by qPCR at baseline, day 28, and day 56, stratified by clinical outcome at 6 months follow-up.

Day	Cure		Relapse		Difference p-value <sup>b</sup>	
	Total N	N	Parasites/mL <sup>a</sup>	N		Parasites/mL <sup>a</sup>
0	143	117	3070 (720-16290)	26	9760 (2574-63195)	0.030*
28	150	123	0 (0-1.5)	27	20 (0-230)	3.91e-06*
56	156	130	0 (0-2.75)	26	270 (59.2-1242)	2.58e-14*

<sup>a</sup>Values are given as median (inter-quartile range).

<sup>b</sup>Wilcoxon test on absolute parasite concentrations.

\* Significant difference when  $p < 0.05$ .

Table 3: Number (%) of positive and negative blood qPCR loads versus microscopy gradings for matching samples at day 0 (N = 143) and day 28 (N = 135). Microscopy gradings >0 were considered positive.

	<b>Day 0</b>		<b>Day 28</b>	
	Microscopy grading Positive	Microscopy grading Negative	Microscopy grading Positive	Microscopy grading Negative
Total (N)	143	0	10	135
Matching blood qPCR loads				
Positive (N (%))	135 (94%)	0 (0%)	7 (70%)	48 (36%)
Negative (N (%))	8 (6%)	0 (0%)	3 (30%)	87 (64%)

## FIGURE LEGENDS

Figure 1: Median absolute parasite load of cured patients (red line) and relapsed patients (blue line) at baseline, day 14, 28 and 56, stratified per treatment arm. Error bars represent the inter-quartile range. Grey dashed lines represent end of treatment.

Figure 2: ROC curves of absolute parasite load as predictor of clinical relapse on day 14, 28, and 56 of follow-up. AUC represents the integrated area under the ROC curve. Green line: day 14 (AUC 0.71). Red line: day 28 (AUC 0.74). Blue line: day 56 (AUC 0.92).

Figure 3: Correlation between log-transformed qPCR blood and tissue parasite load (matching ID/timepoint) determined in bone marrow aspirates (red), lymph nodes (green), and spleen aspirates (blue). Tissue samples include 4 drops of bone marrow aspirate (~200  $\mu$ L), or the remainder in the needle of the spleen or lymph node aspiration. Data below the limit of quantification are shown as 1 p/mL. Linear regression line (solid line) is based on the combined data, excluding data below the limit of quantification:  $y = 1.5 + 0.97x$ .

Figure 4: Correlation between log-transformed qPCR blood parasite load and grading of amastigotes in aspirate smears by microscopy, stratified by parasite load according to tissue source.

Figure 5: Correlation between log-transformed qPCR tissue parasite load and grading of amastigotes in aspirate smears by microscopy, stratified by parasite load according to tissue source. Tissue samples include 4 drops of bone marrow aspirate (~200  $\mu$ L), or the remainder in the needle of the spleen or lymph node aspiration.

Figure 6: Predictive power of blood parasite load is shown for day 28 (left panel) and day 56 (right panel), with clinical cure defined as parasite load  $\leq 20$  p/mL. The difference in cure rate is the difference between the alternative treatment regimens (20%, 40%, 60%, or 80% cure rate) and the reference treatment regimen (90% cure rate). Sample size ranges from  $n=10$  to  $n=50$ . Dotted horizontal line represents the 80% power cut-off.

Figure 1

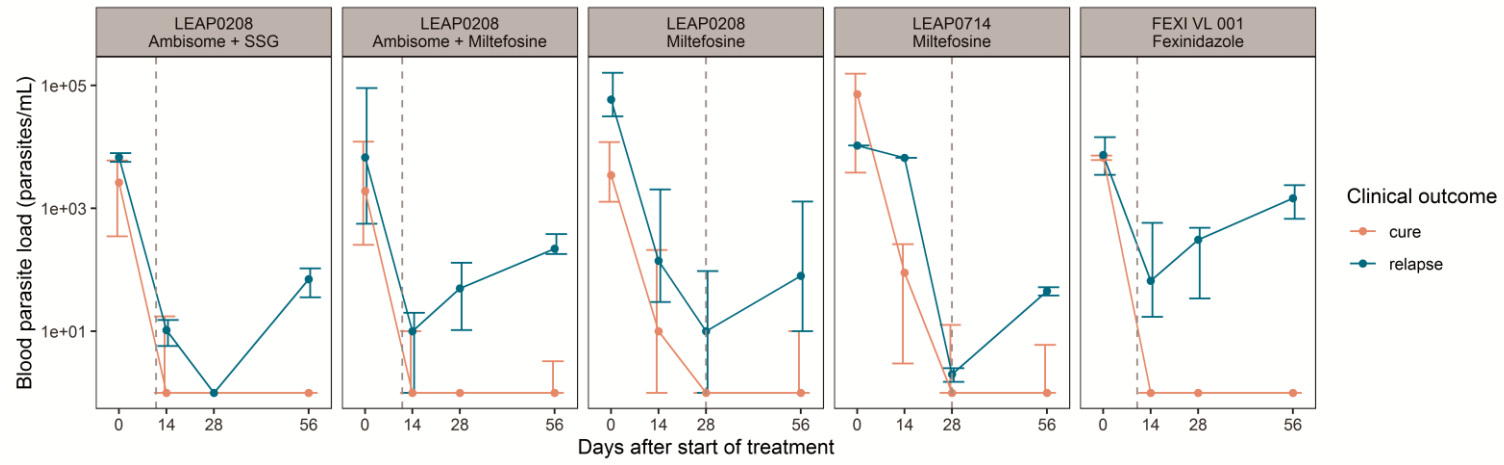




Figure 2

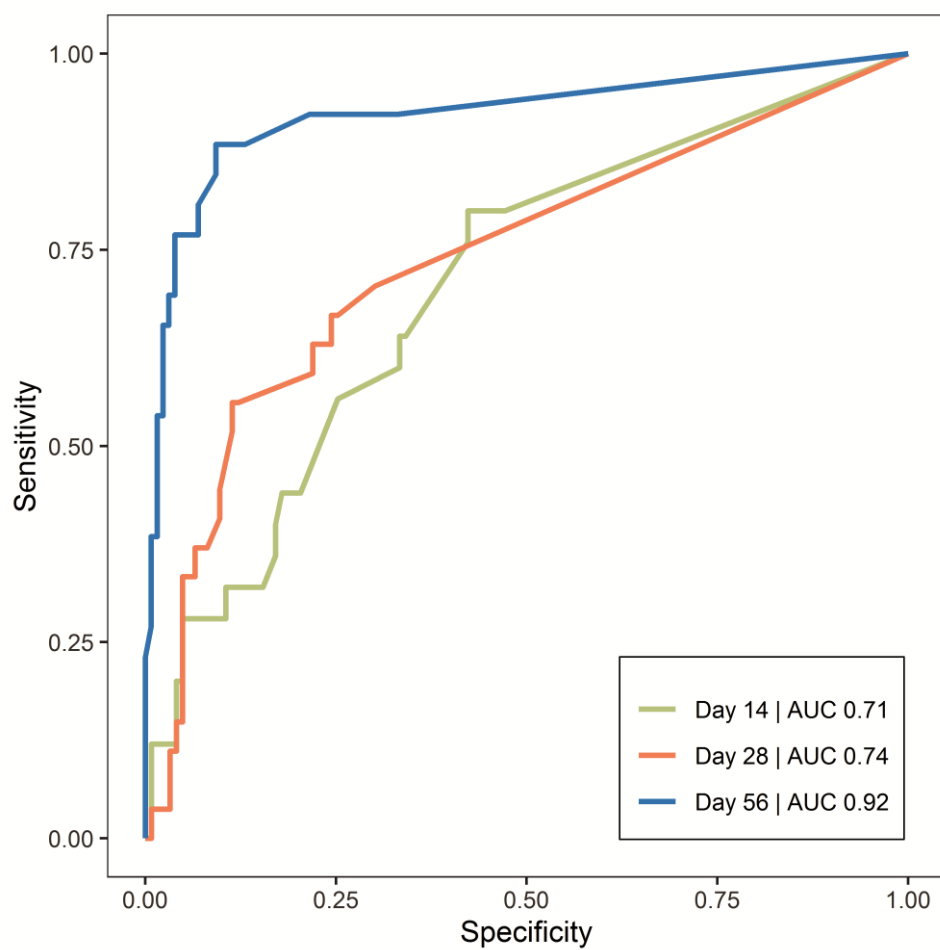


Figure 3

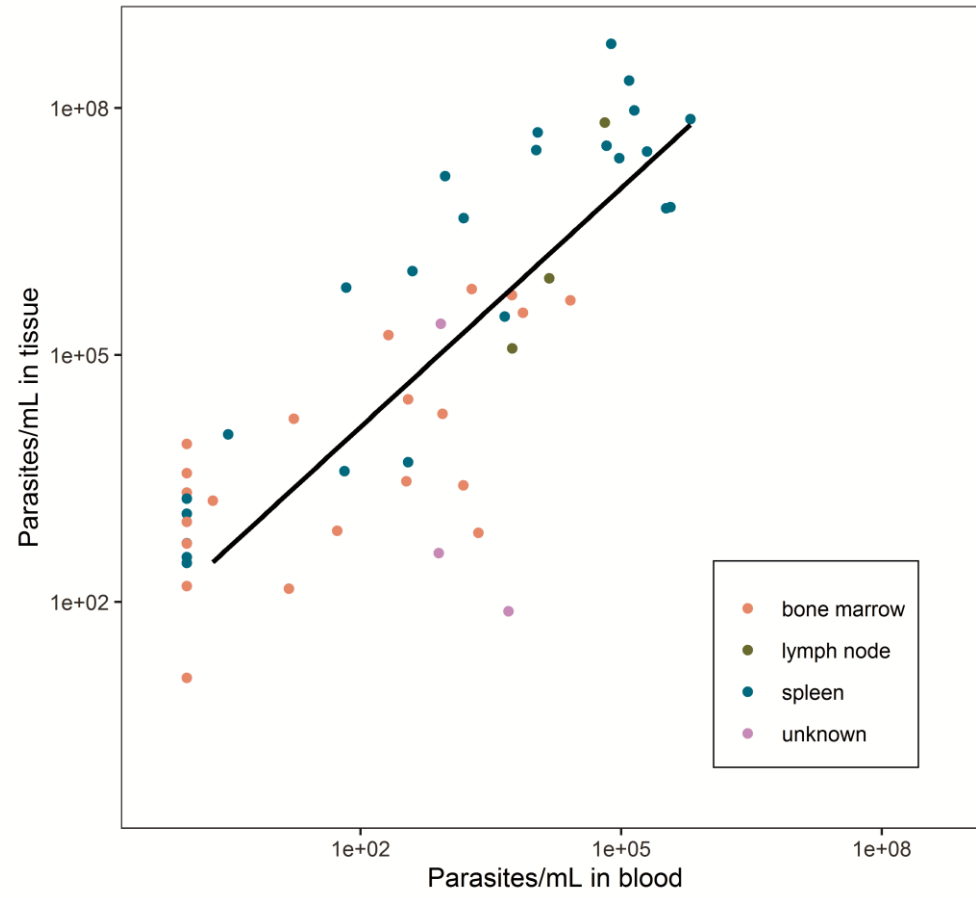


Figure 4

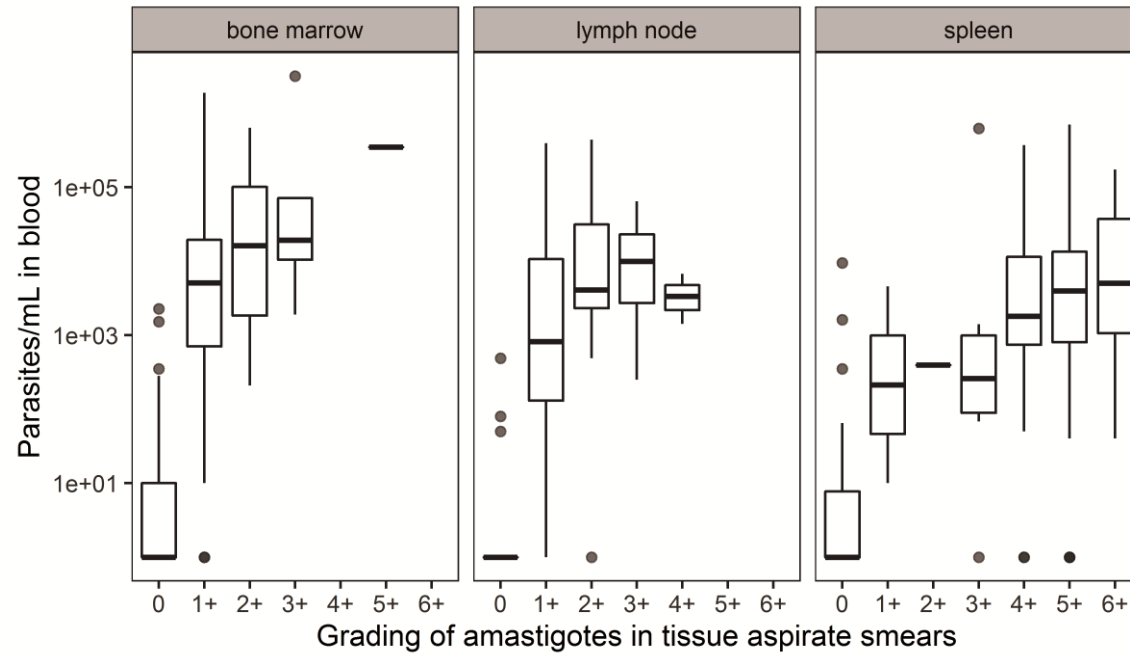


Figure 5

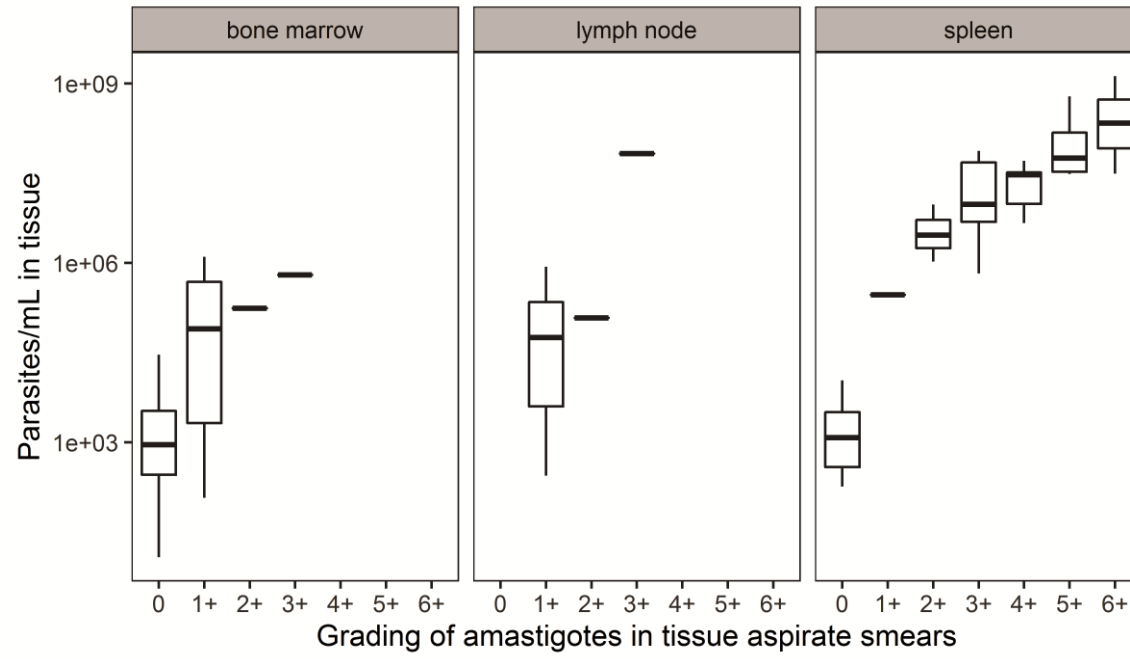


Figure 6

