

# Trypanosome spliced leader RNA for diagnosis of acoziborole treatment outcome in gambiense human African trypanosomiasis: A longitudinal follow-up study



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## Summary

**Background** Detection of spliced leader (SL)-RNA allows sensitive diagnosis of gambiense human African trypanosomiasis (HAT). We investigated its diagnostic performance for treatment outcome assessment.

**Methods** Blood and cerebrospinal fluid (CSF) from a consecutive series of 97 HAT patients, originating from the Democratic Republic of the Congo, were prospectively collected before treatment with acoziborole, and during 18 months of longitudinal follow-up after treatment. For treatment outcome assessment, SL-RNA detection was compared with microscopic trypanosome detection and CSF white blood cell count. The trial was registered under NCT03112655 in [clinicaltrials.gov](https://clinicaltrials.gov).

**Findings** Before treatment, respectively 94.9% (92/97; CI 88.5–97.8%) and 67.7% (65/96; CI 57.8–76.2%) HAT patients were SL-RNA positive in blood or CSF. During follow-up, one patient relapsed with trypanosomes observed at 18 months, and was SL-RNA positive in blood and CSF at 12 months, and CSF positive at 18 months. Among cured patients, one individual tested SL-RNA positive in blood at month 12 (Specificity 98.9%; 90/91; CI 94.0–99.8%) and 18 (Specificity 98.9%; 88/89; CI 93.9–99.8%).

**Interpretation** SL-RNA detection for HAT treatment outcome assessment shows  $\geq 98.9\%$  specificity in blood and 100% in CSF, and may detect relapses without lumbar puncture.

**Funding** The DiTECT-HAT project is part of the EDCTP2 programme, supported by Horizon 2020, the European Union Funding for Research and Innovation (grant number DRIA-2014-306-DiTECT-HAT).

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**Keywords:** Human African trypanosomiasis; *Trypanosoma brucei gambiense*; Treatment outcome; Relapse; RNA; Diagnosis; Blood; Cerebrospinal fluid; Specificity

## Introduction

Human African trypanosomiasis (HAT) is a parasitic disease affecting poor areas of sub-Saharan Africa. Two subspecies of *Trypanosoma brucei* (*T.b.*) are responsible for HAT: *T.b. gambiense* and *T.b. rhodesiense*, which

occur respectively in West- and Central Africa, and in East- and South Africa. They are transmitted to humans by the bite of the tsetse fly vector.<sup>1</sup>

Control of HAT is largely based on diagnosis followed by treatment.<sup>2</sup> If not adequately treated, the

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### Research in context

#### Evidence before this study

Although the medication to treat human African trypanosomiasis has spectacularly improved, a risk for relapse after treatment still exists. As trypanosomes are rarely detectable in the blood of relapses, their diagnosis is mainly based on lumbar puncture followed by examination of the cerebrospinal fluid. Finding of trypanosomes in cerebrospinal fluid confirms relapse but may be delayed, while an increase in white blood cell number is only an indirect indicator. A PubMed search (November 2, 2022) using the terms “gambiense”, “human African trypanosomiasis”, “relapse” and “diagnosis” retrieved 54 publications. The main topics were drug efficacy (31); diagnosis of the neurological disease stage and risk factors for relapse (12); and treatment outcome assessment (11). The latter publications mainly identified cerebrospinal fluid inflammation markers such as white blood cell number, neopterin, IL-10 or other chemokines, total protein, specific antibody or IgM. Most of these markers are not HAT specific and they still require lumbar puncture. Markers allowing a reliable, early and less invasive treatment outcome assessment remain needed. Trypanosomal RNA detection for diagnosis of human African trypanosomiasis is performant, and one study with fragmentary post-treatment follow-up suggested that, for treatment outcome assessment,

specificity of spliced leader-RNA detection in blood is higher than DNA detection.

#### Added value of this study

Through a collaboration with a therapeutic trial, the study systematically collected blood and CSF specimens for RNA detection during the full 18 months follow-up after treatment of 97 HAT patients. Our study showed that specificity of spliced leader-RNA detection in cured patients at 3, 6, 12 and 18 months after treatment ranged from 98.9 to 100% in blood, and was 100% in CSF at 6, 12 and 18 months post-treatment. Furthermore, in a relapse we observed that spliced leader-RNA detection in CSF preceded trypanosome finding by microscopy, and that trypanosomal RNA could be detected in blood.

#### Implications of all the available evidence

Our results support the interest of spliced leader-RNA detection in blood for diagnosis of a relapse. This, together with availability of drugs to treat both stages of HAT such as acoziborole, holds the promise to ban lumbar punctures completely from HAT case management in the future. If sensitivity of RNA detection in blood for diagnosis of relapse is confirmed or could be further increased, it could lead to earlier detection of relapses since patients are less reluctant to undergo blood collection than lumbar puncture.

infection is usually fatal.<sup>3</sup> In the last 2 decades, with the introduction of nifurtimox eflornithine combination therapy (NECT) and fexinidazole, the medication, in particular for treatment of second stage gambiense HAT has significantly improved.<sup>4-6</sup> However, no treatment is 100% effective, and overall around 2–5% of treatment failures do occur.<sup>1,6-9</sup> Early detection of treatment failures is of paramount importance, to avoid the risk for invalidating life-long sequelae or even a fatal outcome. Furthermore, treatment failures, if undetected, might contribute to the transmission of the disease, thus compromising efforts to control the disease.<sup>10</sup> Most relapses are detected late when the individual is showing neurological symptoms. Examination of the blood or lymph for presence of trypanosomes is poorly sensitive for treatment failure diagnosis.<sup>11</sup> Therefore, the cerebrospinal fluid (CSF), obtained after lumbar puncture, needs to be examined.

Occurrence of detectable trypanosomes in CSF may be a late development after treatment failure, and is usually accompanied by very high CSF white blood cell (WBC) counts.<sup>12</sup> The evolution in the CSF of WBC counts, and immunoglobulin M and neopterin concentrations over time are considered as alternative markers for the detection of treatment failure.<sup>13-15</sup> However, these are indirect indicators, reflecting central nervous system inflammation, which are not specific for HAT. In addition, in particular IgM concentrations and WBC

counts in CSF decrease slowly, fail to differentiate between cure and relapse shortly after treatment, and still necessitate lumbar puncture.<sup>16,17</sup> The greatest challenge for treatment outcome assessment remains the identification of non-invasive markers that can avoid lumbar punctures, and are able to determine treatment outcome early after treatment.<sup>3</sup> The introduction of molecular diagnostics raised hope for accurate detection of relapses. However, DNA detection lacks specificity for treatment outcome assessment, as it does not completely differentiate active infection when the parasite is present, from past cleared infection. In some HAT patients, long-term DNA persistence has been observed, despite successful treatment.<sup>18</sup>

The detection of trypanosomal RNA, such as Spliced Leader (SL)-RNA, presents an alternative marker for treatment outcome assessment.<sup>19</sup> Indeed, SL-RNA has been shown to disappear from the blood after effective treatment, unlike DNA.<sup>20</sup> In addition, sensitivity of SL-RNA detection in blood outperforms all traditional techniques for diagnosis of HAT, while in CSF, SL-RNA detection compares in sensitivity to the single modified CSF centrifugation.<sup>21</sup> The detection of SL-RNA in blood could thus offer potential advantages for the non-invasive and earlier detection of treatment failure.

So far, data for SL-RNA in blood of cured patients are limited to a few post-treatment visits, without systematic follow-up.<sup>20</sup> The kinetics of RNA disappearance during

recovery, and RNA reappearance in relapses remains unknown.

We recently performed SL-RNA detection in the blood and CSF of HAT patients in the Democratic Republic of the Congo (DRC).<sup>21</sup> Here, we evaluate the diagnostic performance of SL-RNA detection for gambiense HAT treatment outcome assessment in blood and CSF collected at different time points during the 18 months follow-up of these patients, which were treated with a single dose of acoziborole.<sup>22</sup>

## Methods

### Ethics statement

This study corresponds to the prospective diagnostic trial “Diagnostic tools for elimination and clinical trials for HAT, early tests of cure” (DiTECT-HAT-WP4, [ClinicalTrials.gov](https://clinicaltrials.gov) Identifier: NCT03112655), which received ethical approval from the “Comité Consultatif de Déontologie et d’Ethique” (CCDE) of the French National Institute for Sustainable Development Research (IRD, June 2016 session), from the Institutional Review Board of the Institute of Tropical Medicine in Antwerp (reference 1096/16), and from the Ethics Committees of the Ministry of Health, DRC through the Ngaliema Clinic of Kinshasa (September 2016 session) and the School of Public Health of the University of Kinshasa (reference ESP/CE/136/2020). This diagnostic trial was carried out in conjunction with a prospective therapeutic trial sponsored by DNDi (DNDi-OXA-02-HAT, registered at [clinicaltrials.gov](https://clinicaltrials.gov) as NCT03087955)<sup>22</sup> evaluating the drug acoziborole for HAT treatment, that was approved by the CERSAC, Ethics Committees of the Ministry of Health, DRC through the Ngaliema Clinic of Kinshasa and the CNES (Comité National d’Ethique de la Santé), DRC. Participants first gave their informed consent to participate in the DNDi-OXA-02-HAT trial. Next, a separate written informed consent was obtained for inclusion into the DiTECT-HAT-WP4 study. For minors, an additional written consent was obtained from their legal representative.

### Sample size estimation

The DiTECT-HAT-WP4 study was conducted on a Congolese subset of the HAT patients from the DNDi-OXA-02-HAT therapeutic trial, which took place in Guinea and DRC.<sup>22</sup> The DNDi-OXA-02-HAT trial was planned to take place in 9 HAT treatment centres in DR Congo, with possible extension to Guinea. The sample size was based on the largest number of patients who could be enrolled within a reasonable timeframe. Initially it was expected that 210 late-stage patients (defined as trypanosomes in CSF or  $>20$  WBC/ $\mu$ l) and 150 early-intermediate stage patients (no trypanosomes in CSF and  $\leq 20$  WBC/ $\mu$ l) would be enrolled within

approximately 2 years. Then, following the drastic reduction of the HAT prevalence, the sample size was estimated again to target 155 late stage HAT patients and approximately 50 early-intermediate stage patients. Inclusion for DNDi-OXA-02-HAT took place between October 2016 and March 2019.

### Study sites and inclusion criteria DiTECT-HAT-WP4

Patients for DiTECT-HAT were included consecutively between February 2017 and March 2019, in five Congolese reference district hospitals (out of 12 in the DNDi-OXA-02-HAT trial): Dipumba and Katanda in Kasai Oriental, Ngandajika in Lomami, Masi Manimba in Kwilu and Roi Baudouin in Kinshasa. These hospitals are located in HAT foci (with the exception of Kinshasa), have participated in previous clinical trials on HAT and health staff has been trained extensively in all clinical trial aspects, including diagnosis of HAT. The systematic longitudinal follow-up lasted until September 2020. Confirmed HAT patients were enrolled first in the DNDi-OXA-02-HAT study, where after they were eligible for the DiTECT-HAT-WP4 study. The inclusion criteria for the DNDi-OXA-02-HAT and DiTECT-HAT-WP4 study corresponded: confirmed HAT with *T.b. gambiense* parasites in blood, lymph and/or CSF; age  $\geq 15$  years; give and sign the study consent form; Karnofsky index  $>50$ ; able to take tablets orally; have a permanent address or being retrievable by other people; being able to comply to the follow-up visits schedule and to other constraints of the study and accept to be hospitalized for acoziborole treatment and for the study procedures.

### Diagnosis, treatment and post-treatment follow-up (reference standard)

The detailed procedures for HAT diagnosis have been described previously.<sup>21</sup> Briefly, in all HAT patients, the parasite presence was confirmed by direct lymph examination, by blood examination using the mini-anion exchange centrifugation technique on buffy coat (mAECT-BC) or the mini-haematocrit centrifugation technique (mHCT), or by observation of trypanosomes in the CSF, during the WBC count or after modified single centrifugation. A patient was classified as early or intermediate stage with absence of trypanosomes in the CSF and a WBC count in the CSF of respectively  $\leq 5$  or 6–20 WBC/ $\mu$ l. The late stage of the disease was defined by the presence of trypanosomes in the CSF and/or  $>20$  WBC/ $\mu$ l. After the inclusion in the therapeutic trial, patients were treated with a single dose of acoziborole<sup>23</sup> following the DNDi-OXA-02-HAT trial procedures.<sup>22</sup> To assess treatment outcome, a longitudinal follow-up was carried out with parasitological examinations at day 11 after treatment, and 6, 12 and 18 months post-treatment, and in some cases at month 3. For persons

with swollen cervical lymph nodes, a lymph aspirate was examined. Blood was examined for trypanosome presence by mAECT-BC, or if unfeasible, by mAECT or mHCT. A lumbar puncture was performed (except at three months) and CSF was examined for WBC number and presence of trypanosomes by the modified single centrifugation technique. For quality assurance, presence of trypanosomes, at any moment, was documented by video recording of the microscopic images.

### Blood and CSF collection in PAXgene Blood RNA Tubes

From each HAT patient at the inclusion and during longitudinal follow-up, 2.5 ml of blood were collected in a PAXgene Blood RNA Tube (PreAnalytiX, Switzerland) according to the instructions of the manufacturer. After the collection of CSF for direct examination, 2.5 ml of CSF were collected by holding the PAXgene Blood RNA Tube under the lumbar puncture needle. After collection, the PAXgene Blood RNA Tubes were inverted at least 10 times, and decanted immediately into 10 ml cryotubes. These were stored at  $-20^{\circ}\text{C}$  at the study site for maximum 2 months, and subsequently transported in liquid nitrogen to the Institut National de Recherche Biomédicale in Kinshasa, where they were stored at  $-80^{\circ}\text{C}$  until RNA extraction.

### RNA extraction using the PAXgene Blood RNA Kit

The specimens were thawed at ambient temperature for 4 h and RNA was extracted using a PAXgene Blood RNA Kit following the guidelines of the manufacturer (<https://www.qiagen.com/us/resources/resourcedetail?id=9a4cdd13-8b72-4b33-877c-ba60a83b87be&lang=en>, last accessed on September 5, 2022). As previously described,<sup>21</sup> 4  $\mu\text{l}$  RNA internal extraction control (IEC, Primerdesign Ltd, UK) was added during the lysis step. The obtained RNA extract was stored at  $-80^{\circ}\text{C}$ .

### Reverse transcriptase quantitative PCR for RNA detection

SL-RNA was amplified using SensiFAST SYBR Lo-ROX One-Step Mix (Bioline, UK) with 400 nM of primers 5'-CAATATAGTACAGAACTG-3' (cSL) and 5'-AACAACGCTATTATTAGAA-3' (SL-F) (Integrated DNA Technologies, Belgium) and 4  $\mu\text{l}$  RNA extract in a final volume of 20  $\mu\text{l}$ . All RNA extracts were tested in duplicate, and with (RT) or without (noRT) addition of reverse transcriptase (RT) (Bioline, UK). Cycling conditions using AriaMX Real-Time PCR System Machine (Agilent Technologies, USA) were  $45^{\circ}\text{C}$  for 10 min,  $95^{\circ}\text{C}$  for 2 min, and 40 cycles of  $95^{\circ}\text{C}$  for 2 s,  $50^{\circ}\text{C}$  for 10 s and  $60^{\circ}\text{C}$  for 5 s. The melting curve was recorded between  $45^{\circ}\text{C}$  and  $95^{\circ}\text{C}$ , using increments of  $0.5^{\circ}\text{C}$  for 5 s. Each qPCR run included a positive and negative control consisting of respectively RNA from human blood

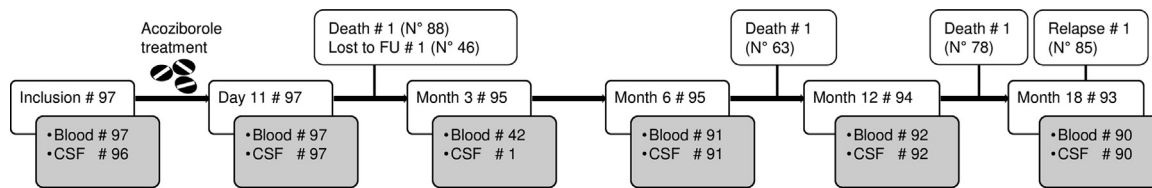
spiked with *T.b. gambiense* LiTat 1.3, and of nuclease-free water. Cq-values were called when the normalized reporter fluorescence (Rn) value minus the Rn value of the baseline signal generated by the instrument ( $\Delta\text{Rn}$ ) surpassed the automatically calculated threshold fluorescence for each run. Melting curve data were calculated based on the negative derivative ( $-\text{Rn}'$ ) of the normalized fluorescence of each well versus the temperature for which the highest peak value was reported as the melting temperature of the amplicon ( $T_m$ ). The IEC was amplified using qScript XLT One-Step RT-qPCR ToughMix, Low ROX, using 1  $\mu\text{l}$  of a 20 fold mix containing primers and a FAM-labelled probe (Primerdesign Ltd, UK) and 5  $\mu\text{l}$  of RNA extract in a 20  $\mu\text{l}$  reaction. Cycling conditions were  $50^{\circ}\text{C}$  for 10 min,  $95^{\circ}\text{C}$  for 1 min and 40 cycles of  $95^{\circ}\text{C}$  for 5 s, and  $60^{\circ}\text{C}$  for 1 min, according to the manufacturer's recommendations (Primerdesign Ltd, UK).

### Data management and analysis

At the study sites, data for the DNDi-OXA-02-HAT trial were entered on paper forms. Scanned paper case report forms were tracked and reconciled before entry into the database management system ClinInfo. Numerical data were entered by a double data entry process. Programmed edit checks were run on the clinical database after new information was entered. Manual queries were generated by the data manager, the monitors, the investigators or the medical responsible. Queries were validated by the data manager and sent to the monitors for further action at the study site level. Data for DiTECT-HAT-WP4 patients were filtered out of the DNDi-OXA-02-HAT database and transferred in Excel. An RNA extract was considered positive for SL-RNA if the Cq-value was below 35 and both RT replicates were associated with a melting temperature ( $T_m$ ) between 65 and  $68^{\circ}\text{C}$ . If Cq was higher than 35 or the  $T_m$  was not correct, SL-RNA detection was considered negative. The noRT reaction was used to calculate the contribution of genomic DNA to the Cq-value of the RT reaction (the % contaminating gDNA is only mentioned in the results if detected), according to the following equation<sup>24</sup>

$$\%gDNA = \left( \frac{2^{-Cq_{noRT}}}{2^{-Cq_{RT}}} \right) \times 100$$

Reference standard results were not known to the reader of the index tests. For each RNA extract, the qPCR results were combined with patient number, clinical sample (blood or CSF), time point (inclusion; day 11; month 3; 6; 12 or 18) and the patients' HAT status (early, intermediate or late stage; parasitological test result in blood and lymph; parasitological test result in CSF; and CSF WBC count/ $\mu\text{l}$ ) before treatment to generate a facet grid graph using the ggplot2 package (v3.3.6) in R (v4.0.2).



**Fig. 1: Flow-chart of DiTECT-HAT-WP4 study conduct.** At each control visit after treatment the number (#) of participants presenting and the number of specimens available for RNA detection is summarized. Events interrupting the follow-up are shown, including corresponding patient number (N°). FU: follow up, CSF: cerebrospinal fluid.

For proportions, GraphPad Prism 9.4.1 was used to calculate 95% Wilson confidence intervals (CI) and for continuous values to calculate the median, 25 and 75 percentiles and maximum. There was no allowance for multiplicity. Confounding between pre-treatment SL-RNA detection and baseline values was assessed by constructing 2x2 tables (continuous variables were dichotomized according to the median value), and their association was assessed with the Fisher exact test.

Sensitivity was calculated before treatment as the number of index test positives over the number of reference test positives (for blood all trypanosome confirmed HAT patients, for CSF either patients with trypanosomes in CSF or with 2nd stage HAT), after exclusion of subjects with missing specimens or with IEC negative specimens. After treatment, specificity at a defined time point was defined as the number of index test negatives over the total number of treated patients that did not relapse (relapse being presence of trypanosomes in any body fluid after treatment), after exclusion of subjects that i) did not present at the given time point or; ii) for whom no specimen was taken or stored that time point or; iii) that had a negative IEC after RNA extraction of their specimen.

### Role of the funding source

The funders had no role in the study design, specimen collection, analysis and interpretation of data, in manuscript writing or the decision to submit the manuscript for publication.

## Results

Out of a total of 208 HAT patients included in the DNDi-OXA-02-HAT trial,<sup>22</sup> 174 were included in DRC, of whom 97 were enrolled in DiTECT-HAT-WP4 (Fig. 1).<sup>21</sup> Fifty HAT patients were female and 47 male, median age was 31 years (interquartile range 24–45, maximum 68). Baseline characteristics of the included HAT patients are summarized in Table 1. Ninety-three (93/97) patients completed the 18 months post-therapeutic follow-up (3 patients died, 1 lost to follow-up), of whom one patient relapsed (N° 85) and all others were considered cured.

Results for each individual, showing C<sub>q</sub> values for RT-qPCR at each time point before and after treatment are presented in Fig. 2 for blood and Fig. 3 for CSF. The corresponding C<sub>q</sub>s of IEC, reflecting the efficiency of RNA extraction in each specimen, are shown in the supporting information figures (Figures S1 and S2).

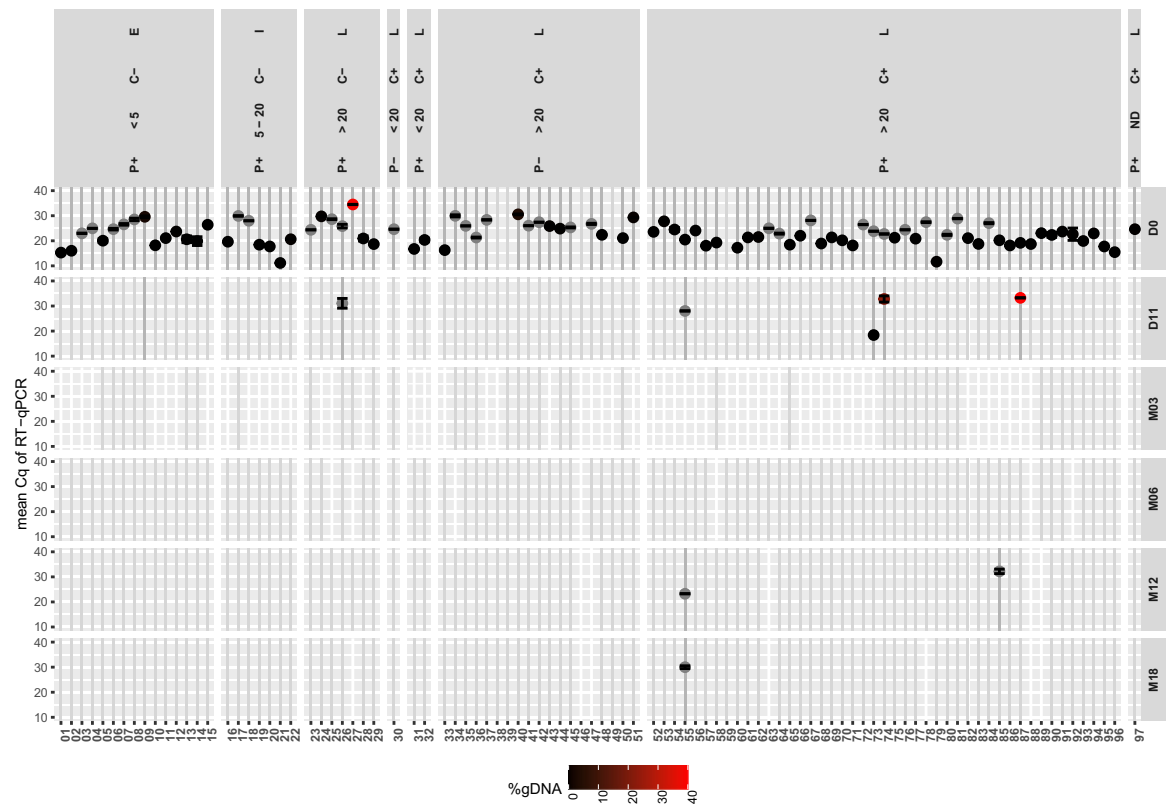
### Results at inclusion, before treatment

All 97 included HAT patients had trypanosomes in one or more body fluids. Lymph or blood were trypanosome positive in 77 of the 97 enrolled HAT patients (79.4%, CI 70.3–86.2%), while 92 tested SL-RNA positive in

Variable	Inclusion (n = 97)
<b>Demographics</b>	
Man	47 (48.6%, CI 38.8–58.3%)
Women	50 (51.6%, CI 41.7–61.2%)
Age (years)	31 (IQR 24–45, max 68)
<b>Hospital</b>	
Dipumba	23 (23.7%, CI 16.4–33.1%)
Katanda	9 (9.3%, CI 5.0–16.7%)
Ngandajika	10 (10.3%, CI 5.7–17.9%)
Masi Manimba	53 (54.6%, CI 44.7–64.2%)
Kinshasa (Roi Baudouin)	2 (2.1%, CI 0.6–7.2%)
Karnofsky index	80 (IQR 70–80, max 100)
Health status – deteriorated	35 (36.1%, CI 27.2–46.0%)
<b>HAT diagnosis</b>	
Presence of trypanosomes	
In lymph	31 (32.0%, CI 23.5–41.8%)
In blood <sup>a</sup>	46/67 (68.7%, CI 56.8–78.5%)
In CSF	68 (70.1%, CI 60.4–78.3%)
White blood cells/ $\mu$ l in CSF	218 (IQR 17–507, max 2705)
<b>Disease stage</b>	
Early	15 (15.5%, CI 9.6–24.0%)
Intermediate	7 (7.2%, CI 3.5–14.2%)
Late	75 (77.3%, CI 68.0–84.5%)

Data are n (%), 95% confidence interval) or median (interquartile range, maximum). Early or intermediate stage was absence of trypanosomes in the CSF and a WBC count in the CSF of respectively  $\leq 5$  or 6–20 WBC/ $\mu$ l, late stage was presence of trypanosomes in the CSF and/or  $>20$  WBC/ $\mu$ l. <sup>a</sup>For 30 patients in whom trypanosomes were detected in lymph, no blood examination was done anymore.

**Table 1: Demography and baseline characteristics of the HAT patients at time of inclusion.**



**Fig. 2: Evolution of Cq values of RT-qPCR SL-RNA detection in blood of 97 gambiense HAT patients before and in function of time after treatment (top to bottom).** The results are grouped according to the patients' HAT status before treatment (D0) [E early, I intermediate and L late stage; parasitological test result in blood and lymph (P); parasitological test result in CSF (C); and CSF WBC count/ $\mu$ l; + trypanosome presence; - absence of trypanosomes; ND not determined]. Time points after treatment, shown from the top to the bottom, were day 11 (D11), month 3 (M03), month 6 (M06), month 12 (M12) and month 18 (M18). White line, specimen not available or internal extraction control undetectable. Dots: average Cq of RT-qPCR SL-RNA in blood with duplicate values indicated, the colour indicates the % gDNA.

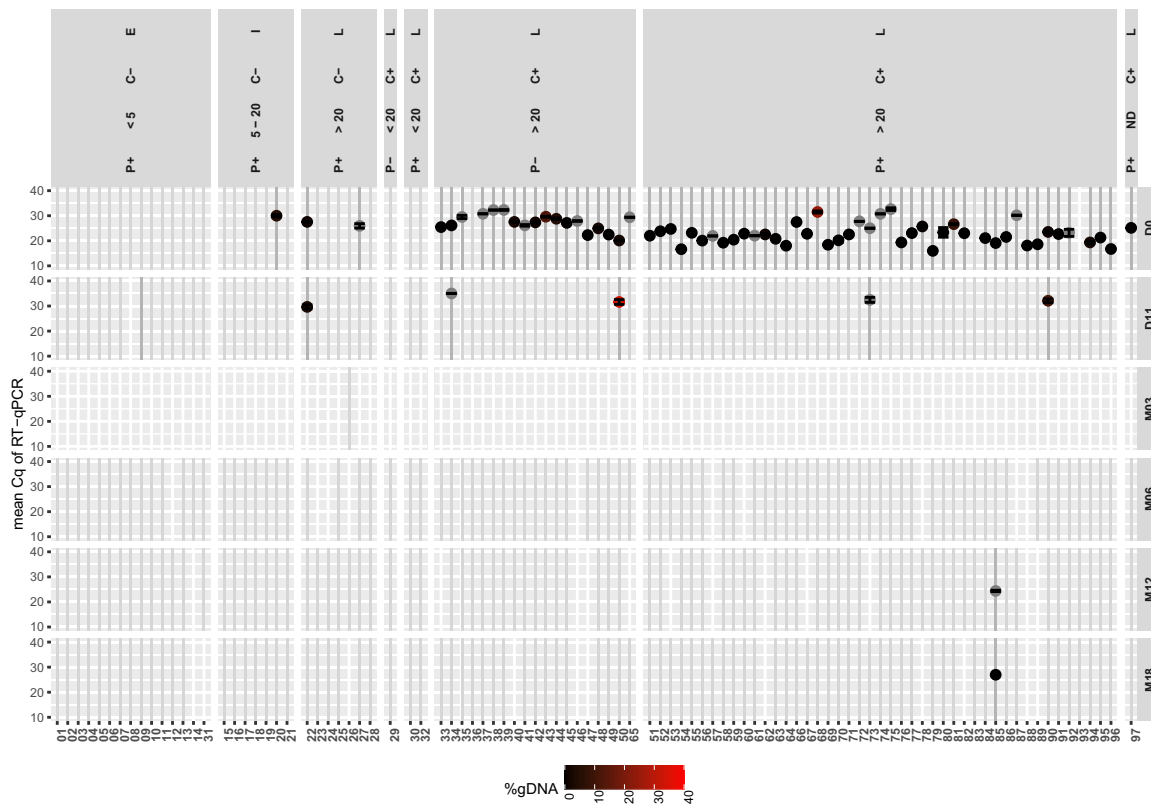
blood (94.9%, CI 88.5–97.8%, Fig. 2). Fifteen HAT patients were classified in early stage (15.5%), 7 in intermediate stage (7.2%) and 75 in late stage (77.3%). The median CSF WBC count was 218/ $\mu$ l (interquartile range 17–507, maximum 2705 WBC/ $\mu$ l). In total 68/97 (70.1%, CI 60.4–78.3%) had trypanosomes in CSF, 65/96 patients were SL-RNA positive in CSF (67.7%, CI 57.8–76.2%, N° 93 CSF specimen missing, Fig. 3). There was an association between CSF SL-RNA positivity and Karnofsky index  $\leq$ 80 ( $p = 0.002$ ), deteriorated health status ( $p = 0.01$ ) and WBC counts  $>$ 218/ $\mu$ l ( $p < 0.0001$ ).

#### Day 11 post-treatment

Eleven days after treatment, blood and CSF were microscopically examined for all 97 patients and lymph for 35 patients. No trypanosomes were detected in any patient suggesting that the infection had been cleared by the treatment. The median CSF WBC count had decreased to 45/ $\mu$ l (interquartile range 10–126,

maximum 946 WBC/ $\mu$ l), a 79.4% reduction compared to the pre-treatment median WBC count.

In blood, 5/97 treated HAT patients still tested SL-RNA positive (5.2%, CI 2.2–11.5%, Fig. 2, Table 2), with average Cqs of 32.8 (patient N° 74, 23.0% of gDNA contamination), 31.1 (N° 26), 28.0 (N° 55), 18.4 (N° 73, 2.8% of gDNA), 33.3 (N° 87, 45.4% of gDNA). In CSF, 96/97 results could be interpreted, one CSF extract had undetectable IEC (N° 08). Five out of 96 CSF extracts (5.2%, CI 2.2–11.6%) still were SL-RNA positive (Fig. 3, Table 2), with average Cqs of 35.0 (patient N° 34), 31.6 (N° 50, 34.0% of gDNA), 29.6 (N° 23, 3.5% of gDNA), 32.1 (N° 90, 11.4% of gDNA), 32.4 (N° 73). One treated HAT patient (N° 73) showed SL-RNA in blood and CSF. All 9 patients (Table 2) remaining SL-RNA positive in blood and/or CSF on day 11 (median CSF WBC 34/ $\mu$ l, interquartile range 24–63, maximum 103 WBC/ $\mu$ l), were in late stage before treatment (median CSF WBC count at inclusion 157/ $\mu$ l, interquartile range 102–336, maximum 487 WBC/ $\mu$ l) and had also tested respectively blood and/or CSF SL-RNA positive at inclusion.



**Fig. 3: Evolution of Cq values of RT-qPCR SL-RNA detection in CSF of 97 gambiense HAT patients before and in function of time after treatment (top to bottom).** The results are grouped according to the patients' HAT status before treatment (D0) [E early, I intermediate and L late stage; parasitological test result in blood and lymph (P); parasitological test result in CSF (C); and CSF WBC count/ $\mu$ l; + trypanosome presence; - absence of trypanosomes; ND not determined]. Time points after treatment of specimen collection were day 11 (D11), month 3 (M03), month 6 (M06), month 12 (M12) and month 18 (M18). White line, specimen not available or internal extraction control undetectable. Dots: average Cq of RT-qPCR SL-RNA in CSF with duplicate values indicated, the colour indicates the % gDNA.

**Month 3 after treatment**

At 3 months after treatment, no routine parasitological examinations or lumbar punctures were foreseen. Of 42 blood specimens collected for RNA detection, none tested

SL-RNA positive (0.0%, CI 0.0–8.4). Upon oral request from the sponsor, one patient underwent a lumbar puncture but was SL-RNA negative (N° 27 with respectively 312, 365 and 46 CSF WBC/ $\mu$ l at inclusion, day 11 and month 3).

N°	Inclusion	Day 11	Month 6	Month 12	Month 18
23	P+; 389; C-; RNA <sup>B,C</sup>	P-; 61; C-; RNA <sup>C</sup>	P-; 13; C-	P-; 4; C-	P-; 8; C-
26	P+; 24; C-; RNA <sup>B</sup>	P-; 17; C-; RNA <sup>B</sup>	P-; 3; C-	P-; 13; C-	P-; 4; C-
34	P-; 67; C+; RNA <sup>B,C</sup>	P-; 2; C-; RNA <sup>C</sup>	P-; 2; C-	P-; 2; C-	P-; 3; C-
50	P-; 318; C+; RNA <sup>B,C</sup>	P-; 35; C-; RNA <sup>C</sup>	P-; 4; C-	P-; 4; C-	P-; 3; C-
55	P+; 2285; C+; RNA <sup>B,C</sup>	P-; 241; C-; RNA <sup>B</sup>	P-; 6; C-	P-; 4; C-; RNA <sup>B</sup>	P-; 3; C-; RNA <sup>B</sup>
73	P+; 165; C+; RNA <sup>B,C</sup>	P-; 67; C-; RNA <sup>B,C</sup>	P-; 4; C-	P-; 5; C-	P-; 3; C-
74	P+; 114; C+; RNA <sup>B,C</sup>	P-; 103; C-; RNA <sup>B</sup>	P-; 13; C-	P-; 6; C-	P-; 4; C-
85	P+; 662; C+; RNA <sup>B,C</sup>	P-; 60; C-	P-; 18; C-	P-; 118; C-; RNA <sup>B,C</sup>	P-; 253; C+; RNA <sup>C</sup>
87	P+; 148; C+; RNA <sup>B,C</sup>	P-; 32; C-; RNA <sup>B</sup>	P-; 9; C-	P-; 3; C-	P-; 1; C-
90	P+; 487; C+; RNA <sup>B,C</sup>	P-; 26; C-; RNA <sup>C</sup>	P-; 6; C-	P-; 4; C-	P-; 5; C-

P: Parasitological test result in blood and lymph; CSF WBC count/ $\mu$ l; C: parasitological test result in CSF; RNA<sup>B,C</sup>: SL-RNA positive in blood (B) or CSF (C); +: trypanosome presence; -: absence of trypanosomes.

**Table 2: Characteristics before and after treatment of 10 patients that tested at least once SL-RNA positive after treatment.**

**Month 6 after treatment**

In total, 95/97 patients accomplished the 6 months control visit. Blood, CSF and lymph were examined in respectively 95, 94 and 12 patients, but no trypanosomes were detected. The median CSF WBC count had further decreased by 96% from the baseline, to 8/μl (interquartile range 4–15, maximum 105 WBC/μl). Only three patients still had >50 WBC/μl in CSF (N° 41, 59 and 79).

Of 91 blood RNA extracts, none tested SL-RNA positive (0.0%, CI 0.0–4.1%). In CSF, RNA extraction was successful in 87/91 specimens while the IEC was undetectable in 4 extracts (N° 12, 61, 78, 87). None of those 87 CSF extracts were SL-RNA positive (0.0%, CI 0.0–4.2%).

**Month 12 after treatment**

In total, 94/97 patients accomplished their 12 months control visit. Blood and CSF were examined for all patients, lymph in 9, and no trypanosomes were detected. The median CSF WBC count was normal at 4 WBC/μl (interquartile range 3–7, maximum 118 WBC/μl), a reduction of 98% compared to pre-treatment. One patient had a CSF WBC to >50/μl, which had increased compared to month 6 (N° 85, Table 2).

Out of 92 blood extracts (2 blood specimens missing), two tested SL-RNA positive in blood (N° 55 and 85, 2.2%, CI 0.6–7.6%, Fig. 2, Table 2), with average Cq values of 23.3 and 32.2 and without detectable gDNA contamination.

Two CSF specimens were missing and no IEC RNA was detectable for 7/92 CSF extracts. With an average Cq of 24.4, SL-RNA was detected (Fig. 3, Table 2) for one out of the remaining 85 CSF extracts (patient N° 85, 1.2%, CI 0.2–6.4%). This individual was also SL-RNA positive in blood and had a WBC count in CSF of 118 cells/μl. The other individual (patient N° 55) with blood SL-RNA Cq 23.3, had no detectable SL-RNA in CSF and had a normal WBC count of 4 cells/μl (Fig. 3, Table 2).

**Month 18 after treatment**

At the final control visit at 18 months, in total, 93/97 patients were re-examined. Respectively 2, 93 and 93

lymph, blood and CSF examinations were performed. The median CSF WBC count remained normal at 3/μl (interquartile range 2–4, maximum 253 WBC/μl). One patient had WBC > 20/μl, individual N° 85, in whom relapse was confirmed, with trypanosomes detected in the CSF and a WBC count of 253/μl.

In blood, one individual (patient N° 55) was blood SL-RNA positive (1/90, 1.1%, CI 0.2–6.0) for a second time with an average Cq of 30.0 (Fig. 2, Table 2), without detectable gDNA contamination.

For 4 out of 90 CSF extracts, the IEC amplification was unsuccessful (N° 14, 15, 40, 82). Only the CSF of the confirmed relapse was SL-RNA positive (N° 85, 1/86, 1.2%, CI 0.2–6.3), with an average Cq of 27.0 (0.17% gDNA, Fig. 3, Table 2). In the blood of this relapse, SL-RNA had become undetectable.

**Diagnostic performance of SL-RNA detection**

Before treatment, SL-RNA detection in blood had a sensitivity of 94.9% (92/97, CI 88.5–97.8%) for HAT (STARD diagram in Figure S3). In CSF, SL-RNA detection was respectively 80.2% sensitive for 2nd stage (1/7 intermediate + 64/74 late = 65/81, CI 70.3–87.5%, 1 late stage CSF specimen missing N° 93), and 92.5% sensitive for trypanosome presence in CSF (62/67, CI 83.7–96.8%, 1 trypanosome positive CSF specimen missing N° 93).

Specificities of SL-RNA detection in blood and CSF at each follow-up time point are shown in Table 3 (STARD diagrams for each study time point in Figures S4–S7). In blood, from month 3 on, specificity ranged from 98.9 to 100% at the individual time points, while in CSF specificity was 100% at 6, 12 and 18 months post-treatment. Only 1 confirmed relapse was observed, rendering sensitivity calculations irrelevant.

**Discussion**

The present study showed that for assessing treatment outcome, specificity of SL-RNA detection at the 6–18 month follow-up visits was ≥98.9% in blood and 100% in CSF. SL-RNA was detectable in blood and CSF of a relapse at the 12 months follow-up visit, while

FU visit	SL-RNA in blood	SL-RNA in CSF
Day 11	94.8% (91/96; CI 88.4–97.8%)	94.7% (90/95; CI 88.3–97.7%)
Month 3	100% (42/42; CI 91.6–100%)	Not applicable
Month 6	100% (90/90; CI 95.9–100%)	100% (86/86; CI 95.7–100%)
Month 12	98.9% (90/91; CI 94.0–99.8%)	100% (84/84; CI 95.6–100%)
Month 18	98.9% (88/89; CI 93.9–99.8%)	100% (85/85; CI 95.7–100%)

The denominator is the number of blood or CSF extracts from non-relapsing patients that were examined. The numerator consists of all of those extracts testing negative for SL-RNA. Were excluded from the specificity calculation: patients that i) did not present at the given time point or; ii) for whom no specimen was taken or stored that time point or; iii) that had a negative IEC after RNA extraction of their specimen. CI: confidence interval; FU: follow-up.

**Table 3: Specificity of SL-RNA detection in blood and CSF for HAT treatment outcome assessment.**



parasitological confirmation was only obtained during CSF examination at 18 months post-treatment.

For this study, we took advantage of collaboration with an ongoing therapeutic trial (DNDi-OXA-02-HAT) taking place in experienced hospitals, which received extensive training.<sup>22</sup> In routine conditions, systematic follow-up after treatment is no longer recommended, and follow-up focuses on symptomatic patients only.<sup>3</sup> In contrast, for clinical trials, post-treatment follow-up with regular control visits at 6, 12 and 18 months after treatment remains mandatory and efforts are made to motivate treated patients to fully comply to follow-up.<sup>25</sup> The DNDi-OXA-02-HAT trial allowed us to study a sub-cohort of well-documented patients, which after treatment were followed-up systematically. Thus, a relatively complete collection of paired blood and CSF specimens, taken at fixed time points after treatment, was established for subsequent SL-RNA detection. To obtain these specimens, no additional lumbar punctures nor venepunctures were required, causing minimal discomfort for the patients. Logistics of frozen specimen storage were shared between both studies.

Despite these advantages, a limitation of the collaboration with a therapeutic trial, was the fact that new drugs being tested in such trials are assumed to be effective, while observation of unacceptably high numbers of relapses would lead to an early stop of the therapeutic trial. Consequently, a low number of relapses could be expected, leading either to inaccurate or impossible estimation of the sensitivity of SL-RNA detection for relapse after treatment. Furthermore, we only studied a subset (# 97) of the therapeutic trial cohort (# 208). This was because the diagnostic SL-RNA detection trial started after the therapeutic trial and it was for logistic reasons limited to the Congolese arm of the therapeutic trial (# 174), while the full trial also included patients in Guinea. The Congolese hospitals where patients were recruited, are HAT diagnosis and treatment reference centres situated in active HAT foci in DR Congo, the country that actually accounts for 70% of all gambiense HAT cases.<sup>26</sup> At the month 3 follow-up visit, although blood was taken to study the pharmacokinetics of acoziborole, no routine parasitological examination was foreseen in the therapeutic trial, nor any lumbar puncture, explaining the reduced number of specimens for SL-RNA detection. An additional limitation is interpretation of missing results. Patients who got lost to follow-up or died for non-HAT related reasons during the follow-up, were considered as cured for specificity calculations at time points where they were still in the cohort, as these patients were not diagnosed as a relapse. Patients with missing specimens, or for whom the IEC in CSF was not detectable, were removed from specificity calculations. Similarly, in the hypothetical case a relapse would occur later than 18 months after treatment, the subject would have been wrongly classified as cured in the actual analysis and sensitivity

of SL-RNA detection would either be overestimated in case of an SL-RNA negative result, or specificity underestimated in case of an SL-RNA positive result. The lack of HAT negative controls could also be considered as a limitation. However, for diagnosis of HAT the specificity of SL-RNA detection in blood had already been determined in previous studies.<sup>19,20</sup> In the present study, we wanted to focus on the accuracy of SL-RNA detection for treatment outcome assessment, within the context of a clinical trial, which did not include HAT negative individuals.

Suboptimal RNA extraction from CSF potentially represented a methodological limitation. This was reflected by overall higher IEC Cq values in CSF (IEC Cq averages of 25.4–32.7, [Figure S2](#)) compared to blood RNA extractions (IEC Cq averages of 20.2–21.9, [Figure S1](#)). Complete loss of the IEC RNA, was observed for only 3.4% (16/466) of the extracted CSF specimens. We previously observed a lower extraction efficacy, in particular in CSF with normal WBC counts.<sup>21</sup> In the present study, the average Cq for the IEC in CSF also increased with a decreasing median CSF WBC count in function of time after treatment ([Figure S2](#), IEC averages of 25.4, 29.3, 29.9, 32.7 and 31.9 at day 0, day 11, months 6, 12 and 18). Suboptimal extraction might negatively impact sensitivity of SL-RNA detection in CSF. Before treatment, SL-RNA positivity in CSF was associated with cell counts >218/μl, Karnofsky index ≤80, and deteriorated health status. Presence of trypanosomes in CSF is known to be associated with increased CSF WBC counts.<sup>12</sup> Also, patients with neurological involvement (defined by increased WBC count and/or presence of trypanosomes in CSF) are more likely to have a low Karnofsky index or a deteriorated health status.

From a methodological point of view, simultaneous detection of SL-RNA and its corresponding gDNA was important. This allowed distinction between active HAT infection witnessed by SL-RNA presence, and traces of past infection represented by detection of the corresponding gDNA.<sup>21</sup> Immediately after treatment, the contributions of contaminating gDNA to the RNA signal were quite elevated, both in blood and CSF. Detection of RNA was carried out to overcome the limited specificity of DNA detection for treatment outcome. Indeed, specificity observed previously with a PCR targeting the 18S ribosomal RNA gene ranged from 80.5 to 92.4% in blood, and 56.3 to 83.7% in CSF between 3 and 24 months after successful treatment.<sup>18</sup> In another study on cured patients, specificity of TBR PCR on blood remained limited to 77.0%, while SL-RNA detection in blood of the same cohort was 98.4–100% specific.<sup>20</sup> In the present study, the SL-RNA RT-qPCR was carried out in duplicate. Observation of a double positive RNA signal allowed unambiguous interpretation of the result as presence of trypanosomal RNA, and doing so, specificities of cured patients between 3 and 18 months after

treatment were as high as 98.9–100% in blood, confirming previous preliminary findings, and 100% in CSF.

At 12 and 18 months after treatment, SL-RNA presence, without DNA contamination, was detected in two patients. In one of those patients (N° 55), at both time points, parasitological examination of blood and CSF was negative and the white blood cell count was normal, and no clinical signs suggesting an infection were present. The patient was therefore classified as cured at the final 18 months follow-up visit. In view of the strength of the signal, and the fact that RNA presence occurred twice in blood in the same patient with an interspace of 6 months, while respective specimens were analyzed separately, the possibility to perform additional blood examinations after the 18 months follow-up visit was explored, to clarify this individual's positive results in SL-RNA. The patient was invited for additional blood examinations to fully exclude the potential occurrence of a late relapse or reinfection but refused and, 32 months after treatment, declared to be in good health. Different explanations for SL-RNA positivity in blood of this patient can be put forward but none can be confirmed or ruled-out in this particular case. First, there is the possibility of a re-infection occurring after successful treatment, since the patient lived in a *T.b. gambiense* endemic area. Second, as SL-RNA is specific for the *Trypanozoon* group, an infection with animal trypanosomes such as *T.b. brucei* cannot be excluded. Third, the two positive tests for this subject were not repeated (nor were any other tests), thus false positivity or contamination cannot entirely be excluded, though we consider it as improbable due to the relatively low Cq values and the fact that positive results for month 12 and 18 were obtained in completely different RNA extraction and amplification reactions.

The second patient in whom a SL-RNA signal was captured after treatment (N° 85), was confirmed as relapse by trypanosome detection in CSF 18 months after treatment, and had abnormal CSF WBC levels at 12 and 18 months. On the one hand, the WBC cell count was interpreted by the investigator as a suspicious relapse at the 12 months control visit, but the patient had no clinical signs.<sup>3</sup> The patient was therefore informed about the possibility of a relapse and was asked to come back as soon as clinical signs occurred. At the 18 month visit, the rescue treatment was administered. On the other hand, a CSF WBC increase above the current thresholds for relapse in HAT might occur in cured patients, although not frequently.<sup>16,17</sup> This is also exemplified by the current cohort, in which, at 6 months after treatment, 3 of the 95 cured patients would have wrongly been considered relapsed based on strict interpretation of the CSF WBC count.<sup>3</sup> Interestingly at 12 months follow-up, 6 months before trypanosomes were observed in the CSF of the suspicious relapsing patient, SL-RNA was detectable in CSF and in blood, underlining the interest of RNA detection for diagnosis

of relapse in blood. Thus, a potential non-CSF based marker has been identified for detecting HAT treatment failure.<sup>11,27</sup> At 18 months post-treatment, SL-RNA in blood became undetectable, while it persisted in CSF. Since the cohort only contained one relapse, no definitive conclusions can be drawn about the diagnostic sensitivity and predictive values of SL-RNA detection for treatment outcome (estimations of negative and positive predictive values are shown in the [Table S9](#), at different relapse rates, taking into account the specificities observed in the actual study and different values for sensitivity). However, presence of SL-RNA in blood should raise concerns about a potential relapse. These results clearly demonstrate the interest to explore target sequences with potentially higher analytical sensitivity for earlier blood based diagnosis of treatment failure.

Surprisingly, at day 11 post-treatment, in 9.3% of patients (9/97, CI 4.8–16.9%) SL-RNA was still detectable in blood and/or CSF, while no trypanosomes were detectable in lymph, blood or CSF. Most bloodstream form trypanosomal mRNAs have half-lives less than 100 min.<sup>28</sup> Acoziborole is a benzoxaborole, a group of drugs displaying activity against *T. brucei*, *Trypanosoma congolense*, *Trypanosoma vivax*, *Trypanosoma cruzi* and *Leishmania*. Benzoxaboroles disturb trypanosomal mRNA polyadenylation and trans-splicing by binding to the active site of the enzyme cleavage and polyadenylation specificity factor 3.<sup>23</sup> The molecule is trypanocidal, seems to exert this activity rapidly and has a long half-life in plasma.<sup>29</sup> *In vitro*, acoziborole used at twice its minimal inhibitory concentration, was demonstrated to kill 50% of *T.b. brucei* within 8 h of exposure, while after 24 h, more than 99% of trypanosomes were killed.<sup>29</sup> Although gDNA SL-RNA detection did contribute to some of the resulting Cq values, the presence of reverse transcribed SL-RNA 11 days after treatment raises questions about the kinetics of trypanosome clearance after a single dose of acoziborole in patients who seemed successfully cured. As a consequence, within 2 weeks after acoziborole treatment, SL-RNA detection might generate positive results that are not indicative for relapse.

We previously showed that SL-RNA detection is more sensitive than the best microscopic parasite detection techniques for diagnosis of HAT. We here demonstrate high specificity of SL-RNA detection for determination of cure. Furthermore, our results support the hypothesis that RNA detection in blood might become a marker of interest for diagnosis of a relapse. This, together with availability of drugs to treat both stages of HAT such as acoziborole, holds the promise to ban lumbar punctures completely from HAT case management. If sensitivity of RNA detection in blood for diagnosis of relapse after treatment is confirmed or could be further increased, it could lead to earlier detection of relapses since patients are less reluctant to undergo blood collection than lumbar puncture.

Despite this advantage, and as already discussed elsewhere,<sup>21</sup> SL-RNA detection is not yet applicable in routine. HAT mainly occurs in remote rural as well as conflict areas of sub Saharan Africa, and diagnostic tests implemented in such contexts should ideally comply with the REASSURED criteria.<sup>30</sup> Complexity of specimen collection and processing, cost and need of specific equipment remain issues preventing large-scale implementation of RT-qPCR. However, for specific purposes, including clinical trials or in patients with suspicion of relapse, RNA detection is a valid option. Biological specimens can be collected on a total nucleic acid stabilizing buffer and expedited within a week to a national or regional reference laboratory where RNA purification and detection can be performed, or where the specimen can be frozen awaiting further analyses. Furthermore, Ebola and COVID-19 epidemics present opportunities for development of new, simplified point of care alternative RNA detection methods including, but not limited to reverse transcription loop-mediated isothermal amplification, reverse transcription recombinase polymerase amplification assays, microfluidic qRT-PCR systems, GeneXpert assays.<sup>31–34</sup>

Other topics for future research include the reason of persistence of SL-RNA in some patients up to 11 days after acoziborole treatment, confirmation of the sensitivity of SL-RNA in blood for diagnosis of relapses, and importantly, evaluation of more sensitive targets for RNA detection allowing earlier detection of relapse in blood, including but not limited to 7SL-derived small RNA or SHERLOCK.<sup>35,36</sup>

#### Contributors

Conceptualization: NVR, DMN, OVM, PB, VL; Verification of the underlying data: INL, NVR; Data curation: INL, PB; Formal analysis: INL, NVR, VL; Funding acquisition: VL; Investigation: INL, WM, DN, SR; Methodology: NVR, SR; Project administration: DMN, EMM, WM, DN, OVM, AT, SR, PB, VL; Resources: JM, PPP, VK, FA, MI, LK, SM, DMM, DMN, EMM, AT, PB, VL; Validation: NVR, PB; Visualization: NVR; Writing original draft: INL, VL; Writing – review & editing: NVR, DMN, EMM, JM, PPP, WM, DN, VK, FA, MI, LK, SM, DMM, OVM, AT, SR, PB. All authors read and approved the final version of the manuscript.

#### Data sharing statement

Metadata, the study protocol, the addendum to the protocol and informed consent forms are available from <https://doi.org/10.23708/MBMROD> (last accessed on 04/11/2022). For scientists performing research relevant to the topic, de-identified data are available for sharing on request, subject to the decision by a data release committee.

#### Declaration of interests

The authors have declared that no competing interests exist.

#### Acknowledgements

The DiTECT-HAT project is part of the EDCTP2 programme supported by Horizon 2020, the European Union Funding for Research and Innovation (grant number DR1A-2014-306-DiTECT-HAT).

We acknowledge all the technicians and health personnel of the General Reference Hospitals of Dipumba, Katanda, Ngandajika, Masi Manimba and Roi Baudouin who have contributed to the study and taken care of the patients. We also acknowledge the personnel of the PNLTHA, INRB and ITM who participated in the study, as well as the assistance of Geneva and Kinshasa DNDi teams, including Christelle Perdrieu, Pere Simarro and Adeline Prêtre.

#### Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.ebiom.2022.104376>.

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