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Short title

Dermal trypanosomes in gHAT cases and suspects

Keywords

Skin, reservoir, Human African Trypanosomiasis, *Trypanosoma brucei gambiense*.

Key points

(37/40 words)

Live trypanosomes can remain undetected in the blood of individuals seropositive for sleeping sickness. Here, we show that they could be infected with parasites in their extravascular dermis, highlighting the skin as a potential reservoir for trypanosomes.

Abstract

(238/250 words)

Background: The diagnosis of *gambiense* Human African Trypanosomiasis (gHAT) typically involves two steps: a serological screen, followed by the detection of living trypanosome parasites in the blood or lymph node aspirate. Live parasites can, however, remain undetected in some seropositive individuals, who we hypothesize are infected with *Trypanosoma brucei gambiense* parasites in their extravascular dermis.

Methods: To test this hypothesis, we conducted a prospective observational cohort study in the gHAT focus of Forecariah, Republic of Guinea. Of the 5,417 subjects serologically screened for gHAT, 66 were enrolled into our study and underwent a dermatological examination. At enrolment, 11 seronegative, 8 unconfirmed seropositive and 18 confirmed seropositive individuals had blood samples and skin biopsies taken and examined for trypanosomes by molecular and immuno-histological methods.

Results: In seropositive individuals, dermatological symptoms were significantly more frequent, relative to seronegative controls. *T.b. gambiense* parasites were present in the blood of all confirmed cases (n=18) but not in unconfirmed seropositive individuals (n=8). However, *T. brucei* parasites were detected in the extravascular dermis of all unconfirmed seropositive individuals and all confirmed cases. Skin biopsies of all treated cases and most seropositive untreated individuals progressively became negative for trypanosomes 6 and 20 months later.

Conclusions: Our results highlight the skin as a potential reservoir for African trypanosomes, with implications for our understanding of this disease's epidemiology in the context of its planned elimination and underlining the skin as a novel target for gHAT diagnostics.

Introduction

The number of new cases of *gambiense* Human African Trypanosomiasis (gHAT or sleeping sickness) has never been so low in the known epidemiological history of the disease, with only ~1,500 new cases reported in 2017 [1, 2], and the World Health Organization (WHO) has targeted gHAT elimination by 2030 [3]. This objective has been encouraged by the success of active surveillance efforts that relies on a two-step diagnosis: an initial serological screen, followed by microscope observation of blood, lymph or cerebrospinal fluid (CSF) to detect extracellular trypanosomes and to confirm the serological diagnosis. However, some seropositive individuals remain without a confirmed parasitological diagnosis for years and have been recently described as being latent cases, raising the question as to whether reservoirs of live parasites persist in these individuals [4].

T. brucei s. l. parasites are found in the extravascular compartment of various tissues of their mammalian hosts, including the skin, albeit mostly under experimental conditions in animal models rather than during the natural progression of the disease [5]. Recent studies have revealed that substantial quantities of trypanosomes persist within the extravascular dermis following experimental infection in mice with *T.b. gambiense* or *T. b. brucei*. These parasites can be transmitted to the tsetse vector, even in the absence of detectable parasites in the host's blood [6]. This study also reported a retrospective screening of archived skin biopsies from a gHAT endemic region, which revealed the presence of some extravascular skin-dwelling trypanosomes [6]. However, the species of these parasites was not identified and no clinical records were available for the screened samples.

These observations raise the question as to whether *T.b. gambiense* might be found in the skin of confirmed gHAT cases, as well as in unconfirmed seropositive individuals, in regions of active disease transmission. To address this question, we performed a prospective

observational study in the Forecariah district in the Republic of Guinea, which is one of the most active gHAT foci in Western Africa.

Methods

More details for material and methods are provided as Supplementary Data.

Ethical approval

All investigations were conducted in accordance with the Declaration of Helsinki and with the approval of the National Ethical Committee of the Republic of Guinea (Study Diag-Cut-THA 032/CNERS/17 and amendment 038/CNERS/19).

Study enrolment, screening and case definitions

From May 2017 to February 2019, a total of 5,417 individuals were screened by the HAT National Control Programme using the card agglutination test for trypanosomiasis, first on whole-blood (CATTwb), then on plasma (CATTp) for validation, in 43 villages in the active gHAT focus of the Forecariah District, Republic of Guinea. All subjects were classified as seronegative, unconfirmed seropositive or confirmed seropositive according to the diagnostic process presented in Table 1. All parasitologically confirmed cases were diagnosed and treated by the HAT National Control Programme according to WHO recommendations and as described previously [7]. All confirmed cases (CATTp ≥1/4 with parasitological confirmation) and all unconfirmed seropositive individuals (CATTp ≥1/4 without parasitological confirmation) were proposed for study enrolment. In total, 40 seronegative controls (39 CATTwb-negative and 1 CATTwb-positive CATTp<1/4) were randomly selected from the 5,417 population, of which the first 29 individuals, enrolled in 2017, were only included in the epidemiological and clinical analysis, and the last 11 individuals, enrolled in 2019, were subjected to the entire protocol. Children under 16 years of age and pregnant women were

excluded from the study. Each participant was informed about the study's objectives and provided written informed consent.

Field procedure and sampling

Participants underwent an epidemiological interview and a clinical examination, during which dermatological symptoms including pruritus (skin itch) and dermatitis (skin inflammation), were assessed at enrolment as well as at each subsequent follow-up at 6 and 20 months after enrolment/treatment. Epidemiological and clinical parameters are detailed in Supplementary Data. The absence of dermatitis lesions at the skin sampling site was verified and a 2mm blood-free skin punch biopsy was sampled from the right back shoulder of all confirmed seropositive cases, all unconfirmed seropositive individuals, and for the final 11 seronegative controls. Touch preparations were obtained by gently rolling the biopsy on a clean glass slide and Giemsa staining in the field. The positivity of a given slide was defined by the detection of at least three trypanosomes. Biopsies were fixed for immuno-histochemistry and molecular analyses. Plasma aliquots from blood samples were also obtained for serological trypanolysis tests [8].

Immunohistochemical detection

Skin biopsy sections were stained with hematoxylin-eosin (HE) and Giemsa stains, and immunolabelled with the *T. brucei*-specific anti-ISG65 antibody that targets the Invariant Surface Glycoprotein 65 expressed at the surface of the mammalian host stages of *T. brucei s.l.* parasites [9], and the *T. brucei*-specific anti-Hsp70 antibody that recognizes the endoplasmic reticulum molecular chaperone heat-shock protein 70 homologue [10]. Slides were blindly assessed by at least two readers. Slides from seronegative controls were mixed with slides from seropositive cases in order to guarantee blind reading. The positivity of a given skin-section slide was defined by the detection of at least three trypanosomes.

PCR detection

DNAs were extracted from paraffin-embedded biopsies and blood samples with tissue-specific commercial kits (Qiagen, Germany). For each sample, at least two PCRs were performed with TBR primers targeting a DNA satellite repeated sequence (10,000 copies per cell) [11], and TgsGP primers directed against the single copy *TgsGP* gene [12], for detecting *T. brucei s. l.* and *T.b. gambiense* DNAs, respectively.

Data analyses

For epidemiological, clinical and diagnostic parameters, differences between seronegative controls versus unconfirmed seropositive individuals and confirmed cases were assessed using the following two-sided tests at 5% confidence: Fisher's exact tests for qualitative data (Tables 2 and 3) and/or Mann-Whitney tests for quantitative data (age in Table 2). For the follow-up analyses, differences between results at enrolment versus results at 6 months and 20 months after treatment/enrolment were assessed for each group using two-sided Fisher's exact tests at 5% confidence (Table 4).

Results

Epidemiological and clinical results

Results of the initial screening of 5,417 individuals are shown in Table 1. Out of 5,377 seronegative subjects (CATTwb-negative or CATTp<1/4), 40 were enrolled as seronegative controls, of whom 11 provided skin biopsies. A total of 40 seropositive individuals (CATTwb-positive and CATTp $\geq$1/4) were identified during the survey, of which 12 tested negative upon parasitological examination (0.22%) and 28 were confirmed as HAT cases (0.52%). Eight non-confirmed seropositive individuals and 18 confirmed HAT cases had no exclusion criteria and accepted to be enrolled in the study.

As shown in Table 2, the occurrence of dermatitis was significantly more frequent in confirmed HAT cases (15/18, 83%, P<0.0001) and non-confirmed seropositive individuals (5/8, 63%, P=0.0166) as compared to seronegative controls (7/40, 18%). Pruritus was the most frequent dermatological sign in confirmed HAT patients (11/18, 61%), as compared to seronegative controls (3/40, 8%). Among the various observed clinical manifestations of localized dermatitis, we unambiguously identified typical cases of intertrigo (in 4/18 confirmed cases versus 2/40 seronegative controls), pityriasis (in 3/18 versus 2/40), scabies (in 3/18 versus 1/40), dermatophytosis (in 3/18 versus 1/40), molluscum (in 3/18 versus 1/40), and ulceration (in 3/18 versus 1/40). The main clinical manifestation in non-confirmed seropositive individuals were eczema (3/8), intertrigo (2/8) and pityriasis (1/8). Apart from general pruritus and intertrigo, all dermatological signs were observed in upper regions of the body, especially on the thorax and arms.

Biological results

Plasma from all confirmed and unconfirmed seropositive cases, and from 11/40 seronegative controls, was assessed using the trypanolysis test, which detects complement-mediated immune responses activated by *T.b. gambiense*-specific antigens. All confirmed cases were positive for the LiTat 1.3 antigen, and 89% (16/18) of these cases were positive for both the LiTat 1.5 and 1.6 antigens (Table 3). Only 25% (2/8) of the unconfirmed seropositive individuals were positive for all antigens, while the others remained negative for all three variants, as seronegative controls.

A skin punch biopsy was sampled from all enrolled confirmed and unconfirmed seropositive cases and from 11/40 seronegative controls. Dermal touch preparations were then generated in the field and full-length trypanosomes were observed on slides from 81% (13/16) of the confirmed cases and from 33% (2/6) of the unconfirmed seropositive individuals (Table 3 and

Supplementary Fig.1). One of the unconfirmed seropositive individuals who tested positive in this dermal test, also tested positive in the trypanolysis test.

The skin biopsy samples were processed for immunohistochemistry analyses (IHC) in the lab. Skin samples obtained from the seronegative controls (11/11) did not test positive for trypanosomes (Table 3). By contrast, all unconfirmed seropositive individuals (8/8) and all confirmed cases (18/18) were found to be positive at least following staining by a *T. brucei*-specific anti-ISG65 antibody (Fig.1, Supplementary Fig.2 and Table 3). In addition, all samples from non-confirmed seropositive individuals and confirmed cases were also found to be positive following either unspecific Giemsa staining and/or unspecific HE staining and/or labelling with a *T. brucei*-specific anti-Hsp70 antibody (Fig.1, Supplementary Fig.2 and Table 3). In positive skin sections, *T. brucei* parasites were evenly distributed in the reticular dermis, and were occasionally associated with edema. No other parasites were detected in any of the skin samples.

To confirm the identity of these skin-dwelling parasites, *T. brucei*-specific PCR (TBR-PCR) assays were performed on total DNA extracted from fresh blood and from paraffin-embedded skin samples. Both blood and skin DNA samples from the seronegative controls (11/11) were found to be negative by the TBR-PCR assays. By contrast, 100% of blood (18/18) and 78% of skin samples (14/18) from confirmed cases tested positively in the TBR-PCR assays. Parasite DNA was only detected in the skin of unconfirmed seropositive individuals (6/8, 75%) but not in their blood (0/8) (Table 3). *T.b. gambiense*-specific TgsGP-PCR assays were performed on the same DNA samples and were positive for only 67% (12/18) of the blood samples of confirmed cases (Table 3). We reasoned that the use of fresh skin biopsies would be more appropriate than paraffin-embedded skin samples for TgsGP-PCR due to the low sensitivity of this method targeting a single-copy gene. To test this hypothesis, we obtained fresh skin

samples from an outgroup of nine additional confirmed cases, who were identified in 2018 in the same district by using the same study protocol (Supplementary Table 1). The fresh skin samples from 89% (8/9) of these confirmed cases were found positive to TBR-PCR, and 33% (3/9) were also found positive to TgsGP-PCR (Supplementary Table 1).

Follow-up results

The same panel of analyses were repeated at 6 and 20 months after study enrolment of the unconfirmed seropositive individuals or after treatment of the confirmed cases (Table 4). In total, 17/18 and 12/18 confirmed cases were followed-up at 6 months and 20 months after treatment, respectively, with 12/18 confirmed cases followed-up 2 times, 5/18 followed-up one time and 1 loss to follow-up (Table 4). Most of the clinical symptoms associated with the stage-2 cases at enrolment, including dermatological signs, significantly decreased in frequency during the first 6 months after treatment. Whereas all parasitological observations and PCR results became negative within 6 months after treatment in all confirmed cases (17/17), trypanosomes were still detected by histological methods in up to 38% of them (5/13 by IHC anti-ISG65). Twenty months after treatment, all CATTp and histological tests became negative (12/12), with 2/3 confirmed cases remaining positive to the trypanolysis test (Table 4).

In total, 5/8 and 4/8 unconfirmed seropositive individuals were followed-up at 6 months and 20 months after enrolment, respectively, with 4/8 unconfirmed seropositive individuals followed-up 2 times, 1/8 followed-up one time and 3/8 losses to follow-up (one death, one pregnancy and one resignation) (Table 4). In 80% (4/5) of the unconfirmed seropositive individuals who were monitored after enrolment, dermatological signs progressively disappeared (Table 4). The four unconfirmed seropositive individuals who were negative to the trypanolysis test at enrolment, became negative to CATTp, TBR-PCR on skin and IHC anti-

ISG65 at the same period. In contrast, the only trypanolysis-positive individual who could be monitored at 6 months maintained a serological reactivity to CATTp. No parasite DNA was detected by TBR-PCR in either blood or skin but the skin biopsy remained positive by IHC-ISG65. Although this individual was lost to follow-up for the 20-month time-point, he was diagnosed as a stage 1 case (CATTp 1/8, mAECT-BC +, CSF - and WBC 4) during an active surveillance campaign that was led in November 2019 (i.e. after the end of this study) and was treated accordingly.

Discussion

Here, we set out to investigate whether *T.b. gambiense* parasites might be found in the skin of confirmed gHAT cases, as well as in unconfirmed seropositive individuals, in regions of active disease transmission. Although this study is somewhat limited to a restricted population and to the detection methods used, 100% of the confirmed cases and unconfirmed seropositive subjects were found to carry extravascular trypanosomes in their skin.

Dermatological signs in gHAT

Our results indicate that dermatological symptoms might be an important aspect of gHAT's clinical presentation. The few reports that exist on this topic in the literature describe a wide array of skin pathologies associated with sleeping sickness, including pruritus, chancre, rashes and localized edemas [13, 14]. However, detailed dermatological profiles of HAT cases have mostly been derived from light-skinned travelers with imported HAT [14]. Whereas chancres and rashes remain anecdotal, pruritus was the most commonly observed dermatological sign in endemic cases (in up to 57% of stage-2 cases) [14]. Here, we observed a higher occurrence of pruritus and dermatitis in unconfirmed seropositive individuals and in confirmed cases, relative to seronegative controls (Table 2). The observed dermatitis profiles included some

252 conditions the etiologies of which might not be directly related to a trypanosome infection.
253 However, it could be hypothesized that the immune status of the infected host skin is
254 somehow altered by the presence of trypanosomes in a way that promotes the outcome of
255 dermatitis caused by other pathogens and/or increases skin sensitivity.

256 Trypanosome detection

257 The direct detection of trypanosomes in the human skin is not well documented in the
258 literature [13]. As there is no gold-standard approach for that purpose, we implemented seven
259 distinct molecular and immuno-histological methods in parallel, yet with their own specific
260 strengths and weaknesses. Here, dermal touch preparations were generated in the field in
261 sub-optimal ambient conditions (31°C at 75% humidity on average), which could explain the
262 unusual morphology of some trypanosomes that were probably altered by osmotic shock
263 while drying. Then, only a limited portion of each parasite is visible in the 2.5μm skin sections
264 because entire trypanosomes do not necessarily lie in the section plan. For the same reason,
265 the parasite nucleus, kinetoplast and flagellum are rarely all visible in the same given cell
266 section. However, the specificities of the anti-ISG65 and anti-Hsp70 antibodies enable to
267 unambiguously detect most *T. brucei* parasites within the extracellular dermal matrix, and this
268 is confirmed by TBR-PCR assays. Considering that *T.b. brucei* are non-infectious to humans and
269 killed within a couple of hours by human serum, the dermal parasites detected here, at least
270 in confirmed cases, are likely to be *T.b. gambiense* parasites, as confirmed by the positivity of
271 some direct TgsGP-PCR assays performed on fresh skin samples from an outgroup. However,
272 further genetic studies would be necessary to rule out the hypothesis of infections with a
273 peculiar *T.b. brucei* strain.

274 The detection of skin-dwelling parasites at enrolment in most of the 2mm skin punch biopsies
275 sampled from seropositive individuals indicates that skin-dwelling parasites might be present

over a considerable proportion of the skin surface. However, the precise dynamics of parasite load and distribution in the extravascular dermal compartment over the course of an infection remains unknown. According to historic (reviewed in [5]) and more recent [6] studies in experimental animal models, skin-dwelling parasites could theoretically be detected in almost the entire skin surface, yet with a variable distribution and at variable local densities.

A dermal reservoir of trypanosomes in non-confirmed seropositive individuals

One possible explanation for the persistence of disease foci in certain regions is the presence of animal reservoirs [15]. Another possibility, as increasing evidence suggests, is that traditionally used diagnostic approaches do not detect some *T.b. gambiense* infections among seropositive cases [15]. Indeed, bloodstream parasite numbers in *T.b. gambiense* infections can periodically fluctuate to less than 100 trypanosomes/ml, falling below the detection limit of the most sensitive methods currently in use [16]. Another study estimated that 20-30% of gHAT cases are missed in active case detection by standard parasitological techniques and are left untreated [17]. These infected individuals might ultimately progress to clinical disease or remain almost asymptomatic until undergoing a possible self-cure [15].

Here, routine molecular analyses confirmed the presence of *T. brucei* parasites in the skin of unconfirmed seropositive individuals, including those testing negative to the LiTat 1.3 trypanolysis test known to be highly specific of *T.b. gambiense*. As previously observed in the same transmission focus [7], trypanolysis-negative individuals rapidly became negative to CATTp and this was associated with the disappearance of detectable dermal trypanosomes. In such subjects, dermal infections could be transient and too short to allow *T.b. gambiense* to invade the bloodstream and express the LiTat 1.3 antigen. Alternatively, these infections could possibly be caused by other trypanosome species cross-reacting with the CATT. More sensitive and extensive molecular analyses will be required to solve this question.

Only two unconfirmed seropositive individuals were positive to the LiTat 1.3 trypanolysis test in this study. One died before the first follow-up and the other was lost to follow-up after six months. Nevertheless, it is noteworthy that this last individual, who was still positive to histological tests at six months, was eventually diagnosed as a stage 1 case during a medical survey almost 2.5 years after enrolment. Systematic characterization and follow-up of dermal trypanosomes in unconfirmed seropositive individuals testing positive to the LiTat 1.3 trypanolysis test would be required to better address the role of these individuals in the transmission of *T.b. gambiense*.

Transmission and epidemiological contribution of dermal trypanosomes

Mathematical modelling recently predicted that, in the absence of any animal reservoirs, these unconfirmed seropositive individuals could contribute to disease transmission by maintaining an overlooked reservoir of skin-dwelling parasites [18]. The infected skin of seropositive unconfirmed individuals could provide a population of parasites that are readily accessible to the tsetse fly. Indeed, this mode of transmission has been demonstrated in experimental animal models, in which skin-dwelling trypanosomes were efficiently transmitted to the tsetse vector, even in the absence of detectable parasitemia [6, 19, 20]. However, the presence of the stumpy parasite forms that are assumed to be most adapted for development in tsetse flies were not investigated here. This is an important question for future studies to address, in order to estimate the actual infectivity potential of human skin-dwelling parasites. Our reported observations should also be confirmed in a larger number of unconfirmed seropositive individuals (including RDT-positive subjects), and the study scaled-up to include other endemic transmission foci in Africa, in order to confidently determine the actual prevalence of dermal trypanosomes.

Our results raise questions about the strategies used to diagnose this disease, which currently focus on detecting parasites in the blood and lymph. If the human skin is indeed a reservoir for trypanosomes, it could represent a novel target for diagnostics, and it could: (i) allow more carriers to be treated; (ii) help to determine a more accurate estimate of the true prevalence of the disease; and (iii) help to identify as yet undetected reservoirs in both human and animal populations. The development of less invasive and field-adapted diagnostic methods to detect extravascular dermal trypanosomes, such as the serological detection of skin-related biomarkers or the identification of specific bio-physical profiles by skin scanning, would benefit to these goals. The current WHO recommendation, based on risk-benefit analyses, is to not treat unconfirmed seropositive individuals without knowing if they have an active infection [1]. Importantly, we observed that the routinely administered trypanocide treatments (Pentamidine for stage-1 and NECT for stage-2 cases) efficiently targeted both bloodstream and dermal trypanosomes in all the patients followed-up over 20 months. With the promise of new cheaper, less toxic and easier to administer drugs on the horizon, the policy of treating unconfirmed seropositive individuals could possibly be reconsidered. Indeed, the new drug Acoziborole, that requires a single oral administration, could hopefully be the next revolutionary treatment against gHAT. As gHAT approaches its elimination targets, we propose from our findings that the current algorithms, used to identify and manage disease cases, could be adapted to include the detection of skin-dwelling parasites, which likely represent a previously unaccounted for anatomical reservoir.

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Author contributions

MarC, AMS and NRKS conduced the clinical study in the field and commented on the manuscript. HI, IS, CT, CC, ACo, ACr, OC, ECA and JMB performed sample analyses and commented on the manuscript. MamC and VJ held logistical aspects, analyzed part of the data and commented on the manuscript. AML, BB and BR designed the study, organized logistical aspects, analyzed the data and wrote the manuscript as co-last authors.

Competing interest

All authors declare no financial relationships with any organizations that might have an interest in the submitted work in the previous three years, no other relationships nor activities that could appear to have influenced the submitted work, and no other relationships or activities that could appear to have influenced the submitted work.

Data and material availability

Upon request, the original protocol and associated forms, as well as an anonymized dataset, could be obtained from the corresponding author rotureau@pasteur.fr.

Transparency statement

The lead author affirms that the manuscript is an honest, accurate and transparent account of the study being reported, that no important aspect of the study has been omitted, and that any discrepancies from the study as originally planned have been explained.

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Figures

Fig. 1. Extravascular trypanosomes in the dermal matrix of human skin biopsies.

For each enrolled study subject, paraffin-embedded skin biopsy sections were stained either with (A) a specific anti-ISG65 antibody (brown) or (B) with Giemsa (purple) and screened at a 100x magnification. Representative trypanosome sections from confirmed stage-1 (subject 1044) and stage-2 cases (subjects 1035, 1036, 1037, 1039 and 1042), as well as from unconfirmed seropositive individuals (subjects 1046, 1065 and 1066) are shown. The scale

bars represent 10μm. More images of extravascular *T. brucei* parasites in human skin biopsies are available in Supplementary Fig2.

Tables

Table 1. Diagnostic process, number of subjects and results.

CATTwb / CATTp: card agglutination test for trypanosomiasis on whole blood / plasma; mAECT BC / LN aspirate: mini anion-exchange column technique on buffy coat / lymph node aspirate; WBC: white blood cells; CSF: cerebrospinal fluid; ND: not determined. *Highest plasma dilution with a positive result.

Groups		Diagnostic process					No. subjects		
		1- Serological screening	2- Serological validation	3- Parasitological confirmation	4- Staging		Screened	Enrolled	Followed-up
		CATTwb / RDT	CATTp*	mAECT BC / LN aspirate observation	Parasites in CSF	No. WBC in CSF			
Seronegative		-	ND	ND	ND		5 377	40	0
		+	< 1/4						
Seropositive		+	≧ 1/4	-	ND		12	8	5
Confirmed	Stage 1	+	≧ 1/4	+	no	0-5	8	4	4
	Stage 2				yes	>5	18	14	13
	ND				ND		2	0	0
	All						28	18	17
Total							**5 417**	**66**	**22**

Table 2. Epidemiological and clinical characteristics of case subjects.

For each group and each parameter, total values correspond to the numbers of subjects for which a value was available (n/total). p values were obtained by comparing one by one the parameters of each group of seropositive subjects (unconfirmed and all confirmed) to those of seronegative controls using two-sided Fisher's exact tests or * two-sided Mann-Whitney tests at 5% confidence. LN: lymph nodes.

Parameters		Groups (n=66)						
		Seronegative (n=40)	Seropositive (n=8)		Confirmed (n=18)			
					Stage 1 (n=4)	Stage 2 (n=14)	All (n=18)	
		n/total (%) or mean (SD)	n/total (%) or mean (SD)	*p values*	n/total (%) or mean (SD)	n/total (%) or mean (SD)	n/total (%) or mean (SD)	*p values*
Epidemiological	Age (n=66)	37.9 (14)	36.6 (18)	*0.7647**	31.0 (17)	35.6 (15)	34.6 (15)	*0.3502**
	Male sex (n=66)	22/40 (55%)	3/8 (38%)	*0.4538*	2/4 (50%)	5/14 (36%)	7/18 (39%)	*0.3950*
	HAT case(s) in the family since 2010 (n=65)	11/40 (28%)	2/7 (29%)	*>0.9999*	2/4 (50%)	5/14 (36%)	7/18 (39%)	*0.5404*
	Occupational risk (n=66)	17/40 (43%)	4/8 (50%)	*0.7155*	2/4 (50%)	5/14 (36%)	7/18 (39%)	*>0.9999*
Clinical	Swollen LN (n=65)	5/39 (13%)	6/8 (75%)	***0.0010***	4/4 (100%)	13/14 (93%)	17/18 (94%)	***<0.0001***
	Any dermatological symptoms (n=66)	8/40 (20%)	5/8 (63%)	***0.0252***	4/4 (100%)	13/14 (93%)	17/18 (94%)	***<0.0001***
	Dermatitis (n=66)	7/40 (18%)	5/8 (63%)	***0.0166***	4/4 (100%)	11/14 (79%)	15/18 (83%)	***<0.0001***
	Pruritus (n=66)	3/40 (8%)	2/8 (25%)	*0.1887*	0/4 (0%)	11/14 (79%)	11/18 (61%)	***<0.0001***
	Asthenia (n=65)	17/39 (44%)	4/8 (50%)	*>0.9999*	4/4 (100%)	14/14 (100%)	18/18 (100%)	***<0.0001***
	Fever (n=63)	6/38 (16%)	1/7 (14%)	*>0.9999*	2/4 (50%)	9/14 (64%)	11/18 (61%)	***0.0013***
	Weight loss (n=61)	6/39 (15%)	3/8 (38%)	*0.1672*	2/4 (50%)	6/10 (60%)	8/14 (57%)	***0.0046***
	Eating disorders (n=66)	4/40 (10%)	1/8 (13%)	*>0.9999*	0/4 (0%)	7/14 (50%)	7/18 (39%)	***0.0250***
	Headache (n=65)	23/39 (59%)	6/8 (75%)	*0.6918*	3/4 (75%)	13/14 (93%)	16/18 (89%)	***0.0322***
	Circadian rhythm disruptions (n=66)	3/40 (8%)	1/8 (13%)	*0.5303*	0/4 (0%)	5/14 (36%)	5/18 (28%)	*0.0925*
	Sexual dysfunctions (n=65)	4/39 (10%)	1/8 (13%)	*>0.9999*	0/4 (0%)	5/14 (36%)	5/18 (28%)	*0.1236*
	Behaviour changes (n=63)	4/39 (10%)	0/7 (0%)	*>0.9999*	0/4 (0%)	3/13 (23%)	3/17 (18%)	*0.6624*

Table 3. Serological, molecular and histological analysis results from blood and skin samples.

For each group and each parameter, total values correspond to the numbers of subjects for which a value was available (n/total). p values were obtained by comparing one by one the parameters of each group of seropositive subjects (unconfirmed and all confirmed) to those of seronegative controls using two-sided Fisher's exact tests at 5% confidence. VAT: variable antigen type; PCR: polymerase chain reaction; TgsGP: *Trypanosoma brucei gambiense* surface glycoprotein; HE: haematoxylin-eosin; IHC: immuno-histochemistry; Hsp70: heat shock protein 70; ISG65: invariant surface glycoprotein 65; ND: not determined.

Parameters		Groups (n=37)						
		Seronegative (n=11)	**Seropositive** (n=8)		**Confirmed** (n=18)			
					Stage 1 (n=4)	Stage 2 (n=14)	All (n=18)	
		n/total (%)	n/total (%)	*p values*	n/total (%)	n/total (%)	n/total (%)	*p values*
Trypanolysis	LiTat 1.3 positive (n=36)	0/10 (0%)	2/8 (25%)	*0.1830*	4/4 (100%)	14/14 (100%)	18/18 (100%)	***<0.0001***
	LiTat 1.5 positive (n=36)	0/10 (0%)	2/8 (25%)	*0.1830*	4/4 (100%)	12/14 (86%)	16/18 (89%)	***<0.0001***
	LiTat 1.6 positive (n=36)	0/10 (0%)	2/8 (25%)	*0.1830*	4/4 (100%)	12/14 (86%)	16/18 (89%)	***<0.0001***
	Positive for all VATs (n=36)	0/10 (0%)	2/8 (25%)	*0.1830*	4/4 (100%)	12/14 (86%)	16/18 (89%)	***<0.0001***
	Negative for all VATs (n=36)	10/10 (100%)	6/8 (75%)	*0.1830*	0/4 (0%)	0/14 (0%)	0/18 (0%)	***<0.0001***
PCR on blood	TBR positive (n=37)	0/11 (0%)	0/8 (0%)	*>0.9999*	4/4 (100%)	14/14 (100%)	18/18 (100%)	***<0.0001***
	TgsGP positive (n=37)	0/11 (0%)	0/8 (0%)	*>0.9999*	2/4 (50%)	10/14 (71%)	12/18 (67%)	***0.0004***
	Negative for all PCRs on blood (n=37)	11/11 (100%)	8/8 (100%)	*>0.9999*	0/4 (0%)	0/14 (0%)	0/18 (0%)	***<0.0001***
PCR on skin	TBR positive (n=37)	0/11 (0%)	6/8 (75%)	***0.0010***	1/4 (25%)	13/14 (93%)	14/18 (78%)	***<0.0001***
	TgsGP positive (n=37)	0/11 (0%)	0/8 (0%)	*>0.9999*	0/4 (0%)	0/14 (0%)	0/18 (0%)	*>0.9999*
	Negative for all PCRs on skin (n=37)	11/11 (100%)	2/8 (25%)	***0.0010***	3/4 (75%)	1/14 (7%)	4/18 (22%)	***<0.0001***
Histology	Dermal touchpreps (n=22, 3 reads)	ND	2/6 (33%)		1/3 (33%)	12/13 (92%)	13/16 (81%)	
	HE section (n=36, 1 read)	0/11 (0%)	6/8 (75%)	***0.0010***	4/4 (100%)	8/13 (62%)	12/17 (71%)	***0.0003***
	Giemsa section (n=37, 2 reads)	0/11 (0%)	4/8 (50%)	***0.0181***	0/4 (0%)	14/14 (100%)	14/18 (78%)	***<0.0001***
	IHC Hsp70 (n=31, 1 read)	0/11 (0%)	1/4 (25%)	*0.2667*	1/4 (25%)	11/12 (92%)	12/16 (75%)	***0.0002***
	IHC ISG65 (n=37, 3 reads)	0/11 (0%)	8/8 (100%)	***<0.0001***	4/4 (100%)	14/14 (100%)	18/18 (100%)	***<0.0001***
	Negative for all reads (n=37)	11/11 (100%)	0/8 (0%)	***<0.0001***	0/4 (0%)	0/17 (0%)	0/18 (0%)	***<0.0001***

Table 4. Clinical, serological, molecular and histological follow-up analyses at 6 and 20 months after enrolment.

Total values correspond to the numbers of subjects for which a value was available (n/total). For each group of subjects, p values were obtained by comparing one by one the parameters recorded at 6 months and 20 months after treatment/enrolment to those obtained at enrolment, using two- sided Fisher's exact tests at 5% confidence. LN: lymph nodes; CATT: card agglutination test for trypanosomiasis; VAT: variable antigen type; PCR: TBR polymerase chain reaction; Hsp70: heat shock protein 70; ISG65: invariant surface glycoprotein 65; ND: not determined.

Parameters		Seropositive					Confirmed								
							Stage 1					Stage 2			
		Enrollment	6 months		20 months		Enrollment	6 months		20 months		Enrollment	6 months		20 months	
		n/total (%)	n/total (%)	*p values*	n/total (%)	*p values*	n/total (%)	n/total (%)	*p values*	n/total (%)	*p values*	n/total (%)	n/total (%)	*p values*	n/total (%)	*p values*
Clinics	Asthenia	3/5 (60%)	1/5 (20%)	*0.5238*	2/4 (50%)	*>0.9999*	4/4 (100%)	0/4 (0%)	***0.0286***	2/3 (67%)	*0.4286*	13/13 (100%)	3/13 (23%)	***0.0001***	3/9 (33%)	***0.0011***
	Swollen LN	4/5 (80%)	1/4 (25%)	*0.2063*	3/4 (75%)	*>0.9999*	4/4 (100%)	2/3 (67%)	*0.4286*	2/3 (67%)	*0.4286*	12/13 (92%)	2/11 (18%)	***0.0005***	3/9 (33%)	***0.0066***
	Any dermatological symptoms	4/5 (80%)	1/5 (20%)	*0.2063*	1/4 (25%)	*0.2063*	4/4 (100%)	1/4 (25%)	*0.1429*	0/3 (0%)	***0.0286***	12/13 (92%)	5/13 (38%)	***0.0112***	3/9 (33%)	***0.0066***
	Fever	1/5 (20%)	1/5 (20%)	*>0.9999*	0/4 (0%)	*>0.9999*	2/4 (50%)	0/4 (0%)	*0.4286*	0/3 (0%)	*0.4286*	9/13 (69%)	0/13 (0%)	***0.0005***	1/9 (11%)	***0.0115***
	Headache	5/5 (100%)	2/5 (40%)	*0.1667*	3/4 (75%)	*0.4444*	3/4 (75%)	0/4 (0%)	*0.1429*	0/3 (0%)	*0.1429*	12/13 (92%)	2/13 (15%)	***0.0002***	4/9 (44%)	***0.0231***
	Pruritus	1/5 (20%)	0/5 (0%)	*>0.9999*	1/4 (25%)	*>0.9999*	0/4 (0%)	0/4 (0%)	*>0.9999*	0/3 (0%)	*>0.9999*	10/13 (77%)	3/13 (23%)	***0.0169***	2/9 (22%)	***0.0274***
	Weight loss	2/5 (40%)	2/5 (40%)	*>0.9999*	0/4 (0%)	*0.4444*	2/4 (50%)	1/4 (25%)	*>0.9999*	0/3 (0%)	*0.4286*	5/9 (56%)	0/13 (0%)	***0.0048***	0/9 (0%)	***0.0294***
	Dermatitis	4/5 (80%)	1/5 (20%)	*0.2063*	1/4 (25%)	*0.2063*	4/4 (100%)	1/4 (25%)	*0.1429*	0/3 (0%)	***0.0286***	10/13 (77%)	5/13 (38%)	*0.1107*	3/9 (33%)	*0.0789*
	Eating disorders	1/5 (20%)	1/5 (20%)	*>0.9999*	1/4 (25%)	*>0.9999*	0/4 (0%)	0/4 (0%)	*>0.9999*	0/3 (0%)	*>0.9999*	6/13 (46%)	0/13 (0%)	***0.0149***	1/9 (11%)	*0.1649*
	Sexual dysfunctions	1/5 (20%)	3/5 (60%)	*0.5238*	0/4 (0%)	*>0.9999*	0/4 (0%)	0/4 (0%)	*>0.9999*	0/3 (0%)	*>0.9999*	5/13 (38%)	5/13 (38%)	*>0.9999*	1/9 (11%)	*0.3330*
	Circadian rhythm disruptions	1/5 (20%)	1/5 (20%)	*>0.9999*	0/4 (0%)	*>0.9999*	0/4 (0%)	0/4 (0%)	*>0.9999*	0/3 (0%)	*>0.9999*	4/13 (31%)	1/13 (8%)	*0.3217*	1/9 (11%)	*0.3602*
	Behaviour changes	0/4 (0%)	1/5 (20%)	*>0.9999*	0/4 (0%)	*>0.9999*	0/4 (0%)	0/4 (0%)	*>0.9999*	0/3 (0%)	*>0.9999*	2/12 (17%)	0/13 (0%)	*0.2200*	0/9 (0%)	*0.4857*
Diagnosis	CATTwb	5/5 (100%)	4/5 (80%)	*>0.9999*	3/4 (75%)	*0.4444*	4/4 (100%)	4/4 (100%)	*>0.9999*	2/3 (67%)	*0.4286*	13/13 (100%)	10/13 (77%)	*0.2200*	4/9 (44%)	***0.0048***
	CATTp	5/5 (100%)	1/5 (20%)	***0.0476***	2/4 (50%)	*0.1667*	4/4 (100%)	0/4 (0%)	***0.0286***	1/3 (33%)	*0.1429*	13/13 (100%)	3/13 (23%)	***0.0001***	0/9 (0%)	***<0.0001***
	Parasitology	0/5 (0%)	0/5 (0%)	*>0.9999*	0/4 (0%)	*>0.9999*	4/4 (100%)	0/4 (0%)	***0.0286***	0/3 (0%)	***0.0286***	13/13 (100%)	0/13 (0%)	***<0.0001***	0/9 (0%)	***<0.0001***
Trypanolysis	LiTat 1.3 positive	1/5 (20%)	1/5 (20%)	*>0.9999*	0/4 (0%)	*>0.9999*	4/4 (100%)	4/4 (100%)	*>0.9999*	3/3 (100%)	*>0.9999*	13/13 (100%)	10/11 (91%)	*0.2200*	5/9 (56%)	***0.0172***
	LiTat 1.5 positive	1/5 (20%)	1/5 (20%)	*>0.9999*	0/4 (0%)	*>0.9999*	4/4 (100%)	4/4 (100%)	*>0.9999*	2/3 (67%)	*0.4286*	11/13 (85%)	8/11 (73%)	*0.6299*	5/9 (56%)	*0.1778*
	LiTat 1.6 positive	1/5 (20%)	1/5 (20%)	*>0.9999*	0/4 (0%)	*>0.9999*	4/4 (100%)	4/4 (100%)	*>0.9999*	0/3 (0%)	***0.0286***	11/13 (85%)	6/11 (54%)	*0.1819*	0/9 (0%)	***0.0002***
	Positive for all VATs	1/5 (20%)	1/5 (20%)	*>0.9999*	0/4 (0%)	*>0.9999*	4/4 (100%)	4/4 (100%)	*>0.9999*	0/3 (0%)	***0.0286***	11/13 (85%)	6/11 (54%)	*0.1819*	0/9 (0%)	***0.0002***
	Negative for all VATs	4/5 (80%)	4/5 (80%)	*>0.9999*	4/4 (100%)	*>0.9999*	0/4 (0%)	0/4 (0%)	*>0.9999*	0/3 (0%)	*>0.9999*	0/13 (0%)	1/11 (9%)	*0.4583*	3/9 (33%)	*0.0545*
TBR PCR	Blood	0/5 (0%)	0/5 (0%)	*>0.9999*	0/4 (0%)	*>0.9999*	4/4 (100%)	0/4 (0%)	***0.0286***	0/3 (0%)	***0.0286***	13/13 (100%)	0/13 (0%)	***<0.0001***	0/9 (0%)	***<0.0001***
	Skin	3/5 (60%)	0/5 (0%)	*0.1667*	0/4 (0%)	*0.1667*	1/4 (25%)	0/4 (0%)	*>0.9999*	0/3 (0%)	*>0.9999*	12/13 (92%)	0/13 (0%)	***<0.0001***	0/9 (0%)	***<0.0001***
Histology	Dermal touchpreps	1/3 (33%)	0/2 (0%)	*>0.9999*	ND		1/3 (33%)	1/4 (25%)	*>0.9999*	ND		11/12 (92%)	2/13 (15%)	***0.0002***	ND	
	IHC Hsp70	1/4 (25%)	ND		0/4 (0%)	*>0.9999*	1/4 (25%)	ND		0/3 (0%)	*>0.9999*	11/12 (92%)	ND		0/9 (0%)	***<0.0001***
	IHC ISG65	5/5 (100%)	1/5 (20%)	***0.0476***	0/4 (0%)	***0.0079***	4/4 (100%)	0/4 (0%)	***0.0286***	0/3 (0%)	***0.0286***	13/13 (100%)	5/13 (38%)	***0.0016***	0/9 (0%)	***<0.0001***
	Negative for all reads	0/5 (0%)	2/5 (40%)	*0.4444*	4/4 (100%)	***0.0079***	0/4 (0%)	2/4 (50%)	*0.4286*	2/3 (67%)	*0.1429*	0/13 (0%)	1/13 (8%)	*>0.9999*	9/9 (100%)	***<0.0001***