Novel Markers for Treatment Outcome in Late-Stage *Trypanosoma brucei gambiense* Trypanosomiasis

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Background. To date, no biological marker for treatment outcome in human African trypanosomiasis (HAT) has been described. The accuracy of biological markers for prediction of treatment outcome of HAT caused by *Trypanosoma brucei gambiense* was assessed.

Methods. Cerebrospinal fluid (CSF) white blood cell (WBC) count and immunoglobulin M (IgM), trypanosome-specific antibody, total protein, and interleukin-10 levels were determined before and up to 24 months after treatment of late-stage HAT.

Results. Treatment failure was experienced by 48 of 260 patients. Pretreatment CSF WBC counts ⩾102 cells/μL, IL-10 concentrations ⩾37 pg/mL, LATEX/IgM end titers ⩾1:32, LATEX/*T. b. gambiense* end titers ⩾1:2, and protein concentrations ⩾674 mg/L were associated with treatment failure. Six months after treatment, patients with CSF WBC counts ⩽5 cells/μL were at low risk of HAT recurrence (negative predictive value, >0.93). After 12 months, the combination of CSF WBC count ⩾8 cells/μL and LATEX/IgM end titer ⩾1:4 predicted treatment failure with 97% specificity and 79% sensitivity. Eighteen months after treatment, each marker accurately predicted treatment outcome. The combination of CSF WBC count ⩾8 cells/μL and LATEX/IgM end titer ⩾1:4 was 100% specific for treatment failure after 18 and 24 months.

Conclusions. HAT-affected patients with elevated pretreatment CSF levels of WBC, interleukin-10, IgM, trypanosome-specific antibody, and total protein are at risk of treatment failure. Six months after treatment, patients with CSF WBC counts ⩽5 cells/μL can be considered to be cured. The assessment of a combination of CSF WBC count and LATEX/IgM level allowed accurate prediction of outcome beginning at 12 months after treatment, as did each individual marker at 18 months after treatment.

Sixty million people in Africa are at risk of contracting human African trypanosomiasis (HAT), and 17,500 cases are reported yearly. Of the 50,000–70,000 patients with HAT, most are infected with *Trypanosoma brucei gambiense* [1].

Diagnosis and treatment are the cornerstones of HAT control. Pentamidine cures hemolymphatic stage *T. b. gambiense* infection with a <10% rate of treatment failure [2]. Melarsoprol was the first-line drug for late-stage HAT when trypanosomes have invaded the CNS [3], but today, difluoromethylornithine (DFMO) is promoted in this indication [4]. Relapse rates are generally <10% but reached 20%–30% with melarsoprol treatment in Uganda, Sudan, and Angola [5–7].

Because of the increase in treatment failures, the infectiousness of relapse cases, the risk of neurological sequelae, and fatal outcome, early and efficient detection of treatment failure is important. Control programs recommend a follow-up of patients with HAT for 2 years after treatment, with examinations every 6 months for the presence of trypanosomes and assessment of the WBC count in CSF. A patient is declared to be cured if no trypanosomes are detected and the WBC count has returned to normal [8]. Detection of trypanosomes constitutes a definite proof of treatment failure, but circulating parasite numbers can be low, and detection techniques are insensitive. Thus, diagnosis of treatment failure is often based on clinical symptoms and WBC count, although persistence or

Received 19 November 2007; accepted 3 March 2008; electronically published 21 May 2008.

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Clinical Infectious Diseases 2008; 47:15–22

1058-4838/2008/4701-0003$15.00
DOI: 10.1086/588668

reappearance of clinical symptoms is no absolute proof of failure, and interpretation of the WBC count is complicated by the absence of precise guidelines [9, 10]. The recommended determination of CSF total protein concentration during follow-up is seldom performed [8], and guidelines for interpretation of the result are lacking. Moreover, a complete follow-up is rarely achieved, because the necessary multiple visits and lumbar punctures represent a burden for patients and their families [11–13].

The long follow-up period, with its low adherence rate and poor cure assessment guidelines, is also a constraint for development of new drug regimens for HAT [14]. Alternative approaches and markers for accurate and early detection of HAT treatment failure are needed.

The CSF of patients with late-stage HAT contains high levels of IgM, and previous studies suggest that cure is correlated with a normalization of CSF IgM levels [10, 15]. A persistently high CSF IgM level might, therefore, be a marker of treatment failure [15, 16]. Likewise, trypanosome-specific antibodies are elevated in the CSF of patients with late-stage HAT and decrease after successful treatment. Several authors suggest assessment of IgM levels and trypanosome-specific antibodies in CSF during patient follow-up [12, 17–19]. Point-of-care tests LATEX/IgM and LATEX/*T. b. gambiense* are available for determination of IgM levels and detection of trypanosome-specific antibodies in CSF [20].

Recently, the counterinflammatory IL-10 was found at high levels in the CSF of patients with late-stage HAT, and it decreased immediately after treatment [21, 22]. High CSF IL-10 levels during treatment follow-up could, therefore, indicate recurring CNS inflammation and treatment failure.

We used data collected during a clinical trial of treatment of late-stage *T. b. gambiense* HAT [23]. The correlation of HAT treatment failure with CSF WBC count and levels of total protein, IL-10, IgM, and trypanosome-specific antibodies before treatment and during treatment follow-up were studied. The aim was to identify which marker most adequately predicted treatment failure.

MATERIALS AND METHODS

Patients and examinations. An open-label, randomized clinical trial to test the equivalence of 3 alternative drug regimens for late-stage HAT to the standard melarsoprol course (3 series of 3×3.6 mg/kg per day) was conducted with 278 patients in the Democratic Republic of Congo, as described elsewhere [23]. Late-stage HAT was defined as a WBC count >20 cells/μL or the presence of trypanosomes in the CSF. Patients were scheduled for follow-up 24 h and 3, 6, 12, 18, and 24 months after completion of treatment. Treatment failure (relapse) was defined as (1) the presence of trypanosomes in the blood or CSF at any follow-up assessment, (2) lack of trypanosomes detected at interim follow-up assessments but a CSF WBC count >20 cells/μL and at least twice as high as that of the previous follow-up assessment, or (3) lack of trypanosomes $\geq$24 months after treatment completion but a CSF WBC count >20 cells/μL [23].

Remaining volumes of diagnostic samples were stored at −20°C for a maximum of 6 months and were shipped to the Institute of Tropical Medicine (ITM; Antwerp) on dry ice and were kept at −80°C for a maximum of 6 years. With the approval of the health authorities and the ethics committee in the Democratic Republic of Congo, the stored CSF samples were examined. Titration in LATEX/IgM (ITM; Antwerp) for IgM quantification, titration in LATEX/*T. b. gambiense* (ITM; Antwerp) for detection of trypanosome-specific antibodies, total protein measurement, and IL-10 quantification were performed on all available pre- and posttreatment CSF samples, as described elsewhere [20, 24, 25].

Statistical analysis. For data analysis, tolerance windows of 2–4, 5–9, 10–16, 17–21, and $\geq$22 months were defined around the expected time of follow-up (i.e., 3, 6, 12, 18, and 24 months) to categorize each follow-up visit [26]. Patients who had not experienced treatment failure at an assessment were considered to be eligible for the next follow-up visit. Patients who had not experienced treatment failure during the total follow-up were considered to be cured.

Baseline characteristics of the treatment arms were compared using the Kruskal-Wallis test. Median pretreatment values of cured versus relapsed patients were compared with the Mann-Whitney 2-sample rank sum test. ORs for treatment failure and 95% CIs were calculated. Pearson's χ^2 or Fisher's exact test was conducted for comparison of proportions.

At each time point (24 h and 3, 6, 12, 18, and 24 months), only first-time treatment failures diagnosed at or after that time and only presenting patients were considered. Accuracy for detection of treatment failure was assessed by the area under the receiver operator characteristics curve (AUC). Ninety-five percent CIs were calculated according to the methods of DeLong et al. [27].

For individual pretreatment markers and candidate markers with good diagnostic accuracy (AUC >0.8) [28], the Youden index was determined for the whole range of cutoffs (Youden index = sensitivity + specificity − 1) [29]. The cutoff with maximal Youden index was retained. At 6, 12, 18, and 24 months after treatment, we explored the accuracy of combinations of markers in so far as they seemed relevant for field use—for example, the combination of WBC and IgM levels. One unique cutoff point for the combination was chosen among the cutoffs for different time points obtained through the above procedure.

The level of significance was set at .05 for all analyses. EpiInfo, version 3.2.2 (Centers for Disease Control and Prevention); and Stata, version 8 (STATA), were used for data analysis.

RESULTS

Study population. Eighteen of 278 randomized patients were excluded from data analysis because no posttreatment CSF specimen was available [23]. Of the 260 remaining patients, 64 had received treated with the standard 3-series melarsoprol regimen, 66 had been treated with a 10-day melarsoprol regimen, 66 had been treated with nifurtimox monotherapy, and 64 had been treated with a melarsoprol-nifurtimox combination. Among patients in the 4 treatment arms, baseline characteristics were similar for sex, clinical and neurological signs [23], median CSF WBC count ($P = .2$), IL-10 concentration ($P = .13$), IgM level ($P = .15$), presence of trypanosome-specific antibodies ($P = .4$), and protein concentration ($P = .2$).

In total, 48 treatment failures were diagnosed: 7 with standard melarsoprol, 17 with 10-day melarsoprol, 24 with nifurtimox, and 0 with the melarsoprol-nifurtimox combination [23]. Table 1 summarizes patient follow-up compliance and number and type of treatment failures at each time point.

Pretreatment values of candidate markers. Patients who experienced treatment failure had a significantly higher median WBC count ($P < .001$), IL-10 concentration ($P < .001$), IgM level ($P < .001$), trypanosome-specific antibody level ($P < .001$), and protein concentration ($P = .002$) in CSF before treatment (figure 1). Pretreatment CSF WBC counts $\geqslant$102 cells/μL, IL-10 concentrations $\geqslant$37 pg/mL, end titers in LATEX/IgM $\geqslant$1:32, end titers in LATEX/*T. b. gambiense* $\geqslant$1:2, and a protein concentration $\geqslant$674 mg/L were significantly associated with treatment failure (table 2). Trypanosome presence in CSF was not.

Evolution of CSF after HAT treatment in cured patients. Marker evolution during follow-up is presented in figure 1. The majority of cured patients showed a sharp decrease in CSF WBC count and CSF IL-10 level—with WBC counts $\leqslant$20 cells/μL (in 119 [57%] of 209 patients) and undetectable levels of IL-10 (i.e., <6.7 pg/mL; in 146 [77%] of 189 patients)—immediately after chemotherapy. A marginal decrease in CSF IgM level and protein concentration was noticed. The trypanosome-specific antibody level remained low. At 6 months after treatment, the CSF of cured patients had normal WBC counts ($\leqslant$5 cells/μL) in 86 (75%) of 114 patients, undetectable levels of IL-10 in 90 (86%) of 105 patients, CSF LATEX/IgM end titers <1:4 in 78 (74%) of 106 patients, undetectable trypanosome-specific antibodies in 95 (90%) of 106 patients, and CSF protein concentrations <500 mg/L in 69 (66%) of 104 patients. These percentages increased to 85%, 90%, 87%, 94%, and 77%, respectively, 12 months after treatment. Twenty-four months after treatment, 96% of patients with definite cure had WBC counts $\leqslant$5 cells/μL, 90% had undetectable CSF IL-10 levels, 96% had CSF LATEX/IgM end titers <1:4, 97% had undetectable CSF trypanosome-specific antibodies, and 84% had a CSF protein concentration <500 mg/L.

Evolution of CSF after HAT treatment in patients who experienced relapse. In patients who experienced treatment failure, there was also a sharp decrease in CSF WBC count and CSF IL-10 level immediately after treatment (figure 1). After this initial decrease, the median WBC count started to increase at 6 months (WBC counts >5 cells/μL in 26 of 32 patients), followed by an increase in CSF IgM level, IL-10 level, and protein concentration at 12 months after treatment and an increase in trypanosome-specific antibodies 18 months after treatment. A decrease in median values was observed 24 months after treatment. At that time, 4 of 12 patients who experienced relapse had trypanosomes in blood only (table 1), 3 of whom showed low CSF values for all markers.

Marker accuracy for treatment outcome. Receiver operator characteristic curves were constructed to assess which marker best discriminated cure from relapse (figure 2). Before treatment and at 24 h after treatment, no single marker had sufficient diagnostic accuracy. Six months after treatment, the WBC count started to have acceptable diagnostic accuracy (AUC >0.80). Twelve months after treatment, the AUC was >0.80 for all markers except for the CSF LATEX/*T. b. gambiense* end titer. Eighteen months after treatment, all markers had sufficient diagnostic accuracy, whereas 24 months after treat-

Table 1. Number and type of treatment failures diagnosed at each follow-up visit and compliance.

	No. of treatment failures determined by			
	Trypanosomes in		No trypanosomes, but increased WBC count	Proportion (%) of patients with follow-up compliance
Assessment time	Blood	CSF		
24 h	0	0	0	260/260 (100)
3 months	4	4	0	188/260 (72)
6 months	3	5	0	147/252 (58)
12 months	0	7	1	135/244 (55)
18 months	0	12	0	102/236 (43)
24 months	4	3	5	165/224 (74)

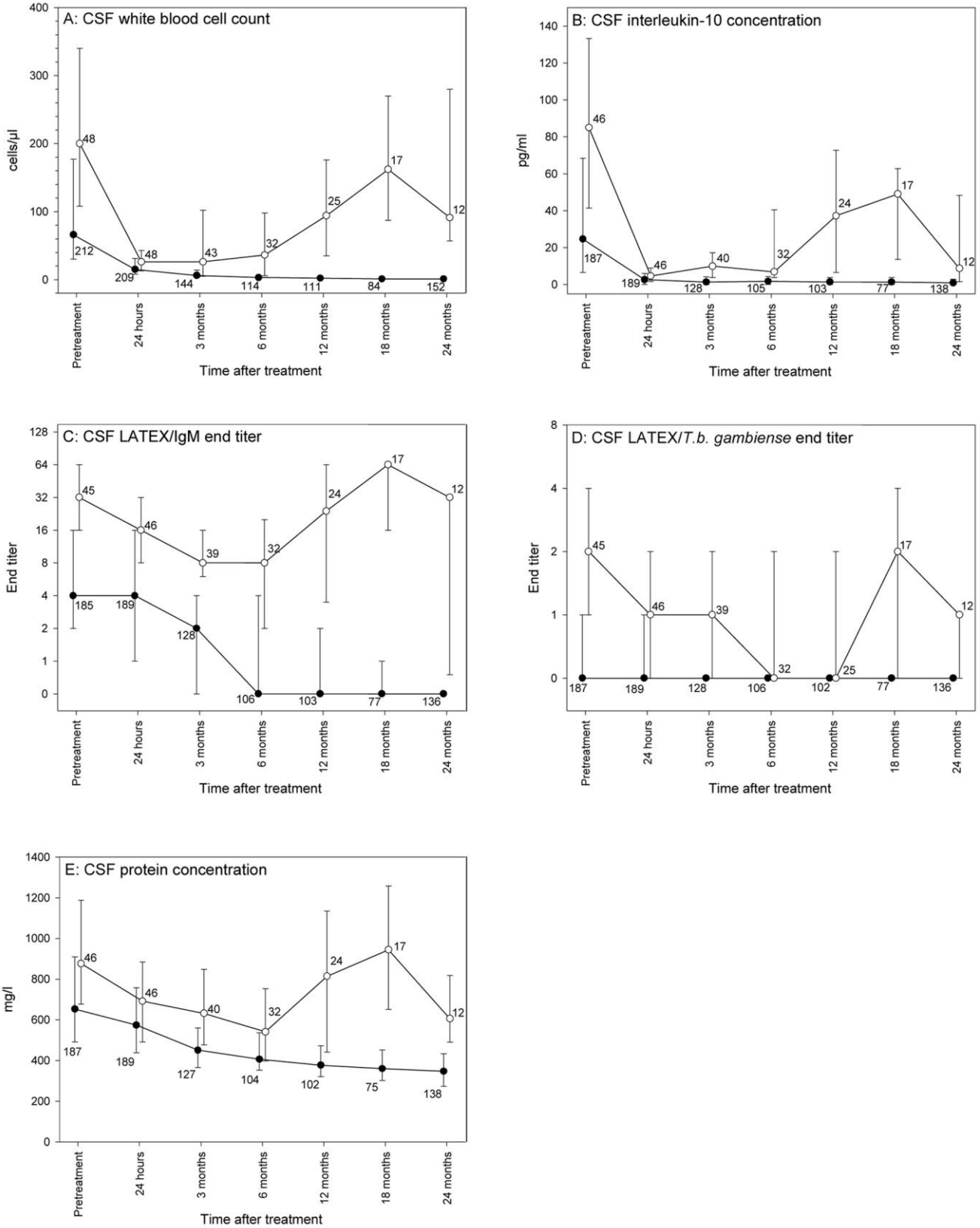

Figure 1. Evolution of CSF WBC count *(A),* CSF IL-10 concentration *(B),* CSF LATEX/IgM end titer *(C),* CSF LATEX/*Trypanosoma brucei gambiense* end titer *(D),* and CSF total protein concentration *(E)* in patients with late-stage human African trypanosomiasis who were cured (●) and who experienced relapse (○) during follow-up. Medians and interquartile ranges are shown, with the number of patients next to the data point.

ment, only WBC count, LATEX/IgM level, and the CSF protein concentration had an AUC >0.80. For parameters with AUC >0.80, the optimal cutoffs and their sensitivities and specificities are shown in table 3. The optimal cutoff for the WBC count is constant at 8–10 cells/μL. The cutoff for CSF end titer in LATEX/IgM was ⩾1:4, except at 24 months.

At 6, 12, 18, and 24 months after treatment, the combination of WBC count ⩾8 cells/μL and LATEX/IgM titer ⩾1:4 increased specificity for treatment failure to 95% (95% CI, 89%–98%), 97% (95% CI, 92%–99%), 100% (95% CI, 95%–100%), and 100% (95% CI, 97%–100%), respectively. Sensitivities for treatment failure became 59% (95% CI, 41%–76%), 79% (95% CI, 58%–93%), 82% (95% CI, 57%–96%), and 67% (95% CI, 35%–90%), respectively.

DISCUSSION

In the cohort under investigation, treatment failure experienced by patients with late-stage *T. b. gambiense* HAT was strongly associated with elevated pretreatment CSF levels of WBC, IL-10, IgM, trypanosome-specific antibody, and total protein. Our results corroborate those of other studies that observed an association between pretreatment WBC counts >100 cells/μL and treatment failure [5, 30, 31]. The association between pretreatment levels of IL-10, IgM, trypanosome-specific antibody, and total protein and treatment failure is demonstrated here for the first time. Thus, treatment failure seems to be related to the pretreatment degree of brain inflammation and counterinflammatory response [21, 22]. However, the prognostic value of these markers in CSF for diagnosis of relapse remains limited, because none of the receiver operator characteristic curves had an AUC >0.80.

The figure is available in its entirety in the online edition of *Clinical Infectious Diseases*.

Figure 2. Receiver operator characteristic curves and area under the curve plots. (The legend is available in its entirety in the online edition of *Clinical Infectious Diseases*.)

An association between the presence of trypanosomes in CSF before treatment and treatment failure has been reported [5, 30, 32] but was not confirmed in our study. In addition, our data (not shown) do not suggest a relationship between treatment failure and sex or age [32].

Immediately after treatment, patient WBC counts and IL-10 concentrations decreased to nearly normal levels. For IL-10, this confirms previous studies [21, 22]. The phenomenon of "liquid storm," a temporary increase in WBC count immediately after treatment, is rare [12, 33]. We observed an increase of 2–10 times in WBC count in only 2% of cured patients (4 of 209 patients). An association between treatment failure and CSF WBC count, protein concentration, and antibody level at the time of hospital discharge has been demonstrated by others [34]. Also, in our patient group, treatment failure was associated with CSF WBC count, IL-10 concentration, IgM level, trypanosome-specific antibody, and total protein concentrations 24 h after treatment (data not shown). Associations are weaker than

Table 2. Pretreatment characteristics of patients with late-stage human African trypanosomiasis.

Variable	No. of patients	No. (%) of treatment failures	OR (95% CI)	*P*
WBC count				
<102 cells/μL	143	10 (7)	…	
⩾102 cells/μL	117	38 (32)	6.4 (3.0–13.5)	<.001
Trypanosomes in CSF				
Absent	143	23 (16)	…	
Present	117	25 (21)	1.4 (0.8–2.7)	.27
IL-10				
<37 pg/mL	123	10 (8)	…	
⩾37 pg/mL	110	36 (33)	5.5 (2.6–11.7)	<.001
LATEX/IgM end titer				
<1:32	164	14 (9)	…	
⩾1:32	66	31 (47)	9.5 (4.6–20)	<.001
LATEX/*Trypanosoma brucei gambiense* end titer				
<1:2	163	16 (10)	…	
⩾1:2	69	40 (58)	12.7 (6.3–26)	<.001
Protein concentration				
<674 mg/L	108	11 (10)	…	
⩾674 mg/L	125	35 (28)	3.4 (1.6–7.2)	<.001

NOTE. Differences in proportions were assessed by Pearson's χ^2 test.

Table 3. Sensitivity and specificity of markers with area under the curve >0.8 for detection of treatment failure.

Time after treatment and marker	No. of treatment failures	No. of cured patients	Cutoff	Youden index	Sensitivity, % (95% CI)	Specificity, % (95% CI)
6 Months						
WBC count	32	114	⩾10 cells/μL	0.578	72 (53–86)	86 (78–92)
12 Months						
WBC count	25	111	⩾9 cells/μL	0.777	84 (64–95)	94 (87–97)
IL-10	24	103	⩾7.3 pg/mL	0.663	75 (53–90)	91 (84–96)
LATEX/IgM	24	103	⩾1:4	0.624	75 (53–90)	87 (79–93)
Protein	24	102	⩾671 mg/L	0.596	63 (41–81)	97 (92–99)
18 Months						
WBC count	17	84	⩾8 cells/μL	0.752	82 (57–96)	93 (85–97)
IL-10	17	77	⩾8.7 pg/mL	0.805	88 (64–99)	92 (84–97)
LATEX/IgM	17	77	⩾1:4	0.869	88 (64–99)	99 (93–100)
LATEX/*Trypanosoma brucei gambiense*	17	77	⩾1:1	0.621	65 (38–86)	97 (91–100)
Protein	17	75	⩾651 mg/L	0.738	76 (50–93)	97 (91–100)
24 Months						
WBC count	12	152	⩾9 cells/μL	0.897	92 (62–100)	98 (94–100)
LATEX/IgM	12	136	⩾1:16	0.659	67 (35–90)	99 (96–100)
Protein	12	138	⩾472 mg/L	0.652	83 (52–98)	82 (74–88)

NOTE. Cutoffs were assessed at a maximal Youden index.

before treatment, and none of the receiver operator characteristic curves had an AUC >0.80.

Among cured patients, Miézan et al. [12] described normalization of total protein concentration and trypanosome-specific antibodies before normalization of the WBC count. In our study, the WBC count was the first to normalize, and the total protein concentration was last to normalize. In both studies, some cured patients showed WBC counts of 5–20 cells/μL 24 months after treatment. As observed elsewhere [15, 35, 36], IgM disappears slowly from the CSF after treatment and reaches normal levels only 6–12 months after treatment. Although specific antibodies are already undetectable in the CSF of 89% of cured patients 3 months after treatment, we cannot make conclusions about their kinetics, because those antibodies were already undetectable in 63% of patients before treatment.

In patients who experience relapse, an early increase in WBC count was followed by an increase in CSF IL-10 concentration, IgM level, and total protein concentration 12 months after treatment and an increase in trypanosome-specific antibodies 18 months after treatment. Persistence of or increase in total IgM level and trypanosome-specific antibodies in CSF is characteristic of relapse [15–19], but we report their sequential activation, to our knowledge, for the first time. The relatively high proportion of relapses detected by blood analysis (in 4 of 12 patients) after 24 months is surprising and might explain the observed decrease in marker median levels.

Final assessment of treatment outcome in HAT is currently scheduled at 24 months after treatment [8]. We investigated the markers' performance for earlier diagnosis of treatment outcome. The earliest diagnostic marker was the WBC count at 6 months after treatment, followed by IL-10 level, LATEX/IgM level, and total protein concentration at 12 months after treatment and LATEX/*T. b. gambiense* levels at 18 months after treatment.

For early diagnosis of relapse and initiation of re-treatment, application of the optimal cutoffs at 6 and 12 months after treatment would imply unnecessary re-treatment of 3%–14% of cured patients. At 18 months after treatment, all single parameters were able to diagnose treatment failure more accurately, with 1%–8% of unnecessary re-treatments in the cured group (positive predictive value [PPV] of 70%–94% and negative predictive value [NPV] of 93%–97%). The combination of WBC count and LATEX/IgM level was assessed because both can be performed in HAT treatment centers. It yielded a specificity of 95% at 6 months (PPV, 79%; NPV, 88%), of 97% at 12 months (PPV, 86%; NPV, 95%), and of 100% at 18 months (PPV, 100%; NPV, 96%) and at 24 months (PPV, 100%; NPV, 97%) after treatment.

In the present study, a normalization of the median WBC count was observed in the group of cured patients at 6 months after treatment, whereas in almost all patients who experienced subsequent treatment failure, the median WBC count was increased at assessment 6 months after treatment. A diagnostic marker for cure, which would enable early discharge of patients at low relapse risk, would allow a more intensive follow-up of high-risk patients [34]. The criterion of a WBC count ⩽5 cells/μL at 6 months after treatment seems promising in this regard; in our data, it categorized a subgroup of 92 (63%) of 146 patients as having "low risk of relapse," which left a 37% subgroup of patients who would require intensive monitoring. The

latter group included 81% (26 of 32 patients) of all relapses (PPV, 48%; NPV, 93%).

Our data have limitations. An important number of patients were lost to follow-up or missed interim control visits, but compliance in this study was as good as that in other published clinical trials with active follow-up [30, 31, 37] and was higher than in routine control activities [12]. The exclusion of relapses from the data set after the time at which they were diagnosed leads to a considerable reduction of number of outcomes and analytical power in function of time, especially 24 months after treatment. The cutoffs and test performances should, therefore, be interpreted carefully.

The assumption that those who did not match the relapse definition within 24 months were cured implies that patients lost to follow-up were considered to be cured and that relapses occurring after the last 24 month posttreatment control visit remained undetected. Finally, parasite detection has limited sensitivity, which might have left relapses undetected. As a consequence, the cured group may have contained false-negative results that led to an underestimation of specificity.

In contrast, the probability that the relapse group contained false-positive results seems low, because 42 (88%) of the 48 relapses were diagnosed parasitologically. Only 6 relapses were defined on the basis of WBC counts of 61–114 cells/μL, of which 5 occurred at 24 months after treatment.

Although PCR has potential for early detection of treatment failure [38, 39], we did not use PCR, because a reliable method for sample preparation was not available to us at the start of the study. Prolonged storage of serum and CSF specimens should not have affected quality of the data, because the markers, including IL-10, are relatively stable under the applied storage conditions [40].

In this study, data on patient groups that received different therapies were pooled, although the treatment may have influenced the evolution of the investigated markers. This is evidenced by the different failure rates of the regimens and the fact that relapses among patients with normal CSF findings but with blood parasites were observed only for those treated with nifurtimox [23]. The sample size per treatment arm did not allow for separate analysis of each marker or for definite conclusions about the independence of marker performance from treatment regimen.

In conclusion, our results confirm that *T. b. gambiense*–infected patients with severe brain inflammation at hospital admission are at high risk of treatment failure. Close monitoring of such patients is recommended. We identified operational criteria that should be validated in larger prospective studies, because they may facilitate the monitoring of patients after treatment. Six months after treatment, a patient group with WBC counts $\leqslant$5 cells/μL was identified as being at low risk of relapse. Twelve months after treatment, the combination of WBC count and LATEX/IgM levels allows accurate detection of subsequent treatment failure. Eighteen months after treatment, each individual marker has acceptable diagnostic accuracy to detect treatment failure, but the combination of LATEX/IgM level and WBC count provides 100% specificity, which is retained at 24 months after treatment. A longitudinal study to validate these markers in a larger group of *T. b. gambiense*–infected patients is performed in the Democratic Republic of Congo to verify the validity and the clinical relevance of findings reported here.

Acknowledgments

We thank F. Balharbi and N. Bebronne for their technical support in analyzing the samples. Logistic, medical, and laboratory support in Congo was provided by the Centre de Développement Intégral (Bwamanda, Democratic Republic of Congo), Medische Missie Samenwerking (Itterbeek, Belgium), and the staff of Bwamanda Hospital—in particular, the late Sister J. Verbunt.

Financial support. Belgian Ministry of Foreign Affairs, Directorate General for Development Cooperation.

Potential conflicts of interest. All authors: no conflicts.

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