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# Neuro-inflammatory risk factors for treatment failure in "early second stage" sleeping sickness patients treated with Pentamidine

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**Abstract**

In a clinical trial on efficacy of Pentamidine in second stage *Trypanosoma brucei gambiense* patients with ≤20 cells/μl in cerebrospinal fluid (CSF), 43% of treatment failures were observed. We hypothesised that unsuccessful treatment was caused by uncured brain infection. The relationship between treatment outcome and CSF cell count, protein concentration, presence of trypanosomes, the intrathecal immune response, and CSF total IgM and trypanosome specific antibodies detected by LATEX/IgM and LATEX/*T.b. gambiense* card agglutination tests was examined. Cell counts of 11–20 cells/μl, intrathecal IgM synthesis, CSF end-titres in LATEX/IgM ≥4 and LATEX/*T.b. gambiense* positive CSF, were associated with treatment failure.

Detection of intrathecal IgM synthesis is valuable for assessment of brain involvement and treatment decision.


*Keywords:* *Trypanosoma brucei gambiense*; Cerebrospinal fluid; Pentamidine; Intrathecal humoral immune response; IgM; Treatment failure; Card agglutination

## 1. Introduction

Infection with *Trypanosoma brucei* (*T.b.*) *gambiense* causes human African trypanosomiasis or sleeping sickness. The disease occurs in West and Central Africa and is transmitted by tsetse flies. After the infective bite, parasites initially proliferate in the hemo-lymphatic system (the first or hemo-lymphatic disease stage), but as the disease advances, the central nervous system (CNS) is invaded (the second or meningo-encephalitic disease stage). Sleeping sickness is fatal if left untreated.

Pentamidine is a relatively safe drug used for first stage treatment, but is inefficient when the CNS has been infected, since it does not sufficiently cross the blood–brain barrier. Treatment of the meningo-encephalitic stage relies almost exclusively on Melarsoprol, which is highly toxic and requires hospitalisation (Pépin and Milord, 1994; Van Nieuwenhove, 1999).

Stage determination is based on the determination of the white blood cell count and detection of trypanosomes in the cerebrospinal fluid (CSF). Patients with >5 cells/μl or trypanosomes in CSF are classified in the second stage and should be treated as such (WHO, 1998). However, successful Pentamidine treatment of second stage patients with up to 20 cells/μl has been reported (Lourie, 1942; Pépin and Milord, 1994). In contrast, in a recent clinical trial conducted in Uganda, 42.9% of Pentamidine treatment failures were observed in such patients (Legros, personal communication).

A large proportion of patients with ≤20 cells/μl, even with CSF trypanosomes, can therefore be cured with Pentamidine, but in some patients, trypanosomes seem to have reached CNS compartments inaccessible for Pentamidine. Before deciding on Pentamidine chemotherapy of second stage sleeping sickness patients with ≤20 cells/μl, additional criteria to exclude CNS involvement should be considered.

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Blood–CSF barrier dysfunction, intrathecal immunoglobulin and trypanosome specific antibody synthesis represent powerful tools for diagnosis of neuro-inflammation (Reiber and Lange, 1991), and are promising markers for CNS involvement in sleeping sickness patients (Bisser et al., 2002; Lejon et al., 2003). In the health centres of rural areas where the disease prevails, their assessment is technically not possible. High levels of IgM and of trypanosome specific antibodies occur in the CSF of second stage sleeping sickness patients (Lucasse, 1964; Greenwood and Whittle, 1973) and can be detected in the field by LATEX/IgM and LATEX/*T.b. gambiense* card agglutination tests (Büscher et al., 1999; Lejon et al., 2002). Positivity in these tests can be considered as indirect evidence of an intrathecal immune response.

Our objective was to examine the relationship between treatment outcome in second stage patients with $\leq 20$ cells/μl treated with Pentamidine and (1) presence of trypanosomes, CSF total protein concentration and CSF cell count (WHO criteria), (2) the intrathecal humoral immune response, (3) detection of the total IgM concentration and trypanosome specific antibodies in CSF by LATEX/IgM and LATEX/*T.b. gambiense*. The same analysis was done on the corresponding Melarsoprol-treated patients, to evaluate whether the identified risk factors for Pentamidine treatment failure are indeed useful for treatment decision.

## 2. Materials and methods

### 2.1. Samples

Paired serum and CSF taken for diagnostic purposes was available from a randomised open clinical equivalence trial on the efficacy of Pentamidine compared to Melarsoprol among second stage patients with $\leq 20$ cells/μl (Legros, unpublished data). The trial was conducted in Arua district, Uganda. Informed consent was obtained from all patients or their parents or companions. Patients had to fulfil the following conditions: [presence of trypanosomes in blood, gland juice or CSF] AND [CSF cell count >5 cells/μl OR presence of trypanosomes in the CSF] AND [CSF cell count $\leq 20$ cells/μl]. Trypanosomes in CSF were detected by double centrifugation (Cattand et al., 1988).

Only patients that had given their informed consent and were not previously treated for sleeping sickness were included. A total of 103 patients, of which 51 were treated with Melarsoprol and 52 with Pentamidine (block randomisation with uniform allocation with the team on the field blinded for blocking procedures), participated in the trial. Five patients were lost to follow-up. A patient was considered cured when trypanosomes were absent and the CSF cell count was $\leq 5$ cells/μl after a follow-up of 2 years. Treatment failure or relapse during follow-up was defined as presence of trypanosomes in blood, CSF or gland juice, more than 20 cells/μl in CSF, or CSF cell count rising on two consecutive follow-up control visits. Respectively 16/49 of the Melarsoprol, and 21/49 of the Pentamidine-treated patients relapsed.

We studied a subset of 33 Pentamidine-treated patients of this trial with known outcome and for which pre-treatment CSF was available. Of all but one of this subgroup of 33 patients, serum samples were also available. Among these 33 patients, 11 relapsed.

Among the Melarsoprol-treated patients with known outcome, CSF samples were available from 33 patients, serum samples from 32 patients. Among these 33 patients, 11 relapsed and 1 patient died during the follow-up period. The latter is also considered as a relapse.

### 2.2. CSF total protein and intrathecal humoral immune response

The total protein concentration in CSF was determined in duplicate with the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL), using the microtiter plate protocol with bovine serum albumin as a standard. Total IgG, total IgM and albumin were determined by nephelometry (BN100, Dade Behring). IgM and albumin were measured by sandwich ELISA if concentrations fell below the detection limit of nephelometry (4 mg/l IgM and 89 mg/l albumin). Trypanosome specific IgG antibodies in serum and CSF were quantified by a semi-quantitative ELISA and their CSF/serum quotient $Q_{\mathrm{IgGsp}}$ was determined (Lejon et al., 1998).

Blood–CSF barrier dysfunction was evaluated by the albumin quotient $Q_{\mathrm{Alb}}$ = CSF/serum albumin. The upper reference limit was $5 \times 10^{-3}$ for patients under 15 years, $6.5 \times 10^{-3}$ for patients aged 16–40 years and $8 \times 10^{-3}$ for patients older than 41 (Andersson et al., 1994).

The intrathecal humoral immune response was quantified using Reibers hyperbolic discrimination curve and calculation of the antibody index (Reiber and Lange, 1991; Reiber and Peter, 2001). The maximum IgG and IgM CSF/serum quotient ($Q_{\mathrm{Lim}}$) in the absence of intrathecal immunoglobulin synthesis was calculated as $Q_{\mathrm{Lim}}=(a/b) \times (Q_{\mathrm{Alb}}^2 + b^2)^{1/2} - c$ with for $Q_{\mathrm{Lim}}$(IgG) $a/b = 0.93$, $b^2 = 6 \times 10^{-6}$, $c = 1.7 \times 10^{-3}$, with for $Q_{\mathrm{Lim}}$(IgM) $a/b = 0.67$, $b^2 = 120 \times 10^{-6}$, $c = 7.1 \times 10^{-3}$. The percentage of the total CSF immunoglobulin concentration derived from intrathecal synthesis, the intrathecal fraction (IF), was $\mathrm{IF_{Ig}}=(1 - Q_{\mathrm{LimIg}}/Q_{\mathrm{Ig}}) \times 100$ with $Q_{\mathrm{Ig}}$ = CSF/serum Ig (Reiber and Peter, 2001). The IF is positive if >0%. The antibody index (AI) for IgGsp was determined as $\mathrm{AI_{IgGsp}} = Q_{\mathrm{IgGsp}}/Q_{\mathrm{IgG}}$ when $Q_{\mathrm{IgG}} < Q_{\mathrm{Lim}}$(IgG), or $\mathrm{AI_{IgGsp}} = Q_{\mathrm{IgGsp}}/Q_{\mathrm{Lim}}$(IgG) when $Q_{\mathrm{IgG}} > Q_{\mathrm{Lim}}$(IgG). The AI is negative if $< 1.5$ and positive if $\geq 1.5$ (Reiber and Lange, 1991).

### 2.3. IgM quantification by LATEX/IgM

The CSF end titre in LATEX/IgM was determined (Lejon et al., 2002). Briefly, two-fold serial dilutions of CSF were

prepared in PBS. 20 μl of LATEX/IgM reagent was mixed with 20 μl of diluted or undiluted CSF and spread over the reaction zone of a test card. The card was rocked at 70 rpm and the agglutination was scored after 5 min. The end titre of a CSF sample—the highest dilution still yielding an agglutination—was determined and expressed as the reciprocal of the dilution factor.

### 2.4. Trypanosome specific antibody detection by LATEX/T.b. gambiense

Briefly, 15 μl of LATEX/*T.b. gambiense* was mixed with 30 μl of CSF on a test card, and rocked for 10 min after which the agglutination was scored (Büscher et al., 1999). All patients with reaction of undiluted CSF in LATEX/*T.b. gambiense* were considered positive.

### 2.5. Statistical analysis

The CSF cell count and total protein concentration were recorded as categorical variables with two categories, above and below the mean values. For LATEX/IgM, cut-off values $\geq 4$, $\geq 8$ and $\geq 16$ were considered separately. Differences in proportions of treatment failures were assessed by Fisher's exact test. Confidence intervals for odds ratios were calculated using logistic regression.

Means were compared by the Student's *t*-test, if the variances within each group were homogeneous (assessed using the Bartlett's test), and Kruskal–Wallis test if not.

## 3. Results

### 3.1. Total patient population characteristics

The mean age of the patients was 26 (standard deviation 13, range 2–59) and male/female ratio was 1:1. Upon clinical examination at admission, the mean body temperature was 37.1 °C (standard deviation 0.5, range 36–38.2). All patients had a normal Glasgow score, a normal tonus, could stand up and walk normally and showed normal coordination. Abnormal movements of the limbs (tremor) were observed in 5 out of 66 patients (8%), but abnormal movements of head, face or trunk did not occur. Serious neurological symptoms or signs were thus not present in any of the patients.

### 3.2. Cerebrospinal fluid analysis of Pentamidine-treated patients

#### 3.2.1. Cell count, trypanosome detection and total protein concentration

The mean CSF cell count was 10.2 cells/μl (standard deviation 5.4 cells/μl). A significant association was observed between a CSF cell count of 11–20 cells/μl and the occurrence of treatment failures (Table 1). Patients with cell counts of 11–20 cells/μl had 7.1 times higher odds to relapse than patients with a lower cell count (95% confidence interval 1.4–36). The mean cell counts of relapsed (13.4 cells/μl) and cured patients (8.6 cells/μl) differed significantly ($p=0.016$).

Trypanosomes were observed in 13 out of 33 patients (39%). The presence of trypanosomes in CSF was not associated with higher odds for treatment failure after Pentamidine treatment (Table 1).

The mean CSF total protein concentration was 319.3 mg/l (standard deviation 167 mg/l). A total protein concentration higher than 320 mg/l was not associated with occurrence of treatment failures (Table 1). The difference between mean CSF protein concentration of relapsed (403 mg/l) and cured (277 mg/l) patients was not statistically significant ($p=0.29$, Kruskal–Wallis).

#### 3.2.2. Intrathecal humoral immune response and blood–CSF barrier function

Due to a missing serum sample for 1 patient, the intrathecal immunoglobulin response and blood–CSF barrier function could be calculated only for 32 patients.

Among the 30 patients with normal blood–CSF barrier function, 9 relapsed (30%, Table 1). Although the 2 patients with blood–CSF barrier dysfunction relapsed after Pentamidine treatment (100%), no significant difference in proportion of treatment failures was observed in patients with or without barrier dysfunction.

Of 32 patients, 17 (53%) showed intrathecal IgM synthesis (Fig. 1). The mean intrathecal IgM fraction of these patients was 45% (standard deviation 26). The two patients with blood–CSF barrier dysfunction had intrathecal IgM fractions of 71% and 86%. The presence of intrathecal IgM synthesis was significantly associated with the occurrence of treatment failures (Table 1). The odds for treatment failures in patients with intrathecal IgM synthesis was 20 times higher than in patients without IgM synthesis (95% confidence interval 2.1–189).

Intrathecal total IgG synthesis occurred in 6/32 patients (19%), 10/32 patients (31%) had a positive trypanosome specific antibody index reflecting intrathecal synthesis of trypanosome specific IgG. Treatment failures did not occur more frequently in patients positive for one of these IgG parameters (Table 1).

#### 3.2.3. Card agglutination tests

The CSF end titers observed with LATEX/IgM ranged between 0 (no reaction with undiluted CSF) and 64. All four patients with a CSF LATEX/IgM end titer $\geq 32$ relapsed. Cut-off CSF end titers $\geq 4$, $\geq 8$ or $\geq 16$ were significantly associated with the occurrence of treatment failures (Table 1). The odds for relapsing in patients with CSF LATEX/IgM end titre $\geq 4$ was 9.6 times higher than in patients with lower end-titres (95% confidence interval 1.6–57), and 12 times higher with CSF end titres of

Table 1
Proportion of treatment failures in function of CSF cell count, presence of trypanosomes, total protein, blood–CSF barrier function, intrathecal IgG and IgM synthesis, the antibody index for IgG ($AI_{IgG}$), LATEX/IgM at different cut-off end titres, and LATEX/*T.b. gambiense*

| Variable | Pentamidine treatment | | Melarsoprol treatment | |
|---|---|---|---|---|
| | Percentage of relapses | $p$ value OR (CI) | Percentage of relapses | $p$ value OR (CI) |
| CSF cell count (cells/μl) | | $p=0.024$* | | $p=0.15$ |
| 0–10 | 16% (3/19) | 7.1 (1.4–36) | 50% (9/18) | 0.25 (0.05–1.2) |
| 11–20 | 57% (8/14) | | 20% (3/15) | |
| Trypanosomes in CSF | | $p=1.00$ | | $p=0.47$ |
| Absent | 35% (7/20) | 0.83 (0.19–3.7) | 27% (4/15) | 2.2 (0.50–9.6) |
| Present | 31% (4/13) | | 44% (8/18) | |
| CSF total protein (mg/l) | | $p=0.71$ | | $p=0.25$ |
| <320 mg/l | 30% (6/20) | 1.5 (0.34–6.3) | 45% (10/22) | 0.27 (0.05–1.5) |
| ≥320 mg/l | 38% (5/13) | | 18% (2/11) | |
| Blood–CSF barrier | | $p=0.11$ | | not applicable |
| No dysfunction | 30% (9/30) | 8516 (0.00–$2\times10^{40}$) | 38% (12/32) | |
| Dysfunction | 100% (2/2) | | (0/0) | |
| Intrathecal IgM synthesis | | $p=0.003$* | | $p=0.29$ |
| Negative | 6.7% (1/15) | 20 (2.1–189) | 27% (4/15) | 2.4 (0.55–11) |
| Positive | 59% (10/17) | | 47% (8/17) | |
| Intrathecal IgG synthesis | | $p=0.39$ | | $p=1.00$ |
| Negative | 31% (8/26) | 2.3 (0.37–14) | 38% (10/26) | 0.80 (0.12–5.2) |
| Positive | 50% (3/6) | | 33% (2/6) | |
| $AI_{IgG}$ | | $p=0.70$ | | $p=1.00$ |
| Negative | 32% (7/22) | 1.4 (0.30–6.7) | 36% (9/25) | 1.3 (0.24–7.3) |
| Positive | 40% (4/10) | | 43% (3/7) | |
| LATEX/IgM | | $p=0.01$* | | $p=0.48$ |
| End titre <4 | 12% (2/17) | 9.6 (1.6–57) | 44% (7/16) | 0.54 (0.13–2.25) |
| End titre ≥4 | 56% (9/16) | | 29% (5/17) | |
| LATEX/IgM | | $p=0.008$* | | $p=0.46$ |
| End titre <8 | 20% (5/25) | 12 (1.8–78) | 43% (9/21) | 0.44 (0.09–2.1) |
| End titre ≥8 | 75% (6/8) | | 25% (3/12) | |
| LATEX/IgM | | $p=0.033$* | | $p=0.43$ |
| End titre <16 | 25% (7/28) | 12 (1.1–126) | 42% (10/24) | 0.40 (0.07–2.3) |
| End titre ≥16 | 80% (4/5) | | 22% (2/9) | |
| LATEX/*T.b. gambiense* | | $p=0.002$* | | $p=1.00$ |
| Negative | 17% (4/24) | 17 (2.6–117) | 36% (9/25) | 1.1 (0.21–5.5) |
| Positive | 78% (7/9) | | 38% (3/8) | |

OR: odds ratio, CI: 95% confidence interval.
*Significant difference in proportion of treatment failures.

≥8 and ≥16 (95% confidence intervals of respectively 1.8–78 and 1.1–126).

The CSF of 9/33 patients (27%) was positive in LATEX/*T.b. gambiense*. Seven of these patients (78%) relapsed. The odds for relapsing in patients with LATEX/*T.b. gambiense* positive CSF was 17 times higher than in patients with LATEX/*T.b. gambiense* negative CSF (95% confidence interval 2.6–117).

### 3.3. Cerebrospinal fluid analysis of Melarsoprol-treated patients

#### 3.3.1. Cell count, trypanosome detection and total protein concentration

The mean CSF cell count was 10.6 cells/μl (standard deviation 5.4 cells/μl). A cell count of 11–20 cells/μl was not significantly associated with treatment failure (Table 1) and the mean cell counts of relapsed (8.9 cells/μl) and cured patients (11.5 cells/μl) did not differ significantly ($p=0.18$, Kruskal–Wallis).

Trypanosomes were observed in 18/33 patients (55%). The presence of trypanosomes in CSF was not associated with treatment failure after Melarsoprol treatment (Table 1).

The mean CSF total protein concentration was 281 mg/l (standard deviation 125 mg/l). A total protein concentration higher than 320 mg/l was not associated with occurrence of treatment failures (Table 1). The difference between mean CSF protein concentration of relapsed (277 mg/l) and cured (284 mg/l) patients was not statistically significant ($p=0.5$, Kruskal–Wallis).

#### 3.3.2. Intrathecal humoral immune response and blood–CSF barrier function

The intrathecal immunoglobulin response and blood–CSF barrier function could be calculated only for 32/33 patients.

None of the 32 patients treated with Melarsoprol showed blood–CSF barrier dysfunction.

Intrathecal total IgM synthesis occurred in 17/32 patients (53%), intrathecal IgG synthesis in 6/32 patients (19%) and

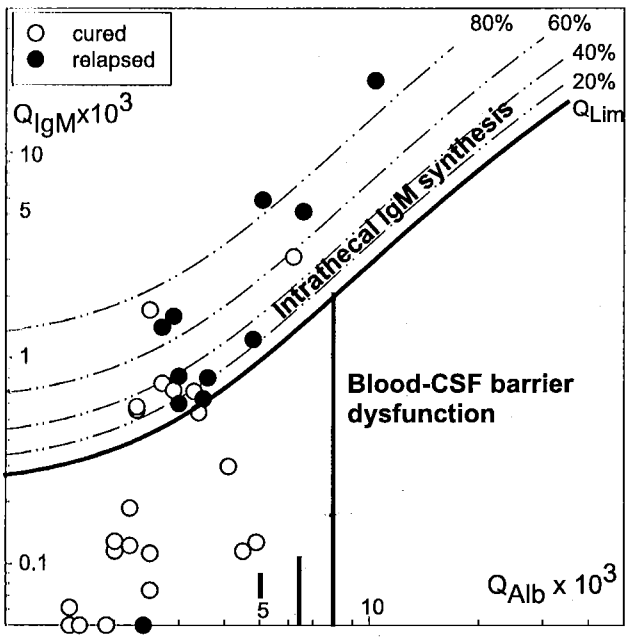


Fig. 1. CSF/serum quotient diagram for IgM with hyperbolic graphs (Reiber and Peter, 2001). The normal range of the blood derived IgM fraction in CSF is under the bold hyperbolic line $Q_{Lim}$. Values above the $Q_{Lim}$ line represent intrathecal synthesis. Intrathecal synthesis is expressed as intrathecal IgM fraction ($IF_{IgM}$) or locally produced IgM as a percent of the total CSF IgM concentration (visualised by dashed lines). The bold vertical lines indicate the upper reference range for normal blood–CSF barrier function for patients aged 15, 40 and 60. Patients cured by Pentamidine treatment are indicated by open circles, relapsed patients by black dots.

intrathecal trypanosome specific IgG synthesis in 7/32 patients (22%). Relapses did not occur more frequently in patients positive for one of these parameters (Table 1).

*3.3.3. Card agglutination tests*

The CSF end titers observed with LATEX/IgM ranged between 0 and 64. There was no association between CSF end titers $\geq 4$, $\geq 8$ or $\geq 16$ and treatment failure (Table 1).

The CSF of 8/33 patients (24%) was positive in LATEX/*T.b. gambiense*. Positivity in LATEX/*T.b. gambiense* was not associated with treatment failure (Table 1).

## 4. Discussion

The WHO parameters for determination of CNS involvement in sleeping sickness are the presence of trypanosomes in CSF, the CSF total protein concentration and the CSF cell count (WHO, 1998).

If trypanosomes are detected in CSF, a patient is classified in second stage and treated with Melarsoprol. However, trypanosomes have been reported in CSF from patients with normal CSF total protein concentration and cell number (Miézan et al., 1998), and such patients have been cured with Pentamidine (Doua et al., 1996). This finding is confirmed here, since 9 out of 13 patients (69%) with trypanosomes in CSF were successfully treated with Pentamidine. This might be explained by early invasion of trypanosomes via areas lacking a blood–brain barrier (Schultzberg et al., 1988). From there, trypanosomes can spread into the CSF and might be detectable upon CSF examination. If at that moment, real CNS infection is not yet established, patients might still be curable with Pentamidine since low concentrations of Pentamidine have been measured in the CSF (Bronner et al., 1991). Alternatively, elimination of trypanosomes from blood might suffice to stop trypanosome leakage into CSF, assuming that survival time of trypanosomes in CSF is limited (Cattand et al., 1988). As a consequence, one can cast doubt about the benefit of trypanosome detection, but also about antigen or DNA detection tests on CSF for stage determination (Nantulya et al., 1992; Truc et al., 1999). The number of examined patients in this study was however not sufficiently high to reject "the presence of trypanosomes in CSF of patients with cell counts $\leq 20$ cells/μl" as a criterion for stage determination in sleeping sickness based on our findings.

Determination of the total protein concentration in CSF is rarely performed in the field. This can be attributed to the need of sophisticated material and the instability of the reagents. In the present study, low total protein concentrations were not associated with reduced risk for Pentamidine treatment failure, and there was no significant difference in the mean CSF total protein concentrations of relapsed and cured patients. As a consequence, protein determination for staging seems superfluous, which confirms earlier observations (Miézan et al., 1998; Lejon et al., 2003).

The protein concentration in CSF is a function of blood–CSF barrier function. Increased protein concentrations in CSF correlate to blood–CSF barrier dysfunction in sleeping sickness patients (Bisser et al., 2002; Lejon et al., 2003). However, blood–CSF barrier dysfunction in trypanosomiasis patients occurs infrequent, is late and mild (Bisser et al., 2002; Lejon et al., 2003). This was confirmed in the present study: both patients with blood–CSF barrier dysfunction relapsed. No significant association between blood–CSF barrier function and treatment failures could be demonstrated due to the limited dataset.

The CSF white blood cell count is most widely used for stage determination. It has been suggested to raise the cut-off of the cell count to 20 cells/μl (Bisser et al., 1997) and patients with up to 20 cells/μl have been cured with Pentamidine (Lourie, 1942; Doua et al., 1996). Although the national control programmes of Côte d'Ivoire and Angola adopted this cut-off (Stanghellini and Josenando, 2001), limited data exist on the results. The high treatment failure rates in the clinical trial in Uganda, which were observed when second stage patients with $\leq 20$ cells/μl were treated with Pentamidine, suggest that the number of patients with CNS involvement is underestimated using this cut-off. A cut-off around 10 cells/μl might be more appropriate, since cell counts of 11–20 cells/μl were associated to

Pentamidine treatment failures. Using this cut-off in a study in Angola, no difference in relapse rates between Pentamidine-treated patients with cell counts of 0–5 or 6–10 cells/μl has been reported (Ruiz et al., 2002).

Demonstration of intrathecal immunoglobulin and antibody synthesis are powerful tools for diagnosis of neurological disorders, as has been shown for multiple sclerosis and several infectious diseases of the CNS (Reiber and Lange, 1991; Felgenhauer and Reiber, 1992; Andersson et al., 1994; Tumani et al., 1995; Reiber and Peter, 2001).

No association between intrathecal IgG synthesis and Pentamidine treatment failure was observed, nor between trypanosome specific IgG synthesis and Pentamidine treatment failure. This can be explained by the lower sensitivity of these parameters for CNS involvement (Lejon et al., 2003) or could result from the limited dataset.

CSF positivity in LATEX/*T.b. gambiense* was significantly associated with treatment failure. LATEX/*T.b. gambiense* detects trypanosome specific antibodies in the CSF, has limited sensitivity but seems rather specific for the second stage (Büscher et al., 1999). If implementation of Melarsoprol treatment in this study would have been based on CSF LATEX/*T.b. gambiense* positivity, 24 patients (73%) would have been treated with Pentamidine and 4/24 (16.7%) of Pentamidine treatment failures would have occurred. When combining cell count (cut of 10 cells/μl) and LATEX/*T.b. gambiense*, 15 patients (45%) would have been treated with Pentamidine and 1 patient (6.7%) in this group would have relapsed (data not shown).

Sleeping sickness is characterised by a predominant intrathecal IgM response, occurring in 95% of patients with CSF cell counts >20 cells/μl (Lejon et al., 2003). The association of an intrathecal IgM response with treatment failures in patients with $\leq 20$ cells/μl confirms the interest of detection of intrathecal IgM synthesis for diagnosis of neurological involvement and treatment decision. If in this study, patients showing intrathecal IgM synthesis would have been treated with Melarsoprol, this would have been the most efficient strategy to avoid treatment failures. Unfortunately, calculation of intrathecal IgM synthesis requires sensitive and precise IgM and albumin determination in serum and CSF, for which a nephelometer and special, rather expensive reagents are necessary. As a consequence, demonstration of intrathecal IgM synthesis remains utopia in African rural settings.

Total IgM in CSF has long been suggested as an indicator of intrathecal IgM synthesis and CNS involvement (Mattern, 1968; Greenwood and Whittle, 1973; Whittle et al., 1977; Lambert et al., 1981; Knobloch et al., 1984). The absolute CSF IgM concentration is additionally influenced by serum IgM and blood–CSF barrier function, and is less accurate than detection of intrathecal IgM synthesis. The development of a card agglutination test for IgM detection in CSF, LATEX/IgM, facilitates IgM detection in the field (Lejon et al., 2002). Cerebrospinal fluid end titers $\geq 4$, 8 or 16 are associated with risk for Pentamidine treatment failure in early second stage sleeping sickness patients, which confirms the interest of LATEX/IgM. Cut-offs of four or eight seem most appropriate if LATEX/IgM is to be used for treatment decision. Application of a cut-off LATEX/IgM end titre of $\geq 4$ in this study would have resulted in Pentamidine treatment of 52% of the patients, with 11.8% of treatment failures. A cut-off of $\geq 8$ for treatment decision in this study would have resulted in Pentamidine treatment of more patients (75%), but also more failures (20%). If the cell count (cut-off 10 cells/μl) plus the LATEX/IgM end titre (cut-off $\geq 4$) would have been combined for treatment decision, 14 patients would have been treated with Pentamidine, reducing the number of treatment failures to 1/14 (7.1%) (data not shown).

In conclusion, this study shows that CSF cell counts of 11–20 cells/μl, presence of intrathecal IgM synthesis, CSF end titres in LATEX/IgM $\geq 4$ or LATEX/*T.b. gambiense* positive CSF are associated with increased risk of Pentamidine treatment failure among second stage patients with $\leq 20$ cells/μl. There is evidence that in sleeping sickness, those parameters may be useful for assessment of CNS involvement, which is incurable by Pentamidine. As expected, such relationship between CNS involvement and treatment failure could not be identified in the Melarsoprol-treated patient group. In contrast to Pentamidine, Melarsoprol is effective when the CNS is infected, and the high number of relapses observed with Melarsoprol in the clinical trial might be due to Melarsoprol refractoriness of trypanosomes (Matovu et al., 2001a,b).

Due to the limited dataset, our study cannot rule out other associations that could be clinically relevant. As a consequence, presence of trypanosomes in CSF, increased total protein in CSF, blood–CSF barrier dysfunction, intrathecal IgG synthesis and intrathecal trypanosome specific IgG synthesis cannot be excluded as possibly important risk factors for treatment failure. Therefore, recommendations towards establishment of a treatment strategy are preliminary.

Our results give hope that Melarsoprol may be replaced with the far less problematic Pentamidine in a significant proportion of the patients treated for sleeping sickness. The presented results require confirmation by appropriately powered comparative trials in diverse settings and patient groups. Moreover, such studies should weight out efficacy, toxicity and ease of application of Pentamidine versus Melarsoprol treatment against each other.

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## References


Andersson, M., Alvarez-Cermeno, J., Bernardi, G., Cogato, I., Fredman, P., Frederiksen, J.L., Frederikson, S., Gallo, P., Grimaldi, L.M., Gronning, M., Keir, G., Lamers, K., Link, H., Magalhães, A., Massaro, A.R., Öhman, S., Reiber, H., Rönnbäck, L., Schluep, M., Schuller, E., Sindic, C.J.M., Thompson, E.J., Trojano, M., Wurster, U., 1994. Cerebrospinal fluid in the diagnosis of multiple sclerosis: a consensus report. J. Neurol. Neurosurg. Psychiatry 57, 897–902.

Bisser, S., Bouteille, B., Sarda, J., Stanghellini, A., Ricard, D., Jauberteau, M.O., Murchan, F., Dumas, M., Breton, J.C., 1997. Apport des examens biochimiques dans le diagnostic de la phase nerveuse de la trypanosomose humaine. Bull. Soc. Pathol. Exot. Fil. 90, 321–326.

Bisser, S., Lejon, V., Preux, P.M., Bouteille, B., Stanghellini, A., Jauberteau, M.O., Büscher, P., Dumas, M., 2002. Blood–cerebrospinal fluid barrier and intrathecal immunoglobulins compared to field diagnosis of central nervous system involvement in sleeping sickness. J. Neurol. Sci. 193, 127–135.

Bronner, U., Doua, F., Ericsson, O., Gustafsson, L.L., Miézan, T.W., Rais, M., Rombo, L., 1991. Pentamidine concentrations in plasma, whole blood and cerebrospinal fluid during treatment of *Trypanosoma gambiense* infection in Côte d'Ivoire. Trans. R. Soc. Trop. Med. Hyg. 85, 608–611.

Büscher, P., Lejon, V., Magnus, E., Van Meirvenne, N., 1999. Improved latex agglutination test for detection of antibodies in serum and cerebrospinal fluid of *Trypanosoma brucei gambiense* infected patients. Acta Trop. 73, 11–20.

Cattand, P., Miezan, B.T., de Raadt, P., 1988. Human African trypanosomiasis: use of double centrifugation of cerebrospinal fluid to detect trypanosomes. Bull. World Health Organ. 66, 83–86.

Doua, F., Miezan, T.W., Sanon, S.R., Boa, Y.F., Baltz, T., 1996. The efficacy of pentamidine in the treatment of early–late stage *Trypanosoma brucei gambiense* trypanosomiasis. Am. J. Trop. Med. Hyg. 55, 586–588.

Felgenhauer, K., Reiber, H., 1992. The diagnostic significance of antibody specificity indices in multiple sclerosis and herpes virus induced diseases of the nervous system. Clin. Investig. 70, 28–37.

Greenwood, B.M., Whittle, H.C., 1973. Cerebrospinal-fluid IgM in patients with sleeping-sickness. Lancet 2, 525–527.

Knobloch, J., Tischendorf, F., König, J., Mehlitz, D., 1984. Evaluation of immunoassays for diagnosis and management of sleeping sickness in Liberia. Tropenmed. Parasitol. 35, 137–140.

Lambert, P.H., Berney, M., Kazyumba, G., 1981. Immune complexes in serum and in cerebrospinal fluid in African trypanosomiasis. Correlation with polyclonal B cell activation and with intracerebral immunoglobulin synthesis. J. Clin. Invest. 67, 77–85.

Lejon, V., Büscher, P., Magnus, E., Moons, A., Wouters, I., Van Meirvenne, N., 1998. A semi-quantitative ELISA for detection of *Trypanosoma brucei gambiense* specific antibodies in serum and cerebrospinal fluid of sleeping sickness patients. Acta Trop. 69, 151–164.

Lejon, V., Legros, D., Richer, M., Ruiz, J.A., Jamonneau, V., Doua, F., Djé, N., N'Siesi, F.X., Bisser, S., Magnus, E., Wouters, I., Konings, J., Vervoort, T., Sultan, F., Büscher, P., 2002. IgM quantification in the cerebrospinal fluid of sleeping sickness patients by a latex card agglutination test. Trop. Med. Int. Health 7, 685–692.

Lejon, V., Reiber, H., Legros, D., Djé, N., Magnus, E., Wouters, I., Sindic, C., Büscher, P., 2003. Intrathecal immune response pattern for improved diagnosis of central nervous system involvement in trypanosomiasis. J. Infect. Dis. 187, 1475–1483.

Lourie, E.M., 1942. Treatment of sleeping sickness in Sierra Leone. Trop. Med. Parasitol. 36, 113–131.

Lucasse, C., 1964. Fluorescent antibody test as applied to cerebrospinal fluid in human sleeping sickness. Bull. Soc. Pathol. Exot. Fil. 57, 283–292.

Matovu, E., Enyaru, J.C.K., Legros, D., Schmid, C., Seebeck, T., Kaminsky, R., 2001a. Melarsoprol refractory *T.b. gambiense* from Omugo, north-western Uganda. Trop. Med. Int. Health 6, 407–411.

Matovu, E., Geiser, F., Schneider, V., Mäser, P., Enyaru, J.C.K., Kaminsky, R., Gallati, S., Seebeck, T., 2001b. Genetic variants of the *TbAT1* adenosine transporter from African trypanosomes in relapse infections following melarsoprol therapy. Mol. Biochem. Parasitol. 117, 73–81.

Mattern, P., 1968. Etat actuel et résultats des techniques immunologiques utilisées à l'Institut Pasteur de Dakar pour le diagnostic et l'étude de la trypanosomiase humaine africaine. Bull. World Health Organ. 38, 1–8.

Miézan, T.W., Meda, H.A., Doua, F., Yapo, F.B., Baltz, T., 1998. Assessment of central nervous system involvement in gambiense trypanosomiasis: value of the cerebro-spinal white cell count. Trop. Med. Int. Health 3, 571–575.

Nantulya, V.M., Doua, F., Molisho, D., 1992. Diagnosis of *Trypanosoma brucei gambiense* sleeping sickness using an antigen detection enzyme-linked immunosorbent assay. Trans. R. Soc. Trop. Med. Hyg. 86, 42–45.

Pépin, J., Milord, F., 1994. The treatment of human African trypanosomiasis. Adv. Parasitol. 33, 2–49.

Reiber, H., Lange, P., 1991. Quantification of virus-specific antibodies in cerebrospinal fluid and serum: sensitive and specific detection of antibody synthesis in brain. Clin. Chem. 37, 1153–1160.

Reiber, H., Peter, J.B., 2001. Cerebrospinal fluid analysis: disease-related data patterns and evaluation programs. J. Neurol. Sci. 184, 101–122.

Ruiz, J.A., Simarro, P.P., Josenando, T., 2002. Control of human African trypanosomiasis in the Quiçama focus, Angola. Bull. World Health Organ. 80, 738–745.

Schultzberg, M., Ambatsis, M., Samuelsson, E.-B., Kristensson, K., Van Meirvenne, N., 1988. Spread of *Trypanosoma brucei* to the nervous system: early attack on circumventricular organs and sensory ganglia. J. Neurosci. Res. 21, 56–61.

Stanghellini, A., Josenando, T., 2001. The situation of sleeping sickness in Angola: a calamity. Trop. Med. Int. Health 6, 330–334.

Truc, P., Jamonneau, V., Cuny, G., Frézil, J.L., 1999. Use of polymerase chain reaction in human African trypanosomiasis stage determination and follow-up. Bull. World Health Organ. 77, 745–748.

Tumani, H., Nölker, G., Reiber, H., 1995. Relevance of cerebrospinal fluid variables for early diagnosis of neuroborreliosis. Neurology 45, 1663–1670.

Van Nieuwenhove, S., 1999. Present strategies in the treatment of human African trypanosomiasis. In: Dumas, M., Bouteille, B., Buguet, A. (Eds.), Progress in Human African Trypanosomiasis, Sleeping Sickness. Springer, Paris, pp. 253–281.

Whittle, H.C., Greenwood, B.M., Bidwell, D.E., Bartlett, A., Voller, A., 1977. IgM and antibody measurement in the diagnosis and management of Gambian trypanosomiasis. Am. J. Trop. Med. Hyg. 26, 1129–1135.

WHO, 1998. Control and surveillance of African trypanosomiasis. WHO Technical Report Series 881, 1–113.