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Review Article: **Cerebrospinal fluid in human African trypanosomiasis: a key to diagnosis, therapeutic decision and post-treatment follow-up**

V. Lejon and P. Büscher

Department of Parasitology, Institute of Tropical Medicine, Antwerpen, Belgium

Summary Human African trypanosomiasis is a lethal parasitic infection with neurological involvement. Examination of the cerebrospinal fluid (CSF) plays an essential role in diagnosis, selection of treatment and post-treatment follow-up. This paper reviews clinical presentation, diagnosis and treatment of the disease, with emphasis on CSF characteristics and interpretation of the CSF results for therapeutic decision and post-treatment follow-up.

keywords human African trypanosomiasis, central nervous system, cerebrospinal fluid, diagnosis, treatment, diagnostic criteria, review

Background

Human African trypanosomiasis (HAT) or sleeping sickness is a lethal disease caused by infection with extracellular protozoan parasites of the *Trypanosoma brucei* (*T.b.*) subspecies. *Trypanosoma brucei rhodesiense* sleeping sickness is found east of the African rift and is a zoonosis (Apted 1970b). In West and Central Africa, sleeping sickness is caused by *T.b. gambiense*, with man as the main reservoir (Scott 1970). Trypanosomes are transmitted by the bites of tsetse flies. After the infective bite, the parasite is initially confined to the hemo-lymphatic system (the first or hemo-lymphatic disease stage), but as the disease advances, the central nervous system (CNS) is invaded which is referred to as the second or meningo-encephalitic disease stage (Dumas & Girard 1978; Greenwood & Whittle 1980). Infection with *T.b. rhodesiense* leads to acute disease with fast CNS invasion. Death may take place within weeks. *Gambiense* infection causes chronic disease that may last for months to years, with slow CNS invasion.

About 60 million people in sub-Saharan Africa are at risk for infection, while less than 4 million of them are under surveillance. Every year, about 30 000 new cases are diagnosed and treated, mainly in Angola, Sudan and R.D. Congo (WHO 1998; Moore & Richer 2001; Stanghellini & Josenando 2001; Van Nieuwenhove *et al.* 2001). The actual number of African trypanosomiasis patients is estimated between 300 000 and 500 000 (WHO 1998).

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Clinical picture

There is considerable variation in the clinical picture of African trypanosomiasis, with many symptoms being non-specific, variable and inconstant (reviewed by Duggan & Hutchinson 1966; Apted 1970a; Dumas & Girard 1978; Edan 1979; Boa *et al.* 1988; Dumas & Bisser 1999; Burri & Brun 2003).

The enlargement of glands, especially in the neck (Winterbottom's sign) is a cardinal sign of *T.b. gambiense* infection. Other clinical signs of the hemo-lymphatic stage are general malaise, headache, fever, pruritus, oedema, splenomegaly, hepatomegaly, and weight loss. As the disease progresses into the meningo-encephalitic stage, signs of nervous system involvement become apparent. Neurological changes are most extensive in *T.b. gambiense* sleeping sickness and include sleep disorders, sensory disturbances, endocrine dysfunction, tone and mobility disorders, abnormal movements, mental changes or psychiatric disorders (Dumas & Bisser 1999). Finally, the patient stops eating, lapses into a semi-coma and dies. Cardiac involvement is more prominent in *rhodesiense* sleeping sickness and in these patients, death may follow from cardiac failure. Anaemia, thrombocytopenia and abnormal liver function may be mild in *gambiense* sleeping

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sickness, but are more marked in *T.b. rhodesiense* infection (Greenwood & Whittle 1980).

Treatment

Sleeping sickness therapy is complicated by the different disease stages. In the meningo-encephalitic stage, the selective permeability of the blood-brain barrier protects trypanosomes in the brain against the action of several drugs, effective in the hemo-lymphatic compartment. Treatment of meningo-encephalitic stage sleeping sickness requires toxic drugs and hospitalization (reviews by Van Nieuwenhove 1999; Legros *et al.* 2002; C. Burri & Brun 2003).

First stage *T.b. rhodesiense* infection is treated with suramin, *gambiense* infection with pentamidine. Both drugs were developed more than 50 years ago. Pentamidine is usually well tolerated, whereas suramin may have severe adverse effects. A new, oral drug for hemo-lymphatic sleeping sickness, DB289, is well tolerated and is currently under phase IIb evaluation (C. Burri, personal communication).

Treatment of the meningo-encephalitic stage relies almost exclusively on melarsoprol, an arsenicum derivative. It is administered intravenously and requires hospitalization for at least 10 days for the short, recently accepted standard course (Burri *et al.* 2000; Schmid *et al.* 2004), or 25–36 days for the old treatment schedule. The worst adverse effect is treatment-associated encephalopathy, which occurs in 5–10% of patients, and is fatal in 50–100% of them (Pépin *et al.* 1994). Alternatives for late stage *T.b. gambiense* sleeping sickness treatment are difluoromethylornithine and nifurtimox. Both drugs are used for treatment of melarsoprol refractory cases, but difluoromethylornithine has been introduced recently as first-line drug by some organizations. In view of the observed increase in melarsoprol resistance, clinical trials on combination therapy are ongoing.

Diagnosis and follow-up

Undiagnosed, a patient will remain untreated and will die sooner or later. In the meantime, he becomes a burden to his family and acts as a reservoir on which tsetse flies can infect themselves. A general flow chart for diagnosis, stage determination, treatment and follow-up of trypanosomiasis patients is presented in Figure 1. Due to their aspecific, variable and inconstant character, clinical signs are insufficient for diagnosis of sleeping sickness. Through detection of trypanosome specific antibodies by agglutination assays, immunofluorescence or ELISA, diagnosis of mainly *T.b gambiense* sleeping sickness is facilitated. The card agglutination test for trypanosomiasis, CATT/*T.b. gambiense* (Magnus *et al.* 1978), is currently used in *T.b. gambiense* endemic areas for mass screening of the population at risk. For reasons related to cost and risks associated with the treatment, definite diagnosis should be obtained by demonstration of the parasite in body fluids. Unfortunately, simple parasitological techniques such as microscopic examination of a lymph node aspirate, or of a wet or thick blood film are insensitive, especially for diagnosis of *T.b. gambiense* infection, where the number of parasites in the blood can be low. Concentration of the trypanosomes by centrifugation using microhematocrit centrifugation (Woo 1971), or by anion exchange chromatography (Lumsden *et al.* 1979), results in slightly improved sensitivity but is more laborious and requires more equipment. Due to the workload, parasitological examination is in practice limited to clinical or serological suspects.

As a consequence of the selective permeability of the blood-brain barrier, and the high toxicity of second stage drugs, an essential step to select optimal treatment with minimal risk for the patient consists of accurate determination of the disease stage. There are no specific clinical signs – except in very advanced cases – nor any clear changes at blood level indicating the evolution from the hemo-lymphatic to the meningo-encephalitic stage (Bisser *et al.* 1997). According to the World Health Organization, stage determination should be performed by examination of the cerebrospinal fluid (CSF) for the presence of trypanosomes, the white blood cell count and the total protein concentration (WHO 1998).

For follow-up after treatment, the blood and the CSF of the patient is re-examined on several occasions (ideally 3, 6, 12, 18 and 24 months after the end of treatment). A patient is considered cured when during this 2 year follow-up period no trypanosomes are detected in the blood, lymph or CSF and when the CSF stayed or returned to normal (WHO 1998).

Thus, examination of the CSF plays a key role in diagnosis, selection of treatment and post-treatment follow-up of sleeping sickness patients (reviews by Van Meirvenne 1999; Büscher & Lejon 2004; Kennedy 2004).

Cerebrospinal fluid in human African trypanosomiasis

For diagnosis of many neurological diseases, the CSF cell count is the cornerstone of CSF examination. In HAT, it is often the only CSF examination performed for staging of the disease. The cut-off value for the white blood cell count in HAT has been set at five cells/μl in CSF. Patients with cell counts higher than 5/μl are considered in the meningo-

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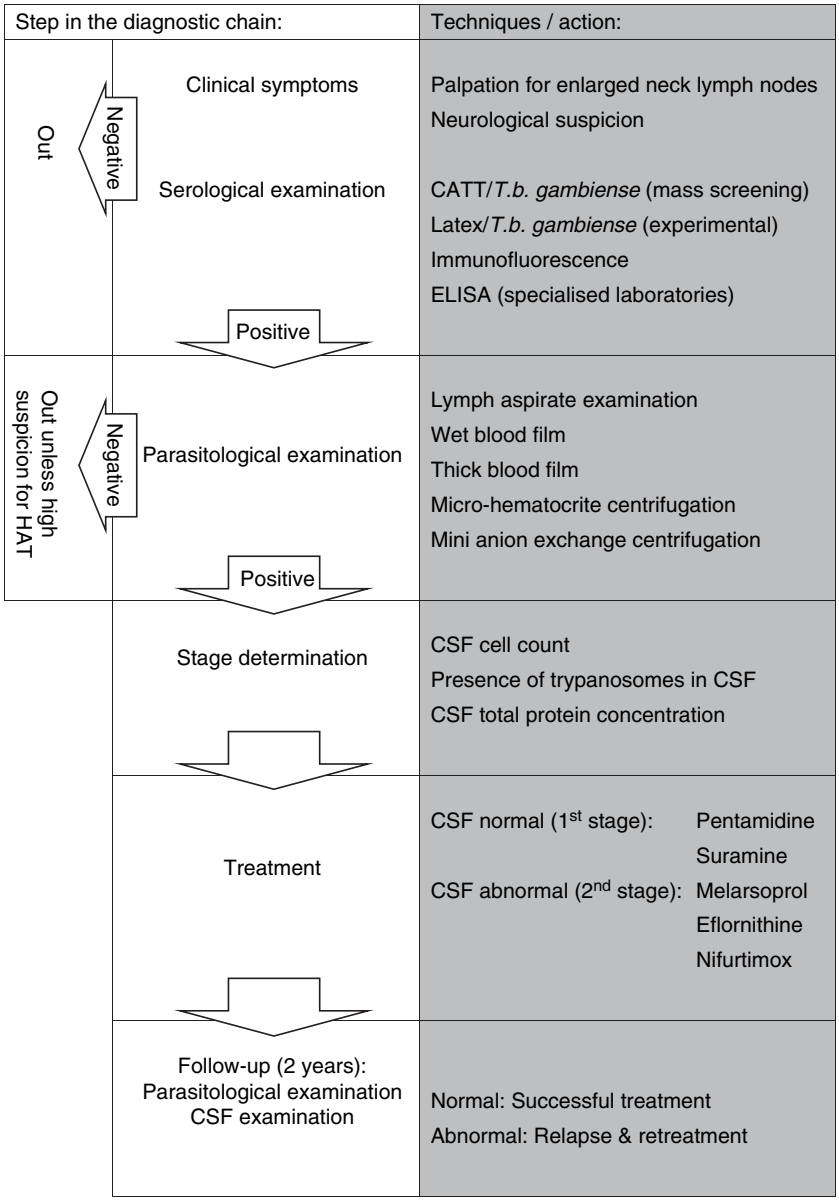

Figure 1 A general flow chart for diagnosis, stage determination, treatment and follow-up of trypanosomiasis patients.

encephalitic stage and should be treated as such (WHO 1998). The number of white blood cells in the CSF of second stage HAT patients varies from normal, to typically around 100–300 cells/μl, and does generally not exceed 1200 cells/μl (Broden & Rodhain 1908; Dumas & Girard 1978; Bisser *et al.* 2002; Miézan *et al.* 2002; Lejon *et al.* 2003a). Pleocytosis is of lymphocytic origin, consisting mainly of B-cells. Some rare eosinophils may be present (Broden & Rodhain 1908; Greenwood *et al.* 1976). A special type of enlarged B-cell, the morular cell of Mott, filled with IgM-containing vacuoles, can also occur in the CSF (Mott 1906; Greenwood & Whittle 1973; Greenwood *et al.* 1976; Greenwood & Whittle 1980). It is often considered pathognomic but may also occur in neuro-infectious diseases of other origin such as neurosyphilis (Kristensson & Bentivoglio 1999). Immediately after treatment a temporary rise in cell count may occur (Dumas & Girard 1978). For follow-up, only few precise guidelines can be found, although cell count is often the only parameter checked. Pépin and Milord (1994) consider cell counts higher than the previous determination and above 50 cells/μl as a relapse, even if the patient is asymptomatic.

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Cell counts higher than the previous one and between 20 and 49 cells/μl are only considered as relapse when symptoms recur. When the cell count is high (>50 cells/μl) but lower than the previous one, they recommend not to treat as it may take months before the cell count returns to normal. Indeed, in the large series of patients studied by Miézan et al., a mean cell count of ≤5 cells/μl was only observed 12 months post-treatment, while 22% of the patients still presented a slightly elevated cell count (<20 cells/μl) 24 months after successful treatment (Miézan *et al.* 2002).

The detection of trypanosomes in the CSF classifies a patient in the meningo-encephalitic stage irrespective of any other parameter. Sometimes trypanosomes can already be seen during the cell counting procedure. In these rare cases the number of trypanosomes exceeds 1/μl, which is often associated with high cell counts. According to Pépin and Milord (1991), trypanosomes are found more often in the CSF of patients with ≥100 cells/μl than in patients with lower CSF cell counts. Increased sensitivity of trypanosome detection can be achieved by single (Laveran & Mesnil 1912) or double centrifugation (Cattand *et al.* 1988) of the CSF. In the latter technique, the CSF sediment obtained after the first centrifugation is resuspended in a small volume of CSF, taken up into two capillaries which are flame sealed, and centrifuged in a microhematocrit centrifuge. Trypanosomes are directly searched for in the bottom of the capillary tube. A modified simple centrifugation of CSF in a sealed Pasteur pipette has recently been proposed as a rapid and simple alternative for the double centrifugation (Miézan *et al.* 2000). Detection of a trypanosome during the follow-up period is obviously considered as the best evidence of treatment failure. In such cases, trypanosomes often remain undetectable in the blood (Miézan *et al.* 2002). The examination of CSF should be performed as soon as possible after lumbar puncture, since CSF seems to be a poor medium for trypanosome survival and trypanosomes start to lyse quickly and become undetectable (Pentreath *et al.* 1992).

Protein concentrations in the CSF of sleeping sickness patients range from 100 to 2000 mg/l (Bisser *et al.* 2002; Lejon *et al.* 2003a). Cut-off values prescribed for sleeping sickness vary according to the method from 250 mg/l (trichloroacetic acid precipitation), to 370 mg/l (colorimetric methods) and 450 mg/l (sulfosalicylic acid precipitation) (WHO 1983). In practice, the protein determination is rarely performed for stage determination or follow-up because of the need of reagents that are often not available in rural health centres, and the belief that protein determination does not provide additional information (Miézan *et al.* 1998). The latter has been confirmed through analysis of the immunoglobulin and albumin concentrations in blood and CSF of sleeping sickness patients (Bisser *et al.* 2002; Lejon *et al.* 2003a). Due to the highly increased immunoglobulin concentrations in serum, the CSF protein concentration in trypanosomiasis patients without central nervous system pathology is already elevated. Blood-CSF barrier dysfunction occurs only in a very advanced stage of the disease and remains moderate. The associated CSF total protein increase (concentrations higher than 750 mg/l) will therefore only occur late, and remain limited. No guidelines have been described for protein concentrations during follow-up after treatment, but the total protein concentration is believed to normalize slowly. Using the Coomassie brilliant blue method, Miézan describes an 'abnormal' CSF protein concentration in 58% of patients before treatment (mean concentration of around 500 mg/l), which is reduced to 6% of patients 24 months after successful treatment (Miézan *et al.* 2002).

The CSF of second stage sleeping sickness patients contains high levels of immunoglobulin, especially of IgM. An increased IgM concentration in CSF has therefore been proposed as a marker of interest for determination of the second disease stage in sleeping sickness already decades ago (Mattern 1968; Greenwood & Whittle 1973; Whittle *et al.* 1977; Lambert *et al.* 1981; Knobloch *et al.* 1984). Two of these early publications report on the intrathecal origin of the CSF immunoglobulins IgG and IgM in HAT. Intrathecal IgG synthesis was demonstrated in 10 *T.b. gambiense* patients using Tourtelottes formula (Lambert *et al.* 1981). The intrathecal origin of IgM was proven by intravenous injections of ^{125}I-labelled IgM of four patients, which could not be traced back in the CSF, and by the demonstration of IgM in plasma cells and morular cells obtained from the CSF of six sleeping sickness patients (Greenwood & Whittle 1973). More recently, the CSF immunoglobulin composition and its origin was studied in detail and on large patient groups by Bisser and Lejon (Bisser *et al.* 2002; Lejon *et al.* 2003a). The elevated immunoglobulin concentrations in CSF – respectively 3- 4- and 40-fold for IgG, IgA and IgM in meningo-encephalitic stage patients compared with hemolymphatic stage patients – were proven to have their origin in intrathecal immunoglobulin synthesis. It was shown that second-stage HAT is characterised by a two or three class immune response in the central nervous system with a predominant IgM synthesis, both in frequency and strength (Lejon *et al.* 2003a). As a consequence, intrathecal IgM synthesis was identified as the most sensitive indicator for neuro-inflammation in sleeping sickness. In contrast, detection of oligoclonal IgG or oligoclonal IgM in CSF may remain negative in second-stage trypanosomiasis patients (Lejon *et al.* 2003b). A recently developed card

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agglutination test, LATEX/IgM, simplifies detection of IgM in CSF of sleeping sickness patients in field conditions and, when fully evaluated, may be of use for staging of the disease (Lejon *et al.* 2002b). Reports on the evolution of IgM concentrations after treatment remain contradictory. According to Knobloch *et al.* (1984), efficacy of treatment is indicated by a decrease of IgM in CSF, and levels of IgM could return to normal after weeks or months (Greenwood & Whittle 1973). On the other hand, Whittle *et al.* (1977) describe a slow decrease of IgM in CSF up to 1 year after treatment. At that moment however, relapses would be characterized by high CSF IgM (Mattern 1967).

The detection of trypanosome specific antibodies in CSF of second-stage patients by indirect immunofluorescence and ELISA has been described extensively (Lucasse 1964; Mattern *et al.* 1965; Whittle *et al.* 1977; Roffi *et al.* 1979; Lambert *et al.* 1981; Knobloch *et al.* 1984; Smith *et al.* 1989; Lejon *et al.* 1998). The possibility to detect trypanosome specific antibodies in CSF by techniques applicable in the field, such as CATT/*T.b. gambiense* and indirect agglutination (LATEX/*T.b. gambiense*), has been explored, but these techniques may lack sensitivity for diagnosis of the second stage (Lemesre *et al.* 1988; Büscher *et al.* 1999). The observed trypanosome specific antibodies are mainly of the IgM and IgG class (IgG1 and IgG3 subtypes) (Lejon *et al.* 1998). Intrathecal synthesis of trypanosome specific IgG was demonstrated in 52% of second stage sleeping sickness patients (Lejon *et al.* 2003a). After successful treatment, CSF antibodies drop down quickly and the decrease of trypanosome specific antibody concentrations in CSF has therefore also been proposed as an interesting parameter for definite cure (Roffi *et al.* 1979; Knobloch *et al.* 1984; Smith *et al.* 1989).

The CSF of sleeping sickness patients also contains antibodies with other affinities. Antibodies against brain specific components such as galactocerebrosides and neurofilament have been detected and may be promising markers for central nervous system involvement (Jauberteau *et al.* 1994; Ayed *et al.* 1997; Bisser *et al.* 2000). Studies to simplify the assays for implementation in the field are ongoing (Courtioux *et al.* 2003). These auto-antibodies could result from the central nervous system tissue damage and immune activation associated with trypanosome invasion. The presence of neurofilament and glial fibrillary acidic protein in the CSF of second stage sleeping sickness patients supports this hypothesis since they are markers of neuronal destruction and astrocyte activation (Lejon *et al.* 1999). Auto-antibodies could also be induced by the polyclonal B-cell activation in sleeping sickness. Anti-endotoxin antibodies and the presence of endotoxin in CSF have also been reported (Pentreath *et al.* 1995, 1997).

Several authors identified the presence of trypanosomal components, such as nucleic acids and proteins, in the CSF. Trypanosomal DNA has been demonstrated in CSF of sleeping sickness patients by PCR, and the high sensitivity of this technique compared with conventional trypanosome detection techniques in CSF was reported (Kirchhoff 1998; Truc *et al.* 1999; Kyambadde *et al.* 2000; Jamonneau *et al.* 2003). During follow-up, CSF may continue to be PCR positive even after successful treatment (Kirchhoff 1998; Truc *et al.* 1999). Trypanosomal antigens were also detected in CSF of *T.b. gambiense* and *rhodesiense* cases (Nantulya 1988; Nantulya *et al.* 1992).

Finally, the concentrations of some cytokines have been studied in the CSF of sleeping sickness patients. In both *gambiense* and *rhodesiense* disease, the interleukin-10 (IL-10) concentration in CSF of second-stage patients is strongly elevated, and returns to normal, undetectable levels immediately after treatment (MacLean *et al.* 2001; Lejon *et al.* 2002a). Due to this immediate negativation, IL-10 could be a potentially interesting marker for treatment-follow up. Other cytokines or chemokines that were shown to be elevated in the CSF of second stage *T.b. gambiense* patients include IL-6, IL-8 and prostaglandin D2 (Pentreath *et al.* 1990).

Interpretation of CSF data and therapeutic decision

At cell counts ≤20 cells/μl, an absence of concordance between different biological data is observed frequently. As a consequence, some controversy exists about the interpretation of CSF data for therapeutic decision at these low cell counts.

Patients classified in the field in the hemo-lymphatic stage, based on cell counts ≤5 cells/μl and the absence of trypanosomes in CSF, may have other CSF abnormalities. Bisser *et al.* and Lejon *et al.* observed neuro-inflammation, i.e. blood-CSF barrier dysfunction, intrathecal IgM synthesis, intrathecal IgG synthesis or intrathecal trypanosome specific antibody synthesis in respectively 11–39 and 8–18% of such patients (Bisser *et al.* 2002; Lejon *et al.* 2003a). Trypanosomal DNA has been detected in the CSF of 23% of hemo-lymphatic stage patients (Jamonneau *et al.* 2003). Inversely, almost half of the patients with cell counts between 6 and 20 cells/μl without trypanosomes in the CSF, classified in the field in meningo-encephalitic stage, may show no signs of neuro-inflammation (Lejon *et al.* 2003a).

These observations prompted several authors to propose an increased cut-off value for the cell count of 10 or 20 cells/μl, possibly in combination with alternative criteria for central nervous system invasion such as intrathecal IgM synthesis (Miézan *et al.* 1998; Bisser *et al.* 2002;

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Lejon *et al.* 2003a). In addition, the meaning of the presence of trypanosomes in CSF with cell counts lower than 20 cells/μl has been questioned.

Indeed, successful pentamidine treatment of second-stage patients with up to 10 or 20 cells/μl has been reported (Lourie 1942; Pépin & Milord 1994; Ruiz *et al.* 2002). Also patients with PCR positive CSF, or with trypanosomes in the CSF detected by *in vitro* culture but with normal CSF cell counts and protein concentrations were reported to be cured with pentamidine (Doua *et al.* 1996; Jamonneau *et al.* 2003). On the other hand, in a recent clinical trial, up to 43% of treatment failures occurred when second-stage patients with less than 20 cells/μl, with or without trypanosomes in the CSF, were treated with pentamidine (Legros *et al.* 2001). In this trial, cell counts of 11–20 cells/μl, intrathecal IgM synthesis, increased CSF end-titres in the IgM detecting card agglutination test LATEX/IgM, and the presence of trypanosome specific antibodies detected with LATEX/*T.b. gambiense*, were associated with treatment failure (Lejon *et al.* 2003c). From this study, it seems therefore advisable to take additional criteria besides cell count into consideration before deciding on pentamidine treatment. Others state that a distinction should be made between stage determination and therapeutic decision, and that the former should not always condition the latter (Jamonneau *et al.* 2003).

Options and perspectives for the future

Modern discovery techniques such as proteomics (Papadopoulos *et al.* 2004) and micro-arrays, open the possibility to identify markers for diagnosis of central nervous system involvement in other compartments than CSF, and might in the long-term lead to less invasive procedures for staging and follow-up in sleeping sickness. Awaiting results from such research, only few practical guidelines for improved stage determination that are applicable in the field are suggested. One proposal is to consider patients with cell counts up to 20 cells/μl, without intrathecal IgM synthesis, in the hemo-lymphatic stage and to treat them with pentamidine (Lejon *et al.* 2003a). In the field, determination of intrathecal IgM synthesis could be replaced by testing the CSF in LATEX/IgM, considering patients with CSF end titres <1:8 in LATEX/IgM to be free of intrathecal IgM synthesis. However, this approach as well as other alternative approaches for stage determination, such as LATEX/*T.b. gambiense*, PCR, detection of auto-antibodies and quantification of cytokines, remain to be validated in sufficiently high patient numbers, of whom the outcome of treatment is known. Also for decision on cure, new tools remain to be documented and validated before implementation.

Such validation first depends on close interaction between laboratory researchers and actors on the field such as national control programmes and non-governmental organizations; and second might require maximum exploitation of the limited resources by combining drug trials and diagnostic trials.

Once validated, improved staging and follow-up algorithms should be implemented, leading to better treatment decision with fewer relapses, fewer complications and significant cost savings and facilitated or shortened post-treatment follow-up.

Conclusion

While awaiting a safe drug that cures both disease stages of HAT, more research is necessary to study the best criteria for treatment choice, taking into account efficacy, toxicity and ease of application of the available drugs.

Correct post-treatment follow-up remains hampered by poor compliance of most patients to scheduled control visits and by slow normalization of the parameters in use to decide on cure. High priority should therefore be given to the identification of parameters that facilitate post-treatment follow-up and that allow earlier cure assessment.

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Authors

Veerle Lejon (corresponding author) and **P. Büscher**, Department of Parasitology, Institute of Tropical Medicine, Nationalestraat 155, 2000 Antwerpen, Belgium. Fax: +32 (0)3 247 63 73; E-mail vlejon@itg.be

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